Investigating Antiviral Tripartite Motif (TRIM) Proteins in the Duck

by

Lee Kaitlynn Campbell

A thesis submitted in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

In

Physiology, Cell and Developmental Biology

Department of Biological Sciences
University of Alberta

Abstract

Ducks are the natural host and reservoir of influenza A virus (IAV). It is currently unknown how ducks can both tolerate and restrict IAV replication. It is likely that ducks have evolved unique transcriptional responses to IAV infection to restrict virus while limiting damage from inflammation, however this is poorly understood. Here we examine global transcriptome changes in tissues from influenza infected ducks to identify differentially expressed genes. We also mined the data for tripartite motif (TRIM) proteins, a group of proteins that arose early in eukaryote evolution of which some members have antiviral functions. TRIM proteins are defined by a conserved N-terminal RING, B-box and coiled-coil domains, and are further subclassified by their variable C-terminal domains. TRIM proteins can inhibit viral replication through either direct targeting of viral proteins, or by augmenting antiviral signaling pathways in the cell. Here we investigate how many TRIM proteins ducks have, which duck TRIM genes change in expression in response to IAV infection or which act as antiviral effectors.

In Aim 1 of my thesis, I investigated the transcriptional responses in spleen, lung and intestines in ducks infected with a low pathogenic avian influenza (LPAI) strain (BC500) and in the lung and spleen in ducks infected with a highly pathogenic avian influenza (HPAI) strain (VN1203). The results of these experiments found that ducks have 65 genes that share upregulation in all tissues sampled when ducks were infected with HPAI or LPAI and that many of these genes were involved in the RIG-I signaling pathway. Tissues involved with IAV replication (lung and intestine) also saw selective downregulation of certain proinflammatory cytokines. The results of this global transcriptome analysis suggest that global and tissue-specific regulation patterns help the duck control viral replication as well as limit some inflammatory responses in tissues involved in replication to avoid damage. In the second Aim of my thesis, I

created a *de novo* transcriptome assembled from Pekin duck RNA-sequencing reads mined from the SRA database on NCBI. From this transcriptome I identified 57 TRIM genes in the duck. I compared these duck TRIM genes to that of the chicken and documented seven TRIM genes that were found in duck but appear absent in chicken, while chickens had two TRIM genes which appear to be missing in duck. TRIM27L and RNF135 are TRIM proteins found in duck but not chicken which increases RIG-I signaling. I also show that many of the MHC-linked TRIM genes arose in a lineage-specific manner in birds and reptiles, and these genes are predominantly expressed in immune relevant tissues such as lung, intestine and spleen. In Aim 3, I investigated the differential expression of the 57 TRIM genes identified in Aim 2, to both VN1203 and BC500. VN1203 caused much more differential expression of duck TRIM genes than BC500 did in all tissues sampled. I investigated if several duck TRIM proteins were able to restrict IAV replication when overexpressed in both duck and chicken cells. TRIM27L, a TRIM protein found in duck but appearing to be missing in chicken, could restrict IAV replication when overexpressed in duck cells only, while TRIM32 and diaTRIM58 could restrict IAV in both chicken and duck cells. Finally, in Aim 4 I investigated the mechanisms behind TRIM27L IAV restriction. Previous work in our lab found TRIM27L could increase IFN-β promoter activity when cotransfected with constitutively active RIG-I (d2CARD) in chicken cells. I determined that TRIM27L was able to increase this promoter activity when cotransfected with d2CARD and dMAVS in both duck and chicken cells, however when cotransfected with duck IRF7 (which is downstream in the RIG-I signaling pathway) TRIM27L inhibited IFN-β promoter activity. TRIM27L uses its RING domain to inhibit IFN-β downstream of IRF7, and its C-terminal PRYSPRY domain to activate IFN-β promoter activity downstream of RIG-I and MAVS. By increasing our knowledge of the functions of TRIM proteins in influenza infection in ducks we

can gain a better understanding of both TRIM protein biology and function in host-pathogen interactions in the reservoir host.

Preface

This thesis is my own original work and contains results of collaborative research that has been published or are being prepared for publication in peer-reviewed journals.

Both Chapter 1 and Chapter 2 act as introductory chapters for this thesis. Chapter 1 outlines the current understanding of pattern recognition receptors and signaling pathways in antiviral response in duck, compared to human and chicken. A version of Chapter 1 has been published as Campbell LK and Magor KE. 2020. Pattern Recognition Receptor Signaling and Innate Responses to Influenza A Viruses in the Mallard Duck, Compared to Humans and Chickens. Frontiers in Cellular and Infection Microbiology 10(209). doi: 10.3389/fcimb.2020.00209. I conducted the original literature search and draft manuscript and Dr. Katharine Magor edited and provided advice for the manuscript.

Chapter 2 is written in a review format and has not been published. I was responsible for all literature searches and drafting the chapter. The title of Chapter 2 is: TRIM protein family evolution in direct and indirect restriction of Influenza A virus.

A version of Chapter 3 is published as: Campbell LK, Fleming-Canepa X, Webster RG and Magor KE. 2021. Title of publication: Tissue Specific Transcriptome Changes Upon Influenza A Virus Replication in the Duck. *Frontiers of Immunology* 12(4653). doi:10.3389/fimmu.2021.786205. I was responsible for data analysis and drafting the manuscript. Ximena Fleming-Canepa was responsible for handling the RNA samples and sending them for sequencing. Dr. Robert Webster provided the facility and expertise for all animal experiments. Dr. Katharine Magor collected all tissues and extracted RNA from infected animals and provided funding, supervision as well as editing of the manuscript. All authors approved the manuscript before publication. I would also like to acknowledge Dr. Rhiannon Peery for providing advice on script and R programming, and thank undergraduate student Sarah Klimchuk for her work on manually adding names of genes from NCBI to our datasets

Chapter 4 of this thesis is currently unpublished, but when published will be authored by: Campbell LK, Peery RM and Magor KE. The title of Chapter 4 is: Evolution and expression of TRIM protein genes in the mallard duck. I was responsible for data analysis and drafting the manuscript. Dr. Rhiannon Peery performed reciprocal blast hits on the data to remove duplicates, provided scripts for analysis and expertise and edited the manuscript. Dr. Katharine Magor edited and provided advice for the manuscript.

Chapter 5 of this thesis is unpublished. The title of Chapter 5 is: The duck TRIM gene repertoire is upregulated in response to highly pathogenic avian influenza virus. I was responsible for data analysis and drafting the manuscript. Dr. Katharine Magor edited and provided advice for the manuscript. I would like to also acknowledge Ximena Fleming-Canepa for running an SDS-page gel and Calla McKague for making cDNA to help determine if TRIM19.2 was expressing in DF-1 cells.

Chapter 6 of this thesis is unpublished. The title of Chapter 6 is: TRIM27L is both a positive and negative regulator of the IFN signaling pathway. I was responsible for data analysis and drafting the manuscript. Dr. Katharine Magor edited and provided advice for the manuscript.

The appendix contains unfinished side projects and detailed instructions for protocols developed during my time as a PhD student. The unfinished projects were developing a duck cell line and identifying *Leucocytozoon ssp* in sick ducks.

.

Acknowledgments

I would like to first thank my esteemed supervisor, Dr. Katharine Magor her tutelage and continuous support during my PhD. Thank you for taking the time to not only talk through the science, as well as making an equal amount of time available to talk horse, give career advice, and just talk about life in general. I would also like to express my sincere gratitude to my committee members Dr. Debbie McKenzie and Dr. Jim Smiley for their guidance, meaningful comments and suggestions which helped improve my thesis work. Thank you to my arm's length external examiner Dr. Maya Shmulevitz and my external examiner Dr. Sonja Best for their helpful comments and suggestions on my thesis. I am also extremely grateful to the members of the K. Magor lab over the years. I would especially like to thank Ximena Fleming-Canepa for her support and friendship, Danyel Evseev for the after-hours science and life talks, and Dexter Barbour for being an amazing duck-surgical assistant. Also, I thank the members of the B. Magor lab for their support. I also acknowledge all the funding sources which allowed me to focus on my thesis research: The University of Alberta FGSR and Biological Sciences Departments, Alberta Innovates, NSERC and the Li Ka Shing Institute for Virology.

I would like to extend my eternal gratitude to my parents for their endless support and patience. I finally have an answer to the question "Are you still in school?". No Mom and Dad, I'm not. A huge thank you to my friends, especially Dr. Rhiannon Peery and Dr. Chandra McAllister. Both of you were instrumental in starting the beginning of my path into big data analysis and are just amazing people in general. Thanks for pushing me when I needed it. I'm also super grateful to my beers club for being there every week, rain or shine.

And finally, thanks to Bruce, Boobear, Mimzabah, Little Princess, Lu, Raffleticket Stewart III, Al, Harley, Floppit, Lucyfur and Houseplant. I couldn't think straight let alone exist, without my fur, scale and claw bearing support system.

Table of Contents

List of Tables

List of Figures

Figure A2.1. Blood smears from 3- and 4-week old Pekin ducks detect Leucocytozoon ssp.

Figure A2.2. Nested PCR confirms presence of haemosporidian parasite cytochrome b gene in visibly ill Pekin and Muscovy ducks.

Figure A2.3. Single nucleotide or amino acid changes in Leucocytozoon simondi *cytb* sequences from Muscovy duck.

Figure A2.4. Single nucleotide or amino acid changes in Leucocytozoon simondi *cytb* sequences from A 3-week old Pekin duck.

Figure A2.5. Single nucleotide or amino acid changes in Leucocytozoon simondi *cytb* sequences from a 4-week old Pekin duck.

Figure A2.6. Leucocytozoon *cytb* clusters based on the breed of duck the *cytb* gene was amplified from.

List of Abbreviations

ALV	-	Avian leukosis virus
ARF	-	ADP ribosylation factor-like
BROMO	-	Bromodomain
BSA	-	Bovine serum albumin
btyTRIM	-	Bloodthirsty TRIM
CARD	-	Caspase activation and recruitment domain
CEF	-	Chicken embryonic fibroblasts
cGAMP	-	2′3′-Cyclic GMP-AMP
cGAS	-	cyclic GMP-AMP synthase
COS	-	C-terminal subgroup one signature
DDX58	-	DExD/H-box helicase 58
DF-1	-	Douglas Foster-1 chicken fibroblast cells
DE	-	Differentially expressed genes
DEE	-	Duck embryonic epithelial
DEF	-	Duck embryonic fibroblasts
DEG	-	Differentially expressed genes
DHV	-	Duck hepatitis virus
DMEM	-	Dulbecco's modified media
DNA	-	Deoxyribonucleic acid
DPI	-	Day post infection
DTMUV	-	Duck Tembusu virus
ECSIT	-	Evolutionarily conserved signaling intermediate in Toll pathways
EIA	-	Equine infectious anemia
EID50	-	50% egg infectious dose
EPC	-	Epithelioma papulosum cyprinid
FBS	-	Fetal bovine serum
FC	-	Fold change
FDR	-	False discovery rate
FIL	-	filamin

finTRIM	-	Fish novel TRIM
FN3	-	Fibronectin, type III
GEE	-	Goose embryonic epithelial
GO BP	-	Gene ontology biological process
HA	-	Hemagglutinin
HBV	-	Hepatitis B virus
HCV	-	Hepatitis C virus
HeLa	-	Henrietta Lacks
HIV	-	Human immunodeficiency virus
HPAI	-	Highly pathogenic avian influenza
HSV	-	Herpes simplex virus
IAV	-	Influenza A Virus
IBV	-	Influenza B virus
IFIT	-	Interferon-induced proteins with tetratricopeptide repeats
IFITM	-	Interferon-inducible transmembrane proteins
IFN	-	Interferon
IFNα	-	Interferon alpha
IFNβ	-	Interferon beta
IHNV	-	Infectious hematopoietic necrosis virus 32-87
IL	-	Interleukin
IRF	-	Interferon regulatory factor
ISG	-	Interferon stimulated gene
IKK	-	Inhibitor of IKB kinase
K	-	Lysine
LECT2	-	Leukocyte cell-derived chemotaxin-2
LGP2	-	Laboratory of genetics and physiology 2
lncRNA	-	Long non-coding RNA
LPAI	-	Low pathogenic avian influenza
LPS	-	Lipopolysaccharide
MAPLC3B	-	Microtubule-associated proteins 1A/1B light chain 3B
MATH	-	Merpin and TRAF homology

MAVS	-	Mitochondrial antiviral-signaling protein
MDA5	-	Melanoma differentiation-associated protein 5
MDCK	-	Madin-Darby canine kidney
MDS	-	Multidimensional scaling plot
MHC	-	Major histocompatibility complex
MuRF	-	Muscle RING finger
MyD88	-	Myeloid differentiation primary response 88
NCOA7	-	Nuclear receptor co-activator 7
ncRNA	-	Non-coding RNA
NDV	-	Newcastle disease virus
NEMO	-	NF-κB essential modulator
NF-κB	-	Nuclear factor kappa B
NHL	-	NCL-1, HT2A and Lin-41 repeats
NLR	-	nucleotide-binding oligomerization domain-like receptors
NLRP3	-	NOD-like receptor family pyrin domain containing 3
NLS	-	Nuclear localization signal
N-MLV	-	N-strain of murine leukemia virus
NOD	-	nucleotide-binding oligomerization domain
NP	-	Nucleoprotein
NS1	-	Non-structural protein 1
OAS	-	Interferon-inducible 2′-5′-oligoadenylate synthase
ORA	-	Over-representation analysis
PA	-	Polymerase acidic protein
PARP	-	poly-ADP-ribose polymerase
PB1	-	Polymerase basic protein 1
PB2	-	Polymerase basis protein 2
pDC	-	Plasmacytoid dendritic cells
PFA	-	Paraformaldehyde
PHD	-	Plant Homeo domain
PKR	-	dsRNA-dependent protein kinase
PML	-	Promyelocytic Leukemia protein

PRYSPRY	-	Pre-SPRY splA kinase and ryanodine receptor
RBP	-	RNA-binding proteins
RD	-	Repressor domain
REV	-	Reticuloendotheliosis virus
RGNNV	-	Red-spotted grouper nervous necrosis virus
RIG-I	-	Retinoic acid-inducible gene I
RING	-	Really interesting new gene
RLR	-	RIG-I-like receptor
RNA	-	Ribonucleic acid
RNA-seq	-	RNA-sequencing
RNF	-	RING finger
SA	-	Sialic acid
SeV	-	Sendai Virus
SGIV	-	Singapore grouper iridovirus
SIV	-	Simian immunodeficiency virus
-ssRNA	-	negative sense single stranded RNA
STING	-	Stimulator of interferon genes
STRING	-	Search tool for the retrieval of interaction genes/proteins
SUMO	-	Small ubiquitin-like modifier
SVCV	-	Spring viremia carp virus
TAK1	-	TGFβ-activated kinase 1
TBK1	-	TANK-binding kinase 1
TGF-β	-	Tumor growth factor beta
TIR	-	Toll/IL-1 receptor
TLR	-	Toll-like receptors
TMM	-	Trimmed mean of M-values
TM	-	Transmembrane
TNF	-	Tumor necrosis factor
TRAF	-	TNF-receptor associated factor
TRIF	-	TIR-domain-containing adaptor-inducing interferon-β
TRIM	-	Tripartite motif

USP14	-	ubiquitin-specific protease 14
VHSV	-	Viral hemorrhagic septicemia virus 07-71
WNV	-	West Nile virus
WSSV	-	White spot syndrome virus
β-TrCP	-	β-transducin repeat-containing protein

CHAPTER 1

Introduction

A version of this chapter has been published as Campbell LK, and Magor KE. 2020. Pattern Recognition Receptor Signaling and Innate Responses to Influenza A Viruses in the Mallard Duck, Compared to Humans and Chickens. Frontiers in Cellular and Infection Microbiology 10(209). doi: 10.3389/fcimb.2020.00209.

Pattern Recognition Receptor Signaling and Innate Responses to Influenza A Viruses in the Mallard Duck, Compared to Humans and Chickens

1.1 Introduction

Influenza A virus (IAV) is a negative sense single stranded RNA (-ssRNA) virus which causes significant disease in both humans and animals. Due to rapid accumulation of mutations during replication, this virus can change surface proteins quickly, thus escape both natural and vaccine-based immunity. These mutations also affect the pathogenicity of individual viral strains. In chickens especially, IAV can cause severe disease and mortality. The virus is classified as low pathogenic or highly pathogenic avian influenza (LPAI and HPAI, respectively) depending on the severity of disease that it causes in chickens (Alexander et al., 1986; Burggraaf et al., 2014). LPAI strains cause mild symptoms, and the birds generally recover within a few days whereas HPAI strains tend to spread systemically and often kill chickens within the first few days of infection.

IAV preferentially replicates in different tissues and organs in the host, and initial infection often depends on the linkage type of terminal sialic acid on glycoproteins expressed on the surface of cells. Viral hemagglutinin (HA) surface proteins bind to glycoprotein-linked sialic acid (SA) on the surface of host cells. The specific linkage of these sialic acids allows the virus to not only become specific to different host species, but also different tissues in these hosts. Humans express sialic acid α-2,6 linked galactose (SA α-2,6-Gal) surface molecules on epithelial cells in the upper airways, which is the site of replication for IAV in humans (Baum and Paulson, 1990; Couceiro et al., 1993). As such, strains of IAV that infect humans replicate in the upper

airways. Birds, however, predominantly express SA α-2,3-Gal in their digestive tracts and lungs (Costa et al., 2012). Strains of IAV which are adapted to replicate in birds preferentially bind these receptors over human SA α-2,6-Gal receptors. Chickens also express α-2,6-Gal in their intestinal tracts and lungs, whereas ducks only express these receptors in their lungs. Chickens also have a predominance of SA α-2,6-Gal in their trachea whereas ducks have SA α-2,3-Gal receptor dominance (Kuchipudi et al., 2009). As IAV has been known to jump host species, as is the case of avian IAV jumping to humans, this suggests that chickens may be responsible for propagating avian strains of influenza that can then infect humans. IAV can use other receptors such as phosphoglycans on host cells to gain entry and seems to depend on more than just SA linkages to enter cells (Byrd-Leotis et al., 2019).

Ducks and migratory waterfowl are thought to be the reservoir hosts of IAV, as they appear to have shared a long evolutionary history with the virus (Webster et al., 1992; Taubenberger et al, 2010). Indeed, phylogenetic analysis has suggested that avian IAV and circulating mammalian strains of IAV share a recent common ancestor of avian origin. So called "dabbling ducks," or more specifically ducks of the genus Anas, are the most frequent host of circulating strains of IAV (Kida et al., 1980; Olsen et al., 2006; Runstadler et al., 2007; Jourdain et al., 2010). For simplicity, we will generalize the term "ducks" to mean mallard ducks (*Anas platyrhynchos*), which also includes the many breeds of domesticated mallard ducks (Zhang et al., 2018). When infected with IAV, ducks generally have no or very mild symptoms, yet surprisingly still replicate and excrete viruses at high titres (Kida et al., 1980). LPAI can replicate in the intestines of ducks for up to 5 days without causing lesions (Daoust et al., 2013). Often called the "Trojan Horse" of infection, these migratory birds can then spread the virus to other ducks in waterways, or to other bird species as they migrate (Kim et al., 2009). HPAI however, preferentially replicates in the lungs of infected ducks, and is more likely to spread systemically in ducks and chickens (Bingham et al., 2009; Vidana et al., 2018). After such a long evolutionary history, the reservoir host likely has evolved adaptations to circumvent damaging effects of prolonged viral replication.

While ducks can control most strains of IAV, some HPAI strains cause significant disease and mortality in ducks, especially those belonging to the H5 subgroup and clade 2.3.2.1 (Sturm-Ramirez et al., 2004; Bingham et al., 2009; Hagag et al., 2015; Haider et al., 2017). It is difficult to generalize, however, because in challenge experiments using viruses belonging to this

clade, ducks demonstrated differences in mortality ranging from 100% lethal to no mortality (Kang et al., 2013; Ducatez et al., 2017). Most strikingly, two viruses from the 2.3.2.1 clade that differed by only 30 amino acids showed complete differences in mortality in mallards, with one virus being 100% lethal while the other causing no mortality (Hu et al., 2013). All of these strains are lethal to chickens and many other species. However, some species may show resistance to some strains. Pigeons are resistant to some strains of H5N1, including to strains belonging to clade 2.3.2 (Smietanka et al., 2011; Yamamoto et al., 2012). However, as summarized in a recent review (Abolnik, 2014), pigeons often do not replicate the virus to significant titres and only shed the virus for a short period of time. We also cannot generalize about all ducks as other types of ducks exhibit varied reactions when infected with H5N1 strains of virus. Gadwall, wigeon, and mallard ducks were asymptomatic, while mandarin duck, tufted ducks, ruddy shelducks, and several species of geese and swans showed signs of morbidity and mortality (Gaidet et al., 2010). In another study, swans and ruddy shelducks showed 100% mortality when infected with HPAI H5N1, whereas mallard ducks had an asymptomatic infection (Kwon et al., 2010). Thus, infection and mortality rates differ between different types of ducks. These studies highlight the difficulty in making generalizations about avian influenza studies but can also pinpoint residues contributing to virulence in each host species. What makes mallard ducks so successful at both limiting viral replication of HPAI virus and resisting damage from replicating virus is currently unknown.

When birds are infected with IAV, the first few days seem to be the most important when determining survival vs. succumbing to infection, highlighting the importance of innate immunity as a protective mechanism. We recently reviewed the immune responses of ducks and chickens to IAV (Evseev et al, 2019). Birds diverged from mammals about 300 million years ago yet have retained many of the same innate immune mechanisms that mammals use to combat viral infections. When viral or pathogen associated molecular patterns (PAMPS) are detected by the host, they are detected by specific pattern recognition receptors (PRRs) in order to elicit antiviral responses including cytokines, chemokines, and upregulation of antiviral effectors. Both immune and non-immune cells contain these PRRs. PRRs of avian species were previously reviewed in 2013 (Chen et al., 2013), however significant advances have been made since that time. In this review, we summarize recent advances in understanding innate signaling pathways in ducks by looking at the similarities and differences between PRR tissue expression in ducks,

chickens, and humans. We also further review new research in characterizing protein function in the signal transduction platform in order to understand how innate signaling pathways differ or are the same in these three species.

The three important PRR signaling pathways responding to influenza infection include toll-like receptors (TLRs), retinoic acid-inducible gene-I (RIG-I)-like receptors (RLRs), and nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) (Figure 1.1). TLRs, RLRs, and NLRs can all be found on the cell surface or in cytosolic compartments in the cell. These PRRs all act to recognize influenza viral components such as double stranded RNA (dsRNA), single stranded RNA (ssRNA), and RNA with a 5′ triphosphate overhang (5′pppRNA) (Yoneyama et al., 2004; Okamoto et al., 2017). Many of these PRRs have signaling pathways that converge downstream to produce interferons (IFNs) or proinflammatory cytokines and utilize similar scaffolding and adaptor proteins to amplify this signal. In this review, we compile recent studies on characterization of these influenza sensors, signaling pathways and their downstream effectors in both chickens and ducks.

Basal expression of these PRRs may also allow different tissues to detect IAV infection earlier. To visualize PRR readiness we show basal expression patterns in different tissues in ducks and chickens (Figure 1.2). Tissues studied include immune relevant organs such as the lung, spleen, bursa, thymus, and intestine as well as other organs such as brain, kidney, and heart.

1.2 RLR Receptors and Their Adaptors

The RIG-I like receptor (RLR) family are select cytosolic RNA helicases which contain conserved DExD/H box domains used in nucleic acid binding (Loo et al., 2011). These PRRs sense non-self RNA from viral pathogens. In contrast to other PRRs like TLRS, RLRs are expressed in immune cells as well as in somatic cell types such as epithelium, thus can protect cell types most targeted by viral infection (Uhlen et al., 2015; Francisco et al., 2019). RLRs involved in IAV recognition include retinoic acid inducible gene I (RIG-I), melanoma differentiation-associated gene 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2) (Figure 1.1). RIG-I and MDA5 share much structural similarity, with both proteins having two caspase activation and recruitment (CARD) domains, a central DEAD helicase domain and a C-terminal repressor domain (RD) (Yoneyama et al., 2005; Zou et al., 2009). While the DExD/H

box helicase domain has the ability to use ATP hydrolysis to aid in binding and unwinding viral RNA, the RD has been implicated in self repression (as in RIG-I). CARD domains are involved in relaying the signal to the downstream adaptor, the mitochondrial antiviral signaling protein (MAVS) (Jacobs et al, 2013; Wu et al, 2015). LPG2 is lacking the CARD domains that RIG-I and MDA5 possess but shares structural similarity in the DExD/H box and RD domains (Pippig et al., 2009). The cytosolic sensor MDA5 preferentially recognizes long dsRNA, whereas RIG-I recognizes shorter dsRNA sequences that are produced during IAV replication (Kato et al., 2008). Once these cytosolic sensors recognize viral RNA, a signal is transduced through MAVS to downstream components to induce type I IFN or proinflammatory cytokine production.

1.2.1 RIG-I

RIG-I is the primary sensor of influenza virus in all cells except plasmacytoid dendritic cells. RIG-I detects dsRNA and viral transcriptional intermediates bearing 5′-pppRNA in infected cells (Hornung et al., 2006; Pichlmair et al., 2006; Schmidt et al., 2009). A panhandle structure, formed by binding of complementary regions in the influenza RNA transcript, is detected by RIG-I (Liu G. et al., 2015). Recently transcriptional intermediates called mini viral RNAs of about 80 nucleotides in length have been shown to act as RIG-I ligands (te Velthuis et al., 2018). Notably, it was recently shown that RIG-I detects viral replication not only in the cytoplasm, but also in a nuclear compartment (Liu G. et al., 2018). This may be particularly relevant for influenza detection since influenza replicates in the nucleus. A recent review considers how dsRNA and viral transcriptional intermediates bearing 5′-pppRNA made in the nucleus are detected by RIG-I in the cytoplasm of infected cells (Liu et al, 2019). It is not known whether RIG-I is capable of nuclear detection in lower vertebrates. In addition, RIG-I (but not MDA5) can act as an antiviral effector protein by directly binding to incoming IAV viral RNA (Weber et al., 2015). RIG-I also has far reaching effects on immune responses. Mice deficient in RIG-I signaling show defects in dendritic cell activation and mobilization, viral antigen presentation and impairment of polyfunctional T cell responses (Kandasamy et al., 2016). More recently, the importance of RIG-I in IAV infection has been questioned. Surprisingly, when RIG-I was knocked out of mice, this did not make mice more susceptible to lethal influenza infection (Wu et al., 2018). These results may stem from mice not being a natural host of IAV or perhaps they rely on different recognition strategies to detect virus.

RIG-I is ubiquitously expressed in human tissues, but ducks have tissue specific basal expression of RIG-I and chickens appear to be missing RIG-I entirely. RIG-I is expressed in most human tissues and does not exhibit tissue specific expression, although there is slightly higher mRNA expression in the thymus, granulocytes, and adipose tissues (Uhlen et al., 2015). A comparison of tissue expression of RLR pathway components between chickens and ducks illustrates the readiness of these tissues to respond to pathogens (Figure 1.2A). In Muscovy ducks, RIG-I is most highly expressed in the trachea and digestive tissues (Cheng et al., 2015a). Chickens appear to have lost RIG-I (Barber et al., 2010). RIG-I gene loss has also been documented in mammals, such as the Chinese tree shrew (Xu et al., 2016). RIG-I knockouts generated in C57BL/6 mice are lethal in the developing embryos (Kato et al., 2005), however this lethality was not seen in mice with a more complex genetic background (Wu et al., 2018).

Duck RIG-I can function in chicken cells, indicating that chickens have the corresponding downstream signaling components. When we overexpressed duck RIG-I in chicken fibroblasts, the cells could detect RIG-I ligand and produce interferon (Barber et al., 2010). We also showed that chicken cells transfected with duck RIG-I produce more IFN-β, augment expression of numerous ISGs, and restrict influenza virus (Barber et al., 2013). Others have demonstrated that chickens detect IAV through the related RLR, MDA5 (Karpala et al., 2011; Liniger et al., 2012). We have speculated that one reason ducks so successfully control influenza virus while chickens do not is partially because of RIG-I. This has been controversial, and we acknowledge that because RIG-I has not been detected does not prove it does not exist. No disrupted gene has been found to confirm its absence. If chicken RIG-I has significantly diverged from duck RIG-I, it would not be detected through hybridization, or PCR. Likewise, it has also been notably absent from the now extensive transcriptome databases available for chickens and other galliform birds. However, if a chicken RIG-I ortholog is expressed in very low amounts or has a very high GC content, it may be difficult to sequence using standard next generation sequencing technology. An interesting experiment to determine the significance of RIG-I in birds would be to knock RIG-I out of ducks or introduce duck RIG-I into chickens. However, some strains of influenza viruses can kill ducks even in the presence of RIG-I, demonstrating that many other factors contribute to successful defense.

RIG-I is upregulated quickly during influenza infection, with a peak at 24 h and expression returning to normal levels in lung, intestine, and spleen when Pekin ducks are

infected with both HPAI and LPAI IAV strains (Fleming-Canepa et al., 2019). In these studies, RIG-I is upregulated much more during HPAI infection than LPAI infection. In Muscovy ducks RIG-I mRNA expression peaked at 2 DPI in brain and spleen, while expression was highest 1 DPI in the lung and bursa (Cheng et al., 2015a). Muscovy ducks are more susceptible to influenza infection than mallard ducks (Phuong do et al., 2011), and this slight delay in RIG-I upregulation may contribute.

1.2.2 MDA5

MDA5 was often thought to be of less importance in IAV infection because of its preference for longer dsRNA, however siRNA knockdown of this host mRNA during IAV infection in mice demonstrated that MDA5 is also an important factor in viral restriction (Benitez et al., 2015). While it appears that chickens have lost RIG-I (Barber et al., 2010), they use the related cytosolic receptor MDA5 to detect IAV and signal through MAVS to induce IFN and proinflammatory cytokine responses (Karpala et al., 2011; Liniger et al., 2012). The tree shrew lineage also appears to have lost RIG-I, and pathogen pressures on tree shrew MDA5 and LGP2 have selected for the ability to detect the RIG-I agonist Sendai virus (SeV) (Xu et al., 2016). Chicken MDA5, unlike mammalian MDA5, preferentially recognizes short dsRNA (Hayashi et al., 2014), and like human MDA5 it can also be stimulated with long polyinosinic-polycytidylic acid (poly (I:C) (Barber et al., 2010). It is currently unknown if duck MDA5 has a dsRNA length preference. Chicken MDA5 also appears to have undergone positive selection and is able to recognize RNA from Newcastle Disease virus (NDV) (Xu et al., 2019). Indeed, when these mutations were introduced into human MDA5, a glutamic acid to a leucine at position 633, the mutant was able to bind NDV RNA. Duck MDA5 has proline at residue 633 (Barber et al., 2010), and thus is not expected to detect NDV RNA.

MDA5 is most highly expressed in the trachea followed by the ileum, duodenum, crop, rectum, and colon in Muscovy ducks (Wei et al., 2014), like basal expression of RIG-I (Cheng et al., 2015a). In healthy adult chickens, MDA5 was most highly expressed in the spleen, followed by the thymus and trachea (Bush et al., 2018). Chicken MDA5 is strongly upregulated in lung, spleen and brain in H5N1 infected birds (Karpala et al., 2011). Duck MDA5 is upregulated in response to IAV infection at 1 DPI in the lung, spleen, and brain, and returns to normal levels at 3 DPI (Wei et al., 2014; Fleming-Canepa et al., 2019). MDA5 was also slightly upregulated in lungs of Pekin ducks infected with LPAI, but not significantly upregulated in intestines of the

same cohort of ducks (Fleming-Canepa et al., 2019).

1.2.3 LGP2

LGP2 is induced in humans during influenza infection. LGP2 seems to function as both a positive and negative regulator of RIG-I and MDA5. This contrary effect on IFN signaling seems to be dose dependent as smaller amounts of LGP2 help increase MDA5 and RIG-I activation while over-expression of LGP2 inhibits it (Rothenfusser et al., 2005; Satoh et al., 2010). In mice infected with IAV, LGP2 attenuates the IFN response, perhaps in an effort to control damaging inflammatory responses (Malur et al., 2012). Recently LGP2 has also been implicated in inhibition of Dicer dependent processing of dsRNA, thus inhibiting RNAi (van der Veen et al., 2018). Muscovy ducks infected with HPAI H5N1 had upregulation of duck LPG2 (duLGP2) in the spleen at 1 DPI (Jiao et al., 2015). In the lung and brain, duLGP2 was upregulated on both 1 and 2 DPI suggesting that duLGP2 is involved in the early response to IAV. This is the same expression pattern seen in geese infected with this strain of H5N1 (Wei L. et al., 2016). No studies have been published to date on duLGP2 interactions with RIG-I during IAV infection. However, duLGP2 was important during duck enteritis virus (DEV) infection through interactions with MDA5 (Huo et al., 2019). Overexpression of chicken LGP2 (chLGP2) reduced IFN signaling in IAV infected cells, however silencing of the LGP2 gene in chicken cells also decreased IFN-β production, suggesting chLGP2 is important for MDA5 signal enhancement at low expression levels (Liniger et al., 2012). It is unknown if duLGP2 augments signaling with duck RIG-I or MDA5.

1.2.4 TRIM25

Tripartite motif protein 25 (TRIM25) can both augment IFN signaling (Gack et al., 2007) and directly restrict virus in mammals (Meyerson et al., 2017). TRIM25 is known to stabilize RIG-I CARD domain interaction with MAVS CARD domains and increase IFN production during an infection (Gack et al., 2007). The CARD domains of RIG-I are exposed when RIG-I recognizes viral RNA, at which point TRIM25 binds to RIG-I CARD domains using its C-terminal PRY-SPRY domain. Using the E3 ligase activity of its RING domain, TRIM25 polyubiquitinates RIG-I, attaching K63-linked ubiquitin chains to lysine residues on RIG-I. Stabilization of the RIG-I CARD domain tetramer allows it to nucleate MAVS filament formation (Peisley et al., 2014). TRIM25 can also physically block vRNA transcription in the nucleus by binding to the vRNP complex (Meyerson et al., 2017). Whether duck TRIM25 has

the ability to restrict viral RNA transcription not yet been examined.

Duck TRIM25 performs much the same function as human TRIM25 in RIG-I stabilization. Human TRIM25 ubiquitinates lysine 172 of human RIG-I CARD domains, but this lysine is not conserved in ducks. Instead, duck TRIM25 ubiquitinates K167 and K193 (Miranzo-Navarro et al, 2014). Mutation of either lysine site alone in the duck did not alter ubiquitination patterns of the CARD domains, however mutation of both sites abrogated covalently attached ubiquitin. Interestingly, duck TRIM25 in our transfection experiments could still activate these double mutants, suggesting unanchored ubiquitin could also stabilize RIG-I in the duck. Chicken TRIM25 augments IFN signaling, however the mechanism is unclear in the absence of RIG-I (Rajsbaum et al., 2012). In human cells, a long non-coding RNA (lncRNA) Lnczc3h7a also contributes to stabilizing the interaction between TRIM25 and RIG-I CARD domains (Lin et al., 2019). Recently, duck lncRNA were analyzed during HPAI and LPAI infection to determine which were differentially expressed and potentially involved in influenza A control (Lu et al., 2019). This study did not assess whether lnczc3h7a is differentially expressed, nor is it known if duck lnczc3h7a can function in the same manner, but this augmentation by lncRNAs may well be conserved.

In healthy chickens, TRIM25 is most highly expressed in the lung, spleen, and thymus and is upregulated in response to NDV in the spleen, thymus, and bursa (Feng et al., 2015). To date, we are unaware of studies looking at TRIM25 basal tissue expression in duck, however we showed TRIM25 is upregulated in the lung of HPAI infected ducks and slightly upregulated in lung of LPAI infected ducks at 1 DPI (Fleming-Canepa et al., 2019).

1.2.5 MAVS

MAVS protein is an adaptor protein that acts as a signaling amplifier during viral infection through interactions with both RIG-I and MDA5 (Figure 1.1). MAVS forms “prion-like” aggregates on the surface of the mitochondria when nucleated by tetramers of CARD domains of RIG-I or MDA5 (Kawai et al., 2005; Hou et al., 2011). The 2CARD domains of RIG-I form a helical tetrameric structure offset by 1 unit, and this helical assembly recruits MAVS CARD monomers (Wu et al., 2014). The helical assembly of tetrameric RIG-I and elongation of MAVS filaments is necessary for signal transduction by MAVS. Although ducks have very different amino acid sequences within these CARD domains compared to mammals, we showed the helical assembly of d2CARD with MAVS leads to signal activation as well (Wu et al., 2014).

Filamentous MAVs then recruits tumor necrosis factor receptor associated factor 3 (TRAF3), which acts as an adaptor protein to phosphorylate TANK-binding kinase 1 (TBK1) and inhibitor of nuclear factor-κB (IκB) kinase (IKK) (Fitzgerald et al., 2003; Liu S. et al., 2015). From there transcription factors such as interferon regulatory factor 3 or 7 (IRF3/IRF7) are activated to induce IFN production.

Duck MAVS expression in healthy tissues varied depending on the age of the ducks tested. In 3-week-old Cherry Valley ducks, MAVS expression was highest in the pancreas, liver and heart (Li N. et al., 2016), while in 2-month-old Cherry Valley ducks, tissue expression was more ubiquitous with slightly higher expression seen in the trachea and heart (Li H. et al., 2016). MAVS basal expression in adult chickens is also more ubiquitous, with only slighter higher expression seen in the spleen, heart, and thymus (Bush et al., 2018). The human protein atlas shows that human MAVS is expressed in almost all tissues, but curiously has the lowest expression in innate immune cells such as dendritic cells, monocytes, T-cells, and B-cells (Uhlen et al., 2015). Pekin duck MAVS is upregulated 1 DPI in both HPAI and LPAI infection in lungs, however no MAVS upregulation was seen in ileum of LPAI infected ducks (Fleming-Canepa et al., 2019).

1.2.6 TBK1

TBK1 activates IFN-β production by phosphorylating IRF3 allowing it to dimerize and translocate to the nucleus and initiate type I IFN production (Fitzgerald et al., 2003; Liu S. et al., 2015) (Figure 1.1). In humans TBK1 (huTBK1) expression is highest in brain tissues, adrenal glands, lungs, and the upper digestive tract (Uhlen et al., 2015). Chickens express TBK1 highest in spleen, lung, and thymus (Wang et al., 2017). This contrasts with 1-month old Cherry Valley ducks, where the highest expression was seen in the liver, heart, and duodenum (Hua et al., 2018). Very little expression was seen in healthy lungs, spleen, or bursa of these ducks. Duck TBK1 (duTBK1) was shown to function similarly to huTBK1 in that overexpression was able to activate IFN-β, NF-κB, and IRF1 promoter activity in duck embryonic fibroblast (DEF) cells. Silencing of endogenous duTBK1 in DEF cells also significantly reduced IFN-β promoter activity in DEF cells. As basal tissue expression of many duck PRR and downstream signaling components seems to favor having reduced expression of these proteins in immune relevant sites, we suggest that this could be another level of immune regulation that is protective to the duck. Experimental dysregulation of basal tissue expression of proteins such as TBK1 and IRF7 could

be done to investigate this question.

1.2.7 TRAF3

TRAF3 operates downstream of both TLRs as well as RLRs to aid in signal transduction and amplification (Hacker et al., 2006) (Figure 1.1). In the RIG-I signaling pathway, TRAF3 acts as an adaptor downstream of MAVS, by recruiting TBK1 and IKKε to phosphorylate the transcription factor IRF3 (Guo et al, 2007). TRAF3 is most highly expressed in lung, spleen, and thymus of 2-week-old chickens (Yang et al., 2015). Duck TRAF3 (duTRAF3) however, has a uniform expression pattern with only slightly higher amounts of TRAF3 expression seen in the brain, and the lowest levels in the lung (Wei et al., 2018). In chicken embryonic fibroblasts (CEF) cells, TRAF3 (chTRAF3) is upregulated in response to poly (I:C) stimulation, NDV infection and poly dA-dT, suggesting it is important in both DNA and RNA viral infections (Yang et al., 2015). Similarly, duTRAF3 is also upregulated in DEF cells stimulated with poly (I:C) and the authors also found that overexpression of duTRAF3 could control both IAV and duck Tembusu virus replication (Wei et al., 2018).

Curiously, a truncated version of duTRAF3 was also found, named duTRAF3-S (splice isoform duck TRAF3) (Wei et al., 2018). This splice variant is missing key N-terminal catalytic domains but can still bind to both TBK1 and MAVS with its C-terminal TRAF domain. DuTRAF3-S can interact with duTRAF3 but not MAVS, thus decreasing IFN-β production. After poly (I:C) stimulation, DEF cells express more duTRAF3 until 9 HPI, at which point duTRAF3 mRNA expression begins to decrease and duTRAF3-S mRNA expression begins to increase. This splice isoform may act to dampen IFN signaling in the later time points of infection to reduce damage from inflammation. In summary, duTRAF3 is most highly expressed in the brain in healthy ducks, while chickens express more in the lung, spleen, and thymus.

1.2.8 IRF7

IRF3 is a known important mediator of the type I interferon system in mammals. IRF3 is ubiquitously expressed, slow to degrade and a potent transcriptional activator of Type I IFN production in mammals (Honda et al, 2006). Birds appear to be missing IRF3, however, they do have IRF7 (Cormican et al., 2009; Huang et al., 2010). Avian IRF7 is structurally like IRF3, suggesting that it may play a similar role to that of IRF3 in mammals. Recent bioinformatics analysis has confirmed that chicken IRF7 clusters more closely to IRF3 of lower vertebrates yet is located in a region with high synteny to mammalian IRF7 (Cheng et al., 2019b).

IRF3, rather than IRF7, is considered more important for the initial response to viral infection. In mammals IRF3 is constitutively expressed in most tissues and seems to have a long half-life (Prakash et al., 2006; Hiscott, 2007). Activation of IRF3 results in increased type I IFN signaling and an eventual increase in transcription of IRF7, which has a very short half-life, comparatively. IRF7, in turn, amplifies both Type I and Type III IFN signaling (Sato et al., 1998). In mice, knockdown of IRF3 is not detrimental to the IFN response to IAV, however knockdown of IRF7 leaves mice much more susceptible to infection and a double knockout of both transcription factors renders mice unable to produce IFN-α or IFN-β (Hatesuer et al., 2017). Humans who have mutations in IRF7 are more susceptible to life threatening infections by IAV (Ciancanelli et al., 2015). There is very little expression of duck IRF7 (duIRF7) in the lung of uninfected ducks, and greater expression seen in the liver and intestine (Chen et al., 2019). Chicken IRF7 (chIRF7) is most highly expressed in the spleen and lung of healthy chickens (Cheng et al., 2019b).

Recent research has focused on the role of IRF7 in inhibition of IAV through IFN mediated responses in chickens and ducks. Chicken IRF7 (chIRF7) is involved in antiviral responses and plays analogous roles to that of mammalian IRF3. Recent studies have found that chIRF7 can be induced to translocate across the nucleus downstream of both chMAVS and chicken stimulator of interferon genes (chSTING), and chIRF7 dimerizes following chTBK1 activation, allowing it to increase IFN-β signaling (Wang et al., 2019). Initial experiments investigating function found that overexpression of chIRF7 increased IFN-β expression (Kim et al, 2015). However, their knockdown of chIRF7 did not significantly change IFN-β expression during poly (I:C) stimulation suggesting other transcription factors may be involved. Contradictory results were published in 2019 showing chirf7−/− DF-1 cells were unable to produce IFN-β, even when transfected with MAVS or STING (Cheng et al., 2019b). DuIRF7 upregulates type I IFNs but does not affect type II IFN expression (Chen et al., 2019). We showed that duIRF7 increases IFN-β signaling when overexpressed in DF-1 cells (Xiao et al., 2018). We also observed duIRF7 translocate to the nucleus upon stimulation with constitutively active RIG-I 2CARD. When chIRF7 is overexpressed in DF-1 cells, it caused increased cell death and resulted in higher levels of viral replication (Kim et al., 2018). With transfection of mCherry-IRF7 (Xiao et al., 2018), we also observed increased cell death.

IRF7 can control viral replication in ducks. A recent study demonstrated that duIRF7 can

control the positive sense RNA virus, duck Tembusu virus in DEF cells (Chen et al., 2019). No studies to date have examined whether duIRF7 controls IAV, or if it increases viral replication, as seen in DF-1 cells. This may be an interesting avenue of study, as Kim and Zhou (2018) suggest that chIRF7 could be a target of IAV.

1.2.9 STING

Stimulator of interferon gene (STING) is a protein on which many PRR pathways converge in order to increase NF-κB and IFN signaling downstream of pathogen pattern recognition (Figure 1.1). It was initially discovered as an adaptor molecule in the cyclic GMP-AMP synthase (cGAS) signaling pathway, which detects viral DNA and subsequently drives the induction of type I IFNs and proinflammatory cytokines (Ishikawa et al., 2009). STING also interacts with both RIG-I and MAVS in mammalian cells and is involved with sensing of RNA viruses (Zhong et al., 2008; Castanier et al., 2010). STING is found on the endoplasmic reticulum and can be closely associated to MAVS on the mitochondrial outer membrane (Zhong et al., 2008; Ishikawa et al., 2009). Acting as a scaffolding protein between TBK1 and IRF3, STING aids in IRF3 phosphorylation and type I IFN induction (Zhong et al., 2008; Tanaka et al., 2012). IAV interferes with STING through its hemagglutinin fusion peptide, effectively preventing STING dimerization and interactions with TBK1 (Holm et al., 2016). In addition, independently of RIG-I or TLR detection, STING also detects RNA viral membrane fusion events and potentiates the IFN response during viral infection (Holm et al., 2012).

Duck STING (DuSTING) shares 43 and 71% identity to human and chicken STING (chSTING), respectively (Cheng et al., 2019a). DuSTING is most highly expressed in the glandular stomach, followed by the trachea, lung, small intestine, spleen, kidney, and bursa (Cheng et al., 2019a). ChSTING is most highly expressed in the thymus, bursa, spleen, lung, and intestine of uninfected chickens (Ran et al., 2018). As chSTING was not analyzed in the glandular stomach or trachea, it is not possible compare expression to ducks. However, it is noteworthy that in ducks, STING is more abundant in the lung than the bursa and spleen. If duSTING is orthologous to mammalian STING, it may react to IAV fusion quicker in these tissues although it is not known if duSTING can detect viral fusion. Human STING shows low tissue specific expression, but has slightly higher mRNA expression in tonsils, lymph nodes, and lung (Uhlen et al., 2015).

Human STING increases IFN-β signaling when overexpressed in 293 T cells (Ishikawa et

al., 2009). MEF cells were shown to require STING but not cGAS to produce IFN after infection with two RNA viruses, NDV and SeV. Similarly, duSTING drastically increased IFN-β promoter activation when overexpressed in DEF cells. However, when the cells were stimulated with poly (I:C), STING was not required to potentiate the IFN response (Holm et al., 2016). DuSTING is highly upregulated in both spleen and lung in ducks infected with a LPAI H9N2. DuSTING was most highly upregulated on day 2 in both these tissues. In lungs, duSTING was only upregulated on day 2, with day 1 showing no significant increase when compared to mock infected birds (Cheng et al., 2019a). This may be because a LPAI strain of virus was used. It would be interesting to look at STING regulation in these tissues during HPAI infection.

1.3 Toll-Like Receptor Pathway

TLRs are important pattern recognition receptors that induce innate immune responses to viral, bacterial, fungal and parasitic pathogens (Kawai et al., 2010). Humans have 10 TLRs (TLR1-10) as do birds, however the TLRs that have been classified in birds are different, as reviewed by several groups (Boyd et al., 2007; Temperley et al., 2008; Brownlie et al., 2011; Chen et al., 2013; Keestra et al., 2013). For example, TLR1 in birds has been duplicated so that birds express TLR1a and TLR1b. Similarly, TLR2 has two paralogous genes, tlr2a and tlr2b. Other homologous TLRs expressed by birds include TLR3, TLR4, TLR5, and TLR7, which leaves TLR8, TLR9, and TLR10 currently unaccounted for in avian species. Birds also have two TLRs which are not found in mammals but have been classified in lower vertebrates: TLR15 and TLR21. TLR15 is upregulated in response to bacterial pathogens in chickens (Nerren et al., 2010), and recognizes a yeast-derived agonist (Boyd et al., 2012) and diacylated lipopeptide from mycoplasma (Oven et al., 2013). TLR21 functions analogously to TLR9 in humans in that it recognizes CpG oligodeoxynucleotides (CpG ODN) in both duck (Cheng D. et al., 2019) and chicken (Brownlie et al., 2009).

TLRs can be expressed both extra and intracellularly, with the cell surface TLRs being more adept at detecting extracellular pathogens (TLR1, 2, 4, 5, and 6) (Hopkins et al, 2005). Likewise, TLRs that are in endosomes, or in other intracellular compartments, are more specialized in detecting intracellular pathogens, such as viruses (TLR3, 7, 8, and 9). Specific TLRs, such as TLR3, TLR7, and TLR8 recognize viral RNA and play important roles in the defense against IAV in mammals (Alexopoulou et al., 2001).

PAMPs are detected through the TLR ectodomain with leucine rich repeats (LRR) and signal downstream to produce IFNs and other cytokines through their cytoplasmic Toll/IL-1 receptor (TIR) domain (Botos et al., 2011). TLRs are activated and different adaptor proteins are recruited to amplify the signal. TIR-domain-containing adapter-inducing interferon-β (TRIF) dependent pathways induce type I IFN production through TBK1 and IRF3 activation (Sato et al., 2003; Yamamoto et al., 2003). Myeloid differentiation primary response 88 (MyD88) dependent pathways induce NF-κB proinflammatory gene expression through recruitment of TRAF6 and eventual activation of the IKK signaling complex (Hemmi et al., 2002; Muroi et al., 2008).

Induction of TLR signaling increases IFN production and cytokine signaling in both mammalian and avian cells. As such, treatment of cells with TLR specific ligands such as poly (I:C), lipopolysaccharide (LPS) and CpG ODN can reduce IAV replication in both mammals (Cluff et al., 2005; Shinya et al., 2011) and chickens (St. Paul et al., 2012; Barjesteh et al., 2014). TLRs can also act synergistically to produce proinflammatory responses. In chicken monocytes, stimulating with the TLR3 ligand poly (I:C) resulted in an increase in mRNA of type I IFNS (He et al., 2012). Co-stimulation of these chicken monocytes with the TLR21 ligand CpG-ODN and poly (I:C) resulted in an even greater increase of proinflammatory cytokines than cells stimulated with a single ligand and biased the cells to a Th1 type response. Since only TLR3 and 7 directly detect IAV during infection in birds, we will focus on these TLRs in the next two sections.

1.3.1 TLR3

TLR3 is an endosomal TLR that recognizes dsRNA or replicating viral intermediates and activates NF-κB signaling in a TRIF dependent signaling pathway (Alexopoulou et al., 2001) (Figure 1.1). In humans, TLR3 is predominantly expressed in the placenta, followed by smaller but still significant amounts in the small intestine and lower amounts in most other tissues (Uhlen et al., 2015). It is also constitutively expressed in bronchial and alveolar epithelial cells (Guillot et al., 2005). Infection of the human cell line A549 (alveolar epithelial cell line) with IAV resulted in an upregulation of TLR3 (Wu et al., 2015). TLR3 stimulation during influenza infection resulted in activation of IRF3 and increased type III IFN production. When TLR3 knockout mice were infected with influenza they had a surprising survival advantage over wildtype mice, despite having higher viral titres in their lungs (Le Goffic et al., 2006), highlighting the complex role of this PRR in influenza restriction.

Tissue expression of TLR3 differs between ducks and chickens (Figure 1.2B). In uninfected tissues, Pekin duck TLR3 is expressed highest in the trachea with lower expression seen in the digestive tissues and the lung (Zhang M. et al., 2015). Muscovy ducks, which are more susceptible to influenza virus infection than Pekin or mallard ducks (Pantin-Jackwood et al., 2013) show higher expression of TLR3 in the trachea, spleen, pancreas, lung, and digestive tissues (Jiao et al., 2012). Thus, Muscovy ducks show high basal expression of TLR3 in many tissues, while Pekin ducks had high expression only in trachea. In chickens, basal TLR3 expression is highest in intestine, liver, and kidney (Iqbal et al., 2005). TLR3 was constitutively expressed in chicken heterophils (Kogut et al., 2005).

After infection with HPAI virus, Muscovy duck TLR3 was upregulated at 24 HPI in the lung and brain, with sustained expression in the brain (even though this is a non-fatal infection in Muscovy ducks) (Jiao et al., 2012). There was no increased expression in the spleen. In contrast, transcriptomic data from Shaoxin mallard ducks infected with a HPAI H5N1 show increased TLR3 expression in the lungs, peaking on day 2 of infection (Huang et al., 2013). This discrepancy between the Muscovy duck and Shaoxin mallard TLR3 expression data may be due to the strains of virus used in the infection (DK212 vs. DK49; both H5N1) but not age of the birds as both experiments used 4-week old ducks. Chickens upregulated TLR3 in the lung during HPAI H5N1 infection when replicating virus was still present in lung tissues (Ranaware et al., 2016). In reovirus infected ducks, TLR3 expression peaked at 72 HPI in the lung, while spleen and bursa showed a sustained response from 24 to 48 h (Zhang M. et al., 2015). These results are of interest as Reovirus infection in Muscovy duck can cause mortality in 20–40% of infected animals (Malkinson et al., 1981; Wozniakowski et al., 2014).

1.3.2 TRIF

TRIF is the adaptor molecule downstream of TLR3 and TLR4 and provides a signaling platform to recruit other adaptor proteins and increase type I IFNs and proinflammatory cytokine expression (Figure 1.1). Similar to humans (Yamamoto et al., 2003), in uninfected tissues, ducks express TRIF most highly in the pancreas and spleen (Wei X. et al., 2016) (Figure 1.2B). Chicken TRIF expression was found to be highest in the cecum, heart, liver, spleen, and kidney (Wheaton et al., 2007). Expression of duck TRIF peaks at 12 h after treatment with poly (I:C), however, it peaks much later at 36 h post infection with IAV (Wei X. et al., 2016), likely due to

viral suppression of IFN signaling pathways in infected cells.

1.3.3 TLR7

Human TLR7 produces a robust type I IFN response upon detection of IAV or other ssRNA viruses using the MyD88-dependent pathway (Diebold et al., 2004; Lund et al., 2004). TLR7 is highly expressed by murine plasmacytoid dendritic cells (pDCs) and is located in endosomal compartments where it can detect incoming viral RNA (Diebold et al., 2004), and produce high levels of IFN-α. RNA from live and inactivated influenza virus can be detected by TLR7 in the endosome of pDCs, provided the hemagglutinin remains intact for receptor-mediated viral entry (Diebold et al., 2004) TLR7 detection is thus known to induce IFN-α, and proinflammatory cytokines (Figure 1.1). Suggesting that the role of TLR7 and RIG-I signaling is complicated in influenza infection, Tlr7−/−Mavs−/− knockout mice succumb quickly to a lethal influenza infection as expected, however infection with a low viral dose revealed that proinflammatory signaling promoted viral replication by recruiting susceptible monocytes (Pang et al., 2013). Oddly, humans have enhanced tissue expression of TLR7 in the brain, with lower expression in mucosal tissues (Uhlen et al., 2015).

Tissue expression of TLR7 is notably different between healthy ducks and chickens (Figure 1.2B). Duck TLR7 is expressed the highest in spleen, bursa, and lung (MacDonald et al., 2008; Kannaki et al., 2018). In chickens, basal TLR7 expression is highest in spleen, bursa, and intestine with very little expression in the lung (Iqbal et al., 2005; Philbin et al., 2005), initially suggesting that this distribution may play a role in chicken susceptibility to HPAI strains that replicate in the lungs. However, the chicken macrophage cell line HD11 expresses high levels of TLR7 (Philbin et al., 2005), and both primary macrophages and heterophils constitutively express TLR7 in other studies (Kogut et al., 2005). The chicken atlas on the BioGPS server agrees with the previous studies in that TLR7 expression is limited in the lung, and higher in tissues such as the spleen, bursa, and immune cells (Bush et al., 2018). It is however worth noting that TLR7 basal expression in chickens is slightly variable depending on the breed and age of chicken sampled. Stimulation using TLR7 agonists decreased viral replication in chicken macrophages (Stewart et al., 2012; Barjesteh et al., 2014; Abdul-Cader et al., 2018), indicating TLR7 can induce IFNs in those cell types. Thus, chicken strains may vary with respect to TLR7 expression. Ducks infected with HPAI upregulate TLR7 most highly in their lungs 2 DPI while chickens infected with the same virus had only a slight increase in expression at 1 DPI

(Cornelissen et al., 2013). In contrast, ducks infected with a LPAI H7N9 had only marginal upregulation of TLR7 In their lungs 0.8 DPI, while chickens had a significant increase in this expression 0.8 DPI (Cornelissen et al., 2012).

1.3.4 MyD88

MyD88 conveys the signal downstream of most of the TLRs, to induce an inflammatory response upon detection of pathogens (Figure 1.1). MyD88 signaling was found to be important for protecting mice during primary influenza infection, as MyD88−/− knockout mice were more susceptible (Seo et al., 2010). MyD88 may also be an important factor in initiating damaging cytokine storms in the host, since there was a significant reduction in proinflammatory cytokines and activated macrophages and neutrophils in the lungs of MyD88−/− mice, but not TRIF−/− mice, following IAV infection (Teijaro et al., 2014). Ducks have two isoforms of the myd88 gene that have been characterized, named DuMyD88-X1 and DuMyD88-X2 (Cheng et al., 2015b). DuMyD88-X2 is a truncated version that encodes a premature stop codon and produces a protein with an interruption in the TIR signaling domain. DuMyD88-X1 is highly expressed in uninfected ducks in all immune relevant tissues including the lung, intestine, and bursa, but it showed the strongest expression in the spleen (Figure 1.2B). DuMyD88-X2 was expressed in these same tissues but to a much lower extent than the X1 isoform. Both isoforms of MyD88 could activate the IL-6 promoter and induce NF-κB activity in duck cells. In ducks challenged with NDV, the X1 isoform was upregulated in liver and spleen. Neither isoform was as highly expressed in the lung during NDV infection, and no studies have looked at the expression of these genes during influenza infection. Three isoforms of MyD88 have been found in chickens (named MyD88-1, 2, and 3) (Qiu et al., 2008). Chicken MyD88 (chMyD88) is the largest of the isoforms, and is ubiquitously expressed, which agrees with previous research on chMyD88 expression although it is of note that these studies demonstrated slightly more chMyD88 expression in the thymus, liver, and spleen than in other tissues tested (Wheaton et al., 2007). ChMyD88 is not significantly upregulated in DF-1 cells infected with influenza (Barber et al., 2013). Upregulation in influenza-infected chicken tissues has not been explored, but MyD88 is upregulated by LPS treatment (Wheaton et al., 2007). As MyD88 plays a role in immune system derived damage during influenza infection in mammals, it would be interesting to know if chMyD88 activation is significantly different from the duck.

1.4 NLR Receptors—the NLRP3 Inflammasome

1.4.1 NLRP3

The NOD-like receptor family pyrin domain containing 3 (NLRP3) can form multi protein complex inflammasomes, which possess autocatalytic activity. This activity can activate caspase-1 and induce the production of proinflammatory cytokines IL-1β and IL-18 (Figure 1.1). NLRP3 inflammasome induction can occur in immune cells such as macrophages (Pirhonen et al., 2001) and dendritic cells (Fernandez et al., 2016) and as well in other cell types such as fibroblasts and epithelial cells (Allen et al., 2009; Pothlichet et al., 2013). Deletion of NLRP3 in mice causes a decrease in immune cell recruitment to the site of infection and poor outcomes when infected with influenza (Allen et al., 2009; Thomas et al., 2009).

Tissue expression of NLRP3 differs between ducks and chickens (Figure 1.2C). NLRP3 is fairly ubiquitously expressed in healthy chicken tissues but most highly expressed in chicken trachea and lung (Ye et al., 2015). Duck NLRP3, however, is most highly expressed in the pancreas with very low expression in the lung and slightly higher expression in the trachea (Li et al., 2018). This expression profile is of interest as NLRP3 inflammasome activation has been associated with contributing to cytokine storms and severe pathology from influenza infection (Teijaro et al., 2014). We are unaware of studies detailing the NLRP3 inflammasome response to influenza infection in either chicken or duck.

1.4.2 IRF1

IRF1 is known to be an activator of IFNs though several mechanisms, but one of importance is its regulation of the NLRP3 inflammasome (Kuriakose et al., 2018) (Figure 1.1). It is thought that by regulating the NLRP3 inflammasome, IRF1 contributes to apoptosis and necroptosis during influenza infection. Kuchipudi et al. (2012) suggest that duck cells are more likely to become apoptotic when infected with IAV than chicken cells. Indeed, DEF cells infected with HPAI strains that are known to cause severe symptoms in infected ducks had decreased apoptosis (Kuchipudi et al., 2012). Thus, IRF1 as a regulator of early apoptotic response is an interesting candidate to study in ducks. Human IRF1 is expressed highest in the spleen and the liver (Uhlen et al., 2015). Duck IRF1 (duIRF1) is most highly expressed in liver and spleen, followed by the pancreas, and digestive tissues such as the stomach and duodenum. Interestingly, it is expressed in very low levels in the lung and trachea (Qian et al., 2018) (Figure 1.2C). The chicken atlas on the BioGPS server indicates that chicken IRF1 (chIRF1) expression

in healthy adult birds is highest in the lung, spleen, and thymus (Bush et al., 2018).

Overexpression of chIRF1 in DF-1 cells caused a significant increase of IFN-β, Mx, and MDA5 mRNA (Liu Y. et al., 2018). chIRF1 mRNA also substantially increased 12 HPI after infection with either IAV or NDV. These transcripts rapidly dropped back down to basal levels after 12 h. Poly (I:C) stimulation of duck fibroblasts resulted in duIRF1 transcripts peaking at 12 HPI and then decreasing, as in chicken cells. However, when these cells were infected with H5N1 the duIRF1 mRNA began to increase at 12 HPI and continued to increase until 48 HPI. The delay in the duck response may be due to strain differences between viruses used (Qian et al., 2018) as the chIRF1 study used A/Chicken/Shanghai/010/2008 (H9N2) while the duIRF1 study used A/Duck/Hubei/hangmei01/2006 (H5N1). DuIRF1 interacts with MyD88 to increase IFN-β independently of IRF7, and overexpression of duIRF1 not only upregulated Type I IFNs but also Type III IFN (IFN-λ) (Qian et al., 2018). When ducks were infected with H6N2, duIRF1 transcripts peaked at 36 HPI, rather late in infection compared to other ISGs or IFNs mentioned in this article. As duIRF1 does not signal downstream of RIG-I, it could be used as a secondary pathway to limit viral replication. Overexpression of duIRF1 also limited H9N6 and H5N1 viral replication.

1.5 Interferon Responses and ISGS

1.5.1 Type I IFNs

Type I interferons include IFN-α and IFN-β, both which are present in birds (Santhakumar et al., 2017). Airway epithelium, macrophages, and pDC are responsible for most of the type I IFNs produced during viral infection (Onoguchi et al., 2007; Khaitov et al., 2009; Crotta et al., 2013). Plasmacytoid dendritic cells are known to produce much of the initial IFN-α (Ito et al., 2005; Liu, 2005), and it is thought that the autocrine action of IFN-α on the pDCs upregulates antiviral factors such as Mx1 and thus protects against influenza infection (Cella et al., 1999). An early IFN response generally provides more positive outcomes in infection, and studies have also implicated type I IFN responses as a factor that can reduce pro-inflammatory cytokine release and thus limit damage (Billiau, 2006; Guarda et al., 2011; Arimori et al., 2013).

Both transcriptomic and qPCR studies have demonstrated that ducks have a robust but short response of type I IFNs in response to HPAI (Cagle et al., 2011; Vanderven et al., 2012;

Saito et al., 2018). Transcriptomic data demonstrated that lungs of ducks infected with a HPAI H5N1 strain had an increase in IFNA expression days 1 and 2 DPI (Huang et al., 2013). While IFNs are most strongly upregulated within the first 24 h, it should be noted that many ISGs have a sustained response for up to 3 DPI (Huang et al., 2013; Smith et al., 2015). Ducks infected with HPAI H5N1 strains A/goose/Guangdong/16568/2016 (GS16568), and A/duck/Guangdong/16873/2016 (DK16873) showed sustained responses of type I IFNs post infection. However, the time points used in these experiments were 12 HPI and 2 DPI (Wu et al., 2019). While these highly pathogenic strains of flu could be eliciting sustained responses, other strains of H5N1 had the peak of IFN upregulation at 1 DPI (Saito et al., 2018). LPAI induces a relatively weak IFN response in ileum of infected ducks (Vanderven et al., 2012).

In ducks infected with HPAI H5N1 strains VN1203 and D4AT, we found that IFN-α and IFN-β were most upregulated 1 DPI in lungs and spleens of infected birds (Saito et al., 2018). The spleen had a greater increase in IFN-α transcripts compared to the lung, while lung showed higher upregulation of IFN-β. This may reflect the relative contribution of different PRRs in these tissues; while TLRs are largely responsible for IFN-α, IFN-β expression is largely RIG-I dependent (Opitz et al., 2007). By day 2 the IFN response had been reduced to mock infection levels. When testing the expression of IFN-α in primary avian cells infected with either H5N1 or H5N9, it was highest in duck cells at 12 and 24 HPI (Jiang et al., 2011). In chicken and turkey cells, IFN-α was most highly expressed at 24 HPI.

Pre-treatment with IFN-α protects duck cells, but not adult ducks from IAV infection. DEF cells treated with IFN-α show a reduced viral load as well as induction of many ISGs (Gao et al., 2018b). Interestingly, pre-treatment of primary chicken lung cells and duck fibroblasts with IFN-α before infection with IAV reduced IFN-α production in both these cell types (Jiang et al., 2011). The protective effects of IFN-α seem to be age dependent in the duck. When looking at survival rates of 2 days vs. 3 weeks old ducklings treated with rIFN-α before infection of HPAI H5N1, the treatment with IFN benefited the 2 days old ducklings but not the 3 weeks old ducks (Gao et al., 2018b). The rIFN-α dose may have been insufficient to protect the older ducks, or alternatively IFN-α is not protective. In contrast, 7 and 33-day old chickens treated with rIFN-α before exposure to a chicken-isolate H9N3 were both found to be protected (Meng et al., 2011). These results are of interest, as generally younger ducks are more susceptible to IAV infection, and protection correlates with onset of RIG-I expression (Londt et al., 2010; Pantin-Jackwood et

al., 2012). The DK383 H5N1 virus used, which is lethal in ducks (Gao et al., 2018b), may impair RIG-I signaling, and IFN-α alone is not sufficient to protect the older ducks. Similarly, IFNB knockout mice are much more sensitive to influenza, suggesting IFN-α cannot fully compensate (Koerner et al., 2007). These results seem to support the hypothesis that an early and quick response is more beneficial to the duck than a sustained type I IFN response.

1.5.2 Type II IFNs

IFN-γ is classified as a type II IFN and is secreted by NK cells, CD8+ lymphocytes and CD4+ T helper cells (Schroder et al., 2004). While IFN-γ has been found in some studies to be protective against influenza (Weiss et al., 2010), other researchers have shown that by knocking out the genes or knocking down gene expression in mice, absence of IFN-γ protected the mice from severe infection with pandemic H1N1 (Califano et al., 2018). Similarly, other studies in mice have shown that IFN-γ negatively regulates the survival of CD8+ T cells during influenza infection and limits the number of influenza specific memory cells available during an infection (Prabhu et al., 2013).

CEFs treated with IFN-γ were more resistant to infection by H9N2 avian influenza virus and H1N1 human influenza virus. Stimulation with IFN-γ also increased IFN-α/β, and Mx transcripts in these cells (Yuk et al., 2016). Likewise, DEF cells treated with recombinant duck IFN-γ showed significant decreases in viral replication with a HPAI H5N1. Two-day old ducks were pre-treated with IFN-γ before being infected with DK383 IAV serotype H5N1. In these experiments 6/10 ducks that were pre-treated survived the infection at 10 DPI, while in PBS treated controls only 2/10 ducks survived (Gao et al., 2018a). As age played a factor in IFN-α pre-treatment reducing viral load in ducks, it would be worthwhile to repeat these experiments in older ducks. To our knowledge no studies have investigated whether duck IFN-γ influences the development of memory T cells during IAV infection.

1.5.3 Type III IFNs

Type III IFNs induce an antiviral state like that of type I IFNs but use different receptors for detection. Additionally, type III IFN receptors are expressed predominantly in airway epithelial cells and intestinal epithelia (Sommereyns et al., 2008), unlike type I IFN receptors, which are more ubiquitously expressed. Ducks and chickens express one kind of type III IFN (IFN-λ) (Karpala et al., 2008; Yao et al., 2014; Santhakumar et al., 2017) whereas other vertebrates produce one to four different type III IFNs, depending on the species (Kotenko et al.,

2003; Chen et al., 2016).

Primary CEF and DEF cells both produce IFN-λ (chIFN-λ and duIFN-λ, respectively) in response to both poly (I:C) stimulation and infection with a mouse-adapted strain of H1N1 (Zhang Z. et al., 2015). Interestingly, DEF cells produce less IFN-λ transcripts when stimulated with poly (I:C) or infected with H1N1 than CEF cells. These same DEF cells also highly upregulate IFN-λ receptor transcripts at 36 HPI whereas the CEF cells highly express the receptor transcripts at 8 HPI and continue to do so until 36 HPI. A separate study found that chIFN-λ was unable to induce an antiviral state in the chicken fibroblast DF-1 cell line when infected with a HPAI H5N1, indeed the cells were not able to respond to recombinant chIFN-λ until they were transfected with the receptor (Reuter et al., 2014). This discrepancy may be due to the use of primary cells in one study and an immortalized cell line in the other. Immortalized cells often drastically change genotype and so the DF-1 cells may have stopped expressing the chIFN-λ receptor. High levels of the chIFN-λ receptor transcripts were found in the lung, trachea and intestine (Zhang Z. et al., 2015), suggesting that like chIFN-λ receptor expression is like that of humans. There is currently very little research on duIFN-λ, its receptor or antiviral activity, making this a promising candidate for future studies into IAV resistance in the duck.

1.6 Other Antiviral proteins of Interest

1.6.1 TRIM proteins

TRIM proteins are a large family of intracellular proteins with diverse functions such as cell cycle regulation, autophagy, proteasomal degradation, development, and immunity which have been comprehensively reviewed (van Gent et al., 2018). Most interestingly, some of these proteins allow species-specific protection from viruses through viral restriction. One of the first TRIM proteins discovered, the alpha isoform of TRIM5 (TRIM5α) was found to restrict HIV in non-human primates, while the human ortholog was unsuccessful in restricting this virus (Stremlau et al., 2004; Sawyer et al., 2005). This highlights the evolutionary relationship these proteins have with pathogens and suggests that members of this protein family might be providing their host species a significant advantage.

A study from 2008 listed 38 TRIM genes in chicken, compared to human, rat, mouse, dog, and cow on their TRIMgene online database (Sardiello et al., 2008). Very few studies have been done on avian TRIM proteins. Avian TRIM25 has a specific role in the activation of RIG-I as discussed above in section TRIM25 (Rajsbaum et al., 2012; Miranzo-Navarro et al., 2014). A

family of related TRIM genes was discovered in the avian MHC-B locus in both chicken (Ruby et al., 2005; Shiina et al., 2007) and duck (Blaine et al., 2015), with the MHC location suggesting this gene expansion may have arisen from pathogen pressures. The set of TRIM proteins in the MHC-B locus of birds all contain the B30.2/PRYSPRY C-terminal domain motif. Proteins containing this domain have recently expanded in TRIM protein evolution (Sardiello et al., 2008). The PRYSPRY domain is thought to be able to recognize specific amino acid sequences rather than peptide motifs, giving it pathogen specific activity (James et al., 2007; D'Cruz et al., 2013). Ducks also have an expanded butyrophilin gene family, proteins which also contain a B30.2/PRYSPRY domain (Huang et al., 2013).

Of the expanded TRIM genes in the duck MHC, TRIM27.1, and TRIM27-L were found to have antagonistic functions in the MAVS signaling pathway (Blaine et al., 2015). TRIM27-L significantly increased IFN-β signaling in a dose dependent manner while TRIM27.1 slightly decreased this same signaling in DF-1 cells. When co-expressed TRIM27-L activity overrode the inhibition of TRIM27.1. Curiously TRIM27-L appears to have been lost in Galliformes while being retained in Anseriformes, other birds and reptiles. As the Galliformes have also lost RIG-I it seems that either TRIM27-L expression was detrimental and thus lost in evolution or provided no benefit. Further, TRIM27.1 expression is higher in infected tissues than TRIM27-L. As the decrease in IFN-β was only slight, it could be that TRIM27.1 is playing another role in infection. TRIM27.1 may be upregulated to inhibit influenza without influencing cytokine signaling, as TRIM32 does in some human cell types (Fu et al., 2015). Of the chicken MHC-B TRIM genes, only TRIM39 has been cloned and tissue expression analyzed, but no function has been determined (Pan et al., 2011).

TRIM23 was identified as a differentially expressed gene in a microarray study from ducks infected with both HPAI and LPAI strains of IAV, as upregulated 5 DPI in LPAI but not HPAI infections (Kumar et al., 2017). TRIM23 is an ancient TRIM with well-conserved structural homology, and uses its ADP-ribosylation factor (ARF) domain to activate TBK1 through GTPase activity (Sparrer et al., 2017). TBK1 then activates selective autophagy, controlling viral replication. This is an interesting observation as LPAI virus can replicate in ducks for many days past initial infection, and the upregulation of TRIM23 suggests it is worth investigating whether it affects viral replication.

Finally, TRIM62 was identified as a retroviral restricting protein in chicken cells (Li et al.,

2019), and until recently TRIM62 was only known to function in innate immune signaling augmentation in fish (Yang et al., 2016). It is not known to be antiviral in mammals. TRIM62 can restrict retroviruses in chickens, but no investigation of anti-IAV potential of this protein has been done in chickens or ducks.

1.6.2 avIFIT

Interferon-induced proteins with tetratricopeptide repeats (IFITs) are a family of proteins which have diverse functions in the cell such as mediating apoptosis, sequestering viral proteins and cell cycle regulation and have been extensively reviewed (Diamond et al., 2013; Fensterl et al., 2015). IFITs have undergone duplication in mammals, fish and frogs, while ducks and chickens only have a single IFIT gene (avIFIT) (Zhou et al., 2013). Evolutionary analysis of duck avIFIT found that it most closely resembled mammalian IFIT5 (Wang et al., 2015; Rong et al., 2018a). Human IFIT5 is effective in restricting RNA virus replication by both interacting with immune signaling components (i.e., RIG-I and MAVS) (Zhang et al., 2013) and by binding 5′-ppp viral RNA (Abbas et al., 2013). In chickens, avIFIT (called IFIT5 by the authors) inhibits viral replication by interacting with 5′-triphosphate viral RNA and blocking subsequent replication steps (Santhakumar et al., 2018), similar to the mechanism of IFIT1 and IFIT5 in mammals (Abbas et al., 2013; Habjan et al., 2013).

Duck avIFIT is constitutively expressed in all tissues at basal levels but shows highest expression in digestive tissues such as intestine and stomach, although the expression levels in these tissues is still relatively low (Wang et al., 2015). To date we are unaware of any data on basal expression levels of avIFIT in the chicken. IFIT5 has low tissue specific expression in humans (Uhlen et al., 2015). Despite the slight differences in expression between humans and ducks, IFIT5/avIFIT is highly upregulated in both these species when induced by IFNs. Similarly, studies have demonstrated that avIFIT is upregulated during influenza infection in chicken intestinal epithelial cells when infected with LPAI (Kaiser et al., 2016) as well as in lungs of chickens infected with HPAI H5N1 (Ranaware et al., 2016).

When both human and chicken IFIT5 were overexpressed in chicken cells, they were found to inhibit viral replication and likewise, when chicken IFIT5 was knocked out from these cells, they were much more susceptible to infection (Santhakumar et al., 2018). Chicken avIFIT is found near the mitochondria in chicken cells, and as human IFIT5 interacts with both RIG-I and MAVS in infected cells, it would be worthwhile to investigate subcellular location of duck

avIFIT. DF-1 cells were depleted of chicken avIFIT and transfected with duck avIFIT (Rong et al., 2018a). Duck avIFIT can inhibit IAV in DF-1 cells and was shown to do so by both upregulating IFNα/β and by binding the viral nucleoprotein (NP) from an H5N1 flu strain. This antiviral activity was not limited to only influenza virus, as in these experiments duck avIFIT also restricted double-stranded RNA and DNA viruses. Interestingly, in these DF-1 cells duck avIFIT also arrested cell growth in both infected and uninfected cells.

1.6.3 Mx

Mx1 is an ISG which is highly upregulated in response to viral infection, whose function and regulation as been recently reviewed (Haller et al., 2015). It acts as an antiviral effector and belongs to a large family of GTPases. Both humans and mice have two Mx genes while birds have one. Mx was found to be protective in laboratory mice, as many lab strains were found to have isoforms of Mx1 with exon deletions that left these mice more susceptible to influenza infection than mice with intact Mx1 (Lindenmann, 1962; Horisberger et al., 1983; Staeheli et al., 1988).

Mx is upregulated strongly in brain, lung and spleen of ducks that show a strong IFN response to infection (Smith et al., 2015; Saito et al., 2018). Mx alleles are highly variable in ducks (Dillon et al., 2010), however only a few of them have been experimentally analyzed for antiviral function. When transfected into mouse or chicken cells, duck Mx was not able to restrict IAV replication (Bazzigher et al., 1993). Chicken Mx weakly inhibits influenza, and that ability is dependent on the breed of chicken that the Mx was cloned from (Ko et al., 2002; Fulton et al., 2014), indicating high diversity in avian Mx. Chicken Mx also appears to be missing the GTPase activity of mammalian Mx proteins, suggesting this may be why antiviral activity has been weak at best in previous studies (Schusser et al., 2011). While more research on allelic variants and their potential to restrict IAV should be done, it is also possible that due to the close evolutionary relationship between IAV and ducks, the virus has evolved the ability to evade avian Mx during infection.

1.6.4 OASL

Interferon-inducible 2′-5′-oligoadenylate synthase (OAS) and OAS-like protein (OASL) are two related ISGs in humans, which are known to restrict influenza. OAS senses and degrades dsRNA through synthesis of oligoadenylates, which in turn switches on RNase L activity (Sarkar et al., 1999a,b; Justesen et al., 2000; Silverman et al., 2014). RNase-L then degrades all mRNA

in the cell (including ribosomal RNA), thus blocking viral replication. OASL inhibits viral replication independently of enzymatic activity by stabilizing the interaction of RIG-I and MAVS in a similar manner to that of ubiquitinylation by TRIM25. OASL has C-terminal ubiquitin-like domains that stabilize RIG-I CARD oligomers, thus potentiating downstream IFN signaling (Zhu et al., 2014; Ibsen et al., 2015). Birds do not appear to have OAS, but have OASL (Sokawa et al., 1984; Tag-El-Din-Hassan et al., 2018). Unlike human OASL, duck OASL has oligoadenylate synthetase activity, as well as the ability to restrict viral RNA in an RNase L independent manner (Rong et al., 2018b). It appears duck OASL functions as both human OAS and OASL, as it can activate both RNase L and RIG-I pathways. Chicken OASL has been found to inhibit WNV in mammalian cells (Tag-El-Din-Hassan et al., 2012). Chicken OASL is highly upregulated in tracheal epithelial cells 24 HPI (Jang et al., 2015). Both ostrich and duck OASL transfected into chicken DF-1 cells could control replication of both HPAI and LPAI influenza virus (Rong et al., 2018b). When OASL was knocked out of DF-1 cells, the cells became more permissive to influenza infection. Consistent with a role in augmenting innate signaling, overexpression of either ostrich or duck OASL also significantly increased the expression of RNase L, as well as other important immune effectors such as IFNα, IFNβ, IRF1, IRF7, Mx, and PKR.

1.6.5 PKR

The double-stranded RNA (dsRNA)-dependent protein kinase (PKR) is an ISG which functions as both an antiviral effector and anti-proliferative protein during infection (Garcia et al., 2006). PKR binds foreign dsRNA in the cytoplasm and auto-phosphorylates in order to become active, at which point it then phosphorylates eukaryotic initiation factor 2 (eIF-2α) causing broad inhibition of protein translation in the cell (Galabru et al., 1987; Hovanessian, 1989). PKR has two N-terminal dsRNA-binding domains, which are both able to recognize viral RNA (Nanduri et al., 1998), and one C-terminal kinase domain.

PKR is an important antiviral effector in mice infected with IAV, as shown by the increased fatality rate of PKR knockout mice when infected with the H1N1 strain WSN (Balachandran et al., 2000). Chicken PKR has been functionally characterized and determined to be antiviral against VSV (Ko et al., 2004). Studies have shown that PKR is upregulated significantly during HPAIV H5N1 infection, even in lethal infections in the chicken where IFN production is limited (Daviet et al., 2009). The non-structural protein 1 (NS1) of IAV inhibits

IFN responses in cells through interactions with OAS and PKR (Ma et al., 2010). Indeed, NS1 from HPAI H5N1 in a HPAI H7N9 background bound and inhibited PKR in chicken embryos.

PKR is upregulated in ducks infected with both HPAI and (to a lesser extent) LPAI virus (Fleming-Canepa et al., 2019) but to date we are unaware of any studies functionally characterizing duck PKR during influenza infection. We previously thought that ducks appeared to be missing the second dsRNA-binding domain (Fleming-Canepa et al., 2019), also confirmed by another group (Liu W. J. et al., 2018). However, through transcriptomic assembly done in our lab we have since found a transcript of the full-length PKR, which contains the second dsRNA-binding domain previously thought to be missing. This find suggests that ducks may predominantly express a splice variant of PKR missing the dsRNA-binding domain, or that this splice variant is preferentially amplified during PCR. Interestingly, it has been suggested that NS1 needs to bind both the kinase domain of PKR and residues 170–230 to keep PKR in an inactive conformation and prevent it from responding to dsRNA (Li et al., 2006). These residues correspond to the second RNA binding domain and the linker region of the protein. The two variants of duck PKR may allow ducks to respond to viral RNA despite NS1 antagonism. Duck PKR needs to be functionally characterized to determine not only its antiviral potential, but also expression levels of the full-length transcript.

1.6.6 Viperin

Viperin (RSAD2) is highly induced by Type I IFN, and many RNA virus infections. Viperin inhibits IAV by perturbing lipid rafts and thus inhibiting viral budding (Wang et al., 2007). Duck viperin is most highly expressed in blood, intestine, lung, and spleen in healthy birds (Zhong et al., 2015). Chicken viperin was upregulated in both spleen and lung of IAV infected birds after 24 h (Goossens et al., 2015). It was also upregulated in chicken splenocytes as early as 6 h after poly (I:C) stimulation. In Newcastle disease (NDV) infected ducks, viperin was found to be highly upregulated after 24 h in the blood and peaked in expression in the lung and brain at 72 HPI (Zhong et al., 2015). Viperin is one of the most highly upregulated genes in duck lungs in response to H5N1 HPAI infection (Fleming-Canepa et al., 2019), however, the levels of viperin expression in chickens infected with the same strain of H5N1 was not mentioned (Smith et al., 2015). Ducks also significantly upregulated viperin in response to LPAI in the lung, but curiously not in the ileum (Fleming-Canepa et al., 2019).

1.6.7 IFITMs

Interferon-inducible transmembrane proteins (IFITMs) are upregulated upon viral infection, and have antiviral activity (Diamond et al., 2013). This viral restriction usually happens during entry in either the early or late endosomes. Human IFITM1, IFITM2, and IFITM3 have all been shown to restrict IAV in vitro (Brass et al., 2009). The naming of the avian IFITMs has been complicated by the evolutionary history of gene duplication in this region during speciation, but sites for post-translational modifications identify IFITM3 as the gene next to B4GALNT4 (Smith et al., 2013), and the duck orthologs follow the same synteny (Blyth et al., 2016).

IFITM3 restricts IAV in both duck and chicken cells. Ducks upregulated all IFITMs including IFITM1, IFITM2, and IFITM3 in both lung and ileum during infection with HPAI, whereas chickens showed minimal upregulation of IFITMs (Smith et al., 2015). When duck IFITM1, IFITM2, IFITM3, and IFITM5 were overexpressed in DF-1 cells and challenged with LPAI, only IFITM3 significantly decreased viral infection (Blyth et al., 2016). Chicken IFITM3 is also able to restrict both IAV and lyssa virus in DF-1 cells (Smith et al., 2013). As IFITM1 and IFITM2 also control IAV in humans, it may be that host-pathogen co-evolution has allowed the virus to evade these proteins in ducks. Notably, duck IFITM1 has an insertion in exon 1, which changes the sub-cellular localization of the protein (Blyth et al., 2016), or it would restrict influenza. A 2017 study found that when duck IFITM2 was transfected into DF-1 cells it could control the replication of avian Tembusu virus (Chen et al., 2017). Avian Tembusu virus is a positive sense RNA virus belonging to the Flaviviridae family (Zhang et al., 2017). As IFITM2 restricts this virus but not IAV, it is possible that either the mammalian IFITM2 developed the ability to restrict IAV later in evolution, or that the avian strains we tested have evolved to escape from IFITM2. The upregulation of IFITM2 during IAV infection is most likely due to interferon stimulation and is not virus specific.

1.7 A Note on Missing Genes and Dark DNA

Throughout this review we have spoken about genes that are presumed missing from ducks, chickens or birds in general. Because bird genomes contain many GC rich areas (Hron et al., 2015), they are notoriously hard to amplify using PCR based methods. As such, genes may be presumed missing in next generation sequencing applications, as well as with exploratory PCR based methods. This leaves many genes thought to not exist in birds, simply undiscovered.

Such was the case with tumor necrosis factor alpha (TNFα), which for years was thought to not exist in chickens. It was recently cloned and characterized from chickens and found to have very low homology to mammalian orthologs, as well as have a high GC content (Rohde et al., 2018). We have also updated the full-length sequence of PKR that was formerly thought to be missing specific domains. One method to help with finding undiscovered genes from next gen sequencing data is using more advanced de novo assembly methods on combined RNA-seq data. With advances in NGS technology and new software development to analyze fragmented GC rich RNA-seq data, we will be able to better mine transcriptomes for genes as well as gain insight into avian immune system evolution. Furthermore, a wealth of genome information from many avian species is becoming available.

1.8 Conclusions

In this review, we summarize recent advances in understanding PRR in ducks, comparing them to chicken PRR, and analyzing their downstream signaling adaptors. We also investigated tissue expression of these innate immune components to try to gain insight into where these proteins were most expressed. Higher tissue expression of PRRs and their effectors may allow ducks to respond more quickly to IAV in a tissue-specific manner. A rapid and robust response that is quickly dampened could allow ducks to limit damage from inflammatory sequelae. Duck RIG-I and MDA5 are most highly expressed in the trachea, lung and intestines, areas of both HPAI and LPAI influenza replication (Figure 1.2A). However, the downstream adaptor molecules TBK1, TRAF3 and IRF7 are mostly expressed in digestive tissues with very little basal expression in the lung. This contrasts with chickens, which have high expression of these proteins in the lung. This pattern may circumvent out-of-control inflammatory reactions to HPAI and be protective to the duck, but further investigation is needed to confirm this. Duck TLR3 is most highly expressed in the trachea, while duck TLR7 is highest in the lung, fitting a similar pattern to the RLRs (Figure 1.2B). There appears to be a similar pattern of low lung expression of the adaptor of TLR3, TRIF, but there is no data on duck tracheal expression of TRIF to confirm this. Likewise, chicken TRIF basal expression has not yet been looked at in respiratory tissues. NLRP3 has high relative expression in chicken lungs, whereas ducks have higher basal expression in their hearts (Figure 1.2C). It is interesting to note that ducks seem to express PRRs at a high basal level in areas where influenza replicates, but the adaptor molecules are much less expressed in lung and respiratory tissues, the areas of HPAI replication. As more tissues are

investigated, transcriptome mining for expression levels of these PRRs and adaptors, where missing, may help a complete picture to emerge.

Throughout this review we summarize the function and regulation of PRRs in chickens, ducks, and humans during IAV infection. While the differences in the RLR pathway are well-studied in ducks, there are currently few studies on TLR and NLR and their adaptor molecules in ducks during IAV infection. As these pathways converge and co-regulate each other, this is a very important piece of the story that is missing. Likewise, many proteins mentioned in this paper have been studied at the regulation level, but very few have been functionally and biochemically characterized.

We acknowledge that much of the work is yet to be done characterizing adaptor proteins in IFN and pro-inflammatory cytokine signaling networks. Investigation of these regulatory proteins in ducks and other birds, will allow us to see the conserved mechanisms, and find those that are not. Further, we acknowledge the bias that most immunological research looks at positive regulators of innate signaling. However, as ducks are equally adept at initiating and shutting down inflammatory responses, we should also begin to investigate inhibitory proteins and their expression and function. It should also be noted that functional studies in innate immunity in both ducks and chickens are limited. As such, data on PRR tissue expression and upregulation is often limited to small sample groups. Tissue expression can vary with age and breed of animals, and all studies discussed here used domestic breeds of ducks. When looking at tissue expression of genes as a potential route of resistance, it may be beneficial to also look at gene expression in wild mallards, which are constantly adapting and evolving with IAV. Indeed, it would be worthwhile understanding the allelic diversity of PRR genes and variation in function across many species of wild ducks. This may give us more insight into detection and resistance to IAV in its natural host and reservoir, the mallard duck.

1.9 References

Abbas, Y.M., Pichlmair, A., Gorna, M.W., Superti-Furga, G., and Nagar, B. (2013). Structural basis for viral 5'-PPP-RNA recognition by human IFIT proteins. *Nature* 494(7435)**,** 60-64. doi: 10.1038/nature11783.

Abdul-Cader, M.S., De Silva Senapathi, U., Nagy, E., Sharif, S., and Abdul-Careem, M.F. (2018). Antiviral response elicited against avian influenza virus infection following activation of toll-like receptor (TLR)7 signaling pathway is attributable to interleukin (IL)-1β production. *BMC Research Notes* 11(1)**,** 859. doi: 10.1186/s13104-018-3975-4.

Abolnik, C. (2014). A current review of avian influenza in pigeons and doves (Columbidae). *Veterinary Microbiology* 170(3)**,** 181-196. doi: https://doi.org/10.1016/j.vetmic.2014.02.042.

Alexander, D.J., Parsons, G., and Manvell, R.J. (1986). Experimental assessment of the pathogenicity of eight avian influenza A viruses of H5 subtype for chickens, turkeys, ducks and quail. *Avian Pathol* 15(4)**,** 647-662. doi: 10.1080/03079458608436328.

Alexopoulou, L., Holt, A.C., Medzhitov, R., and Flavell, R.A. (2001). Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* 413(6857)**,** 732-738. doi: 10.1038/35099560.

Allen, I.C., Scull, M.A., Moore, C.B., Holl, E.K., McElvania-TeKippe, E., Taxman, D.J., et al. (2009). The NLRP3 inflammasome mediates in vivo innate immunity to influenza A virus through recognition of viral RNA. *Immunity* 30(4)**,** 556-565. doi: 10.1016/j.immuni.2009.02.005.

Arimori, Y., Nakamura, R., Yamada, H., Shibata, K., Maeda, N., Kase, T., et al. (2013). Type I interferon limits influenza virus-induced acute lung injury by regulation of excessive inflammation in mice. *Antiviral Res* 99(3)**,** 230-237. doi: 10.1016/j.antiviral.2013.05.007.

Balachandran, S., Roberts, P.C., Brown, L.E., Truong, H., Pattnaik, A.K., Archer, D.R., et al. (2000). Essential role for the dsRNA-dependent protein kinase PKR in innate immunity to viral infection. *Immunity* 13(1)**,** 129-141. doi: 10.1016/s1074-7613(00)00014-5.

Barber, M.R., Aldridge, J.R., Jr., Webster, R.G., and Magor, K.E. (2010). Association of RIG-I with innate immunity of ducks to influenza. *Proc Natl Acad Sci U S A* 107(13)**,** 5913-5918. doi: 10.1073/pnas.1001755107.

Barber, M.R.W., Aldridge, J.R., Jr., Fleming-Canepa, X., Wang, Y.-D., Webster, R.G., and Magor, K.E. (2013). Identification of avian RIG-I responsive genes during influenza infection. *Molecular immunology* 54(1)**,** 89-97. doi: 10.1016/j.molimm.2012.10.038.

Barjesteh, N., Behboudi, S., Brisbin, J.T., Villanueva, A.I., Nagy, E., and Sharif, S. (2014). TLR ligands induce antiviral responses in chicken macrophages. *PloS one* 9(8)**,** e105713-e105713. doi: 10.1371/journal.pone.0105713.

Baum, L.G., and Paulson, J.C. (1990). Sialyloligosaccharides of the respiratory epithelium in the selection of human influenza virus receptor specificity. *Acta Histochem Suppl* 40**,** 35-38.

Bazzigher, L., Schwarz, A., and Staeheli, P. (1993). No enhanced influenza virus resistance of murine and avian cells expressing cloned duck Mx protein. *Virology* 195(1)**,** 100-112. doi: 10.1006/viro.1993.1350.

Benitez, A.A., Panis, M., Xue, J., Varble, A., Shim, J.V., Frick, A.L., et al. (2015). In Vivo RNAi Screening Identifies MDA5 as a Significant Contributor to the Cellular Defense against Influenza A Virus. *Cell Rep* 11(11)**,** 1714-1726. doi: 10.1016/j.celrep.2015.05.032.

Billiau, A. (2006). Anti-inflammatory properties of Type I interferons. *Antiviral Res* 71(2-3)**,** 108-116. doi: 10.1016/j.antiviral.2006.03.006.

Bingham, J., Green, D.J., Lowther, S., Klippel, J., Burggraaf, S., Anderson, D.E., et al. (2009). Infection studies with two highly pathogenic avian influenza strains (Vietnamese and Indonesian) in Pekin ducks (Anas platyrhynchos), with particular reference to clinical disease, tissue tropism and viral shedding. *Avian Pathol* 38(4)**,** 267-278. doi: 10.1080/03079450903055371.

Blaine, A.H., Miranzo-Navarro, D., Campbell, L.K., Aldridge, J.R., Jr., Webster, R.G., and Magor, K.E. (2015). Duck TRIM27-L enhances MAVS signaling and is absent in chickens and turkeys. *Mol Immunol* 67(2 Pt B)**,** 607-615. doi: 10.1016/j.molimm.2015.07.011.

Blyth, G.A., Chan, W.F., Webster, R.G., and Magor, K.E. (2016). Duck interferon-inducible transmembrane protein 3 mediates restriction of influenza viruses. *J Virol* 90(1)**,** 103-116. doi: 10.1128/JVI.01593-15.

Botos, I., Segal, D.M., and Davies, D.R. (2011). The structural biology of Toll-like receptors. *Structure* 19(4)**,** 447-459. doi: 10.1016/j.str.2011.02.004.

Boyd, A., Philbin, V.J., and Smith, A.L. (2007). Conserved and distinct aspects of the avian Toll-like receptor (TLR) system: implications for transmission and control of bird-borne

zoonoses. *Biochemical Society transactions* 35(Pt 6)**,** 1504-1507. doi: 10.1042/bst0351504.

Boyd, A.C., Peroval, M.Y., Hammond, J.A., Prickett, M.D., Young, J.R., and Smith, A.L. (2012). TLR15 Is Unique to avian and reptilian lineages and recognizes a yeast-derived agonist. *The Journal of Immunology* 189(10)**,** 4930. doi: 10.4049/jimmunol.1101790.

Brass, A.L., Huang, I.C., Benita, Y., John, S.P., Krishnan, M.N., Feeley, E.M., et al. (2009). The IFITM proteins mediate cellular resistance to influenza A H1N1 virus, West Nile virus, and dengue virus. *Cell* 139(7)**,** 1243-1254. doi: 10.1016/j.cell.2009.12.017.

Brownlie, R., and Allan, B. (2011). Avian toll-like receptors. *Cell Tissue Res* 343(1)**,** 121-130. doi: 10.1007/s00441-010-1026-0.

Brownlie, R., Zhu, J., Allan, B., Mutwiri, G.K., Babiuk, L.A., Potter, A., et al. (2009). Chicken TLR21 acts as a functional homologue to mammalian TLR9 in the recognition of CpG oligodeoxynucleotides. *Molecular immunology* 46(15)**,** 3163-3170. doi: 10.1016/j.molimm.2009.06.002.

Burggraaf, S., Karpala, A.J., Bingham, J., Lowther, S., Selleck, P., Kimpton, W., et al. (2014). H5N1 infection causes rapid mortality and high cytokine levels in chickens compared to ducks. *Virus Res* 185**,** 23-31. doi: 10.1016/j.virusres.2014.03.012.

Bush, S.J., Freem, L., MacCallum, A.J., O'Dell, J., Wu, C., Afrasiabi, C., et al. (2018). Combination of novel and public RNA-seq datasets to generate an mRNA expression atlas for the domestic chicken. *BMC Genomics* 19(1)**,** 594. doi: 10.1186/s12864-018-4972-7.

Byrd-Leotis, L., Jia, N., Dutta, S., Trost, J.F., Gao, C., Cummings, S.F., et al. (2019). Influenza binds phosphorylated glycans from human lung. *Science Advances* 5(2)**,** eaav2554. doi: 10.1126/sciadv.aav2554.

Cagle, C., To, T.L., Nguyen, T., Wasilenko, J., Adams, S.C., Cardona, C.J., et al. (2011). Pekin and Muscovy ducks respond differently to vaccination with a H5N1 highly pathogenic avian influenza (HPAI) commercial inactivated vaccine. *Vaccine* 29(38)**,** 6549-6557. doi: 10.1016/j.vaccine.2011.07.004.

Califano, D., Furuya, Y., Roberts, S., Avram, D., McKenzie, A.N.J., and Metzger, D.W. (2018). IFN-gamma increases susceptibility to influenza A infection through suppression of

group II innate lymphoid cells. *Mucosal Immunol* 11(1)**,** 209-219. doi: 10.1038/mi.2017.41.

Castanier, C., Garcin, D., Vazquez, A., and Arnoult, D. (2010). Mitochondrial dynamics regulate the RIG-I-like receptor antiviral pathway. *EMBO Rep* 11(2)**,** 133-138. doi: 10.1038/embor.2009.258.

Cella, M., Salio, M., Sakakibara, Y., Langen, H., Julkunen, I., and Lanzavecchia, A. (1999). Maturation, activation, and protection of dendritic cells induced by double-stranded RNA. *J Exp Med* 189(5)**,** 821-829. doi: 10.1084/jem.189.5.821.

Chen, S., Cheng, A., and Wang, M. (2013). Innate sensing of viruses by pattern recognition receptors in birds. *Vet Res* 44(1)**,** 82. doi: 10.1186/1297-9716-44-82.

Chen, S., Wang, L., Chen, J., Zhang, L., Wang, S., Goraya, M.U., et al. (2017). Avian interferon-inducible transmembrane protein family effectively restricts avian Tembusu virus infection. *Front Microbiol* 8(APR)**,** 672. doi: 10.3389/fmicb.2017.00672.

Chen, S., Wang, T., Liu, P., Yang, C., Wang, M., Jia, R., et al. (2019). Duck interferon regulatory factor 7 (IRF7) can control duck Tembusu virus (DTMUV) infection by triggering type I interferon production and its signal transduction pathway. *Cytokine* 113(January 2018)**,** 31-38. doi: 10.1016/j.cyto.2018.06.001.

Chen, S.N., Zhang, X.W., Li, L., Ruan, B.Y., Huang, B., Huang, W.S., et al. (2016). Evolution of IFN-λ in tetrapod vertebrates and its functional characterization in green anole lizard (Anolis carolinensis). *Developmental & Comparative Immunology* 61**,** 208-224. doi: https://doi.org/10.1016/j.dci.2016.04.004.

Cheng, D., Wu, X., Jia, R., Wang, M., Chen, S., Liu, M., et al. (2019a). CpG oligodeoxynucleotide-specific duck TLR21 mediates activation of NF-κB signaling pathway and plays an important role in the host defence of DPV infection. *Molecular immunology* 106**,** 87-98. doi: 10.1016/j.molimm.2018.12.019.

Cheng, Y., Huang, Q., Ji, W., Du, B., Fu, Q., An, H., et al. (2015a). Muscovy duck retinoic acid-induced gene I (MdRIG-I) functions in innate immunity against H9N2 avian influenza viruses (AIV) infections. *Vet Immunol Immunopathol* 163(3-4)**,** 183-193. doi: 10.1016/j.vetimm.2014.12.009.

Cheng, Y., Liu, Y., Shi, S., Niu, Q., Zhu, W., Wang, Z., et al. (2019b). Functional Characterization of Duck STING in IFN-beta Induction and Anti-H9N2 Avian Influenza

Viruses Infections. *Front Immunol* 10(September)**,** 2224. doi: 10.3389/fimmu.2019.02224.

Cheng, Y., Wang, H., Yan, Y., Ding, C., and Sun, J. (2015b). Two myeloid differentiation factor 88 (MyD88) isoforms identified in ducks. *Dev Comp Immunol* 52(2)**,** 144-154. doi: 10.1016/j.dci.2015.03.015.

Cheng, Y., Zhu, W., Ding, C., Niu, Q., Wang, H., Yan, Y., et al. (2019c). IRF7 Is involved in both STING and MAVS mediating IFN-beta signaling in IRF3-lacking chickens. *J Immunol* 203(7)**,** 1930-1942. doi: 10.4049/jimmunol.1900293.

Ciancanelli, M.J., Huang, S.X., Luthra, P., Garner, H., Itan, Y., Volpi, S., et al. (2015). Infectious disease. Life-threatening influenza and impaired interferon amplification in human IRF7 deficiency. *Science* 348(6233)**,** 448-453. doi: 10.1126/science.aaa1578.

Cluff, C.W., Baldridge, J.R., Stöver, A.G., Evans, J.T., Johnson, D.A., Lacy, M.J., et al. (2005). Synthetic Toll-Like Receptor 4 Agonists Stimulate Innate Resistance to Infectious Challenge. *Infection and Immunity* 73(5)**,** 3044. doi: 10.1128/IAI.73.5.3044-3052.2005.

Cormican, P., Lloyd, A.T., Downing, T., Connell, S.J., Bradley, D., and O'Farrelly, C. (2009). The avian Toll-Like receptor pathway—Subtle differences amidst general conformity. *Developmental & Comparative Immunology* 33(9)**,** 967-973. doi: https://doi.org/10.1016/j.dci.2009.04.001.

Cornelissen, J.B., Vervelde, L., Post, J., and Rebel, J.M. (2013). Differences in highly pathogenic avian influenza viral pathogenesis and associated early inflammatory response in chickens and ducks. *Avian Pathol* 42(4)**,** 347-364. doi: 10.1080/03079457.2013.807325.

Cornelissen, J.B.W.J., Post, J., Peeters, B., Vervelde, L., and Rebel, J.M.J. (2012). Differential innate responses of chickens and ducks to low-pathogenic avian influenza. *Avian Pathology* 41(6)**,** 519-529. doi: 10.1080/03079457.2012.732691.

Costa, T., Chaves, A.J., Valle, R., Darji, A., van Riel, D., Kuiken, T., et al. (2012). Distribution patterns of influenza virus receptors and viral attachment patterns in the respiratory and intestinal tracts of seven avian species. *Vet Res* 43**,** 28. doi: 10.1186/1297-9716-43-28.

Couceiro, J.N., Paulson, J.C., and Baum, L.G. (1993). Influenza virus strains selectively recognize sialyloligosaccharides on human respiratory epithelium; the role of the host

cell in selection of hemagglutinin receptor specificity. *Virus Res* 29(2)**,** 155-165. doi: 10.1016/0168-1702(93)90056-s.

Crotta, S., Davidson, S., Mahlakoiv, T., Desmet, C.J., Buckwalter, M.R., Albert, M.L., et al. (2013). Type I and type III interferons drive redundant amplification loops to induce a transcriptional signature in influenza-infected airway epithelia. *PLoS Pathog* 9(11)**,** e1003773. doi: 10.1371/journal.ppat.1003773.

D'Cruz, A.A., Babon, J.J., Norton, R.S., Nicola, N.A., and Nicholson, S.E. (2013). Structure and function of the SPRY/B30.2 domain proteins involved in innate immunity. *Protein Sci* 22(1)**,** 1-10. doi: 10.1002/pro.2185.

Daoust, P.Y., van de Bildt, M., van Riel, D., van Amerongen, G., Bestebroer, T., Vanderstichel, R., et al. (2013). Replication of 2 subtypes of low-pathogenicity avian influenza virus of duck and gull origins in experimentally infected Mallard ducks. *Vet Pathol* 50(3)**,** 548-559. doi: 10.1177/0300985812469633.

Daviet, S., Van Borm, S., Habyarimana, A., Ahanda, M.L., Morin, V., Oudin, A., et al. (2009). Induction of Mx and PKR failed to protect chickens from H5N1 infection. *Viral Immunol* 22(6)**,** 467-472. doi: 10.1089/vim.2009.0053.

Diamond, M.S., and Farzan, M. (2013). The broad-spectrum antiviral functions of IFIT and IFITM proteins. *Nat Rev Immunol* 13(1)**,** 46-57. doi: 10.1038/nri3344.

Diebold, S.S., Kaisho, T., Hemmi, H., Akira, S., and Reis e Sousa, C. (2004). Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* 303(5663)**,** 1529-1531. doi: 10.1126/science.1093616.

Dillon, D., and Runstadler, J. (2010). Mx gene diversity and influenza association among five wild dabbling duck species (Anas spp.) in Alaska. *Infection, Genetics and Evolution* 10(7)**,** 1085-1093. doi: https://doi.org/10.1016/j.meegid.2010.07.004.

Ducatez, M., Sonnberg, S., Crumpton, J.C., Rubrum, A., Phommachanh, P., Douangngeun, B., et al. (2017). Highly pathogenic avian influenza H5N1 clade 2.3.2.1 and clade 2.3.4 viruses do not induce a clade-specific phenotype in mallard ducks. *Journal of General Virology* 98(6)**,** 1232-1244. doi: https://doi.org/10.1099/jgv.0.000806.

Evseev, D., and Magor, K.E. (2019). Innate immune responses to avian influenza viruses in Ducks and Chickens. *Vet Sci* 6(1)**,** 5-5. doi: 10.3390/vetsci6010005.

Feng, Z.Q., Cheng, Y., Yang, H.L., Zhu, Q., Yu, D., and Liu, Y.P. (2015). Molecular characterization, tissue distribution and expression analysis of TRIM25 in Gallus gallus domesticus. *Gene* 561(1)**,** 138-147. doi: 10.1016/j.gene.2015.02.025.

Fensterl, V., and Sen, G.C. (2015). Interferon-induced IFIT proteins: their role in viral pathogenesis. *J Virol* 89(5)**,** 2462-2468. doi: 10.1128/JVI.02744-14.

Fernandez, M.V., Miller, E., Krammer, F., Gopal, R., Greenbaum, B.D., and Bhardwaj, N. (2016). Ion efflux and influenza infection trigger NLRP3 inflammasome signaling in human dendritic cells. *J Leukoc Biol* 99(5)**,** 723-734. doi: 10.1189/jlb.3A0614-313RRR.

Fitzgerald, K.A., McWhirter, S.M., Faia, K.L., Rowe, D.C., Latz, E., Golenbock, D.T., et al. (2003). IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. *Nat Immunol* 4(5)**,** 491-496. doi: 10.1038/ni921.

Fleming-Canepa, X., Aldridge, J.R., Jr., Canniff, L., Kobewka, M., Jax, E., Webster, R.G., et al. (2019). Duck innate immune responses to high and low pathogenicity H5 avian influenza viruses. *Vet Microbiol* 228**,** 101-111. doi: 10.1016/j.vetmic.2018.11.018.

Francisco, E., Suthar, M., Gale, M., Jr., Rosenfeld, A.B., and Racaniello, V.R. (2019). Cell-type specificity and functional redundancy of RIG-I-like receptors in innate immune sensing of Coxsackievirus B3 and encephalomyocarditis virus. *Virology* 528**,** 7-18. doi: 10.1016/j.virol.2018.12.003.

Fu, B., Wang, L., Ding, H., Schwamborn, J.C., Li, S., and Dorf, M.E. (2015). TRIM32 senses and restricts influenza A virus by ubiquitination of PB1 polymerase. *PLoS Pathog* 11(6)**,** e1004960. doi: 10.1371/journal.ppat.1004960.

Fulton, J.E., Arango, J., Ali, R.A., Bohorquez, E.B., Lund, A.R., Ashwell, C.M., et al. (2014). Genetic variation within the Mx gene of commercially selected chicken lines reveals multiple haplotypes, recombination and a protein under selection pressure. *PLoS One* 9(9)**,** e108054. doi: 10.1371/journal.pone.0108054.

Gack, M.U., Shin, Y.C., Joo, C.H., Urano, T., Liang, C., Sun, L., et al. (2007). TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity. *Nature* 446(7138)**,** 916-920. doi: 10.1038/nature05732.

Gaidet, N., Cappelle, J., Takekawa, J.Y., Prosser, D.J., Iverson, S.A., Douglas, D.C., et al. (2010). Potential spread of highly pathogenic avian influenza H5N1 by wildfowl:

dispersal ranges and rates determined from large-scale satellite telemetry. *Journal of Applied Ecology* 47(5)**,** 1147-1157. doi: 10.1111/j.1365-2664.2010.01845.x.

Galabru, J., and Hovanessian, A. (1987). Autophosphorylation of the protein kinase dependent on double-stranded RNA. *J Biol Chem* 262(32)**,** 15538-15544.

Gao, P., Fan, L., Du, H., Xiang, B., Li, Y., Sun, M., et al. (2018a). Recombinant duck interferon gamma inhibits H5N1 influenza virus replication in vitro and in vivo. *J Interferon Cytokine Res* 38(7)**,** 290-297. doi: 10.1089/jir.2018.0034.

Gao, P., Xiang, B., Li, Y., Li, Y., Sun, M., Kang, Y., et al. (2018b). Therapeutic effect of duck interferon-alpha against H5N1 highly pathogenic avian influenza virus infection in Peking ducks. *J Interferon Cytokine Res* 38(4)**,** 145-152. doi: 10.1089/jir.2017.0116.

Garcia, M.A., Gil, J., Ventoso, I., Guerra, S., Domingo, E., Rivas, C., et al. (2006). Impact of protein kinase PKR in cell biology: from antiviral to antiproliferative action. *Microbiol Mol Biol Rev* 70(4)**,** 1032-1060. doi: 10.1128/MMBR.00027-06.

Goossens, K.E., Karpala, A.J., Rohringer, A., Ward, A., and Bean, A.G. (2015). Characterisation of chicken viperin. *Mol Immunol* 63(2)**,** 373-380. doi: 10.1016/j.molimm.2014.09.011.

Guarda, G., Braun, M., Staehli, F., Tardivel, A., Mattmann, C., Forster, I., et al. (2011). Type I interferon inhibits interleukin-1 production and inflammasome activation. *Immunity* 34(2)**,** 213-223. doi: 10.1016/j.immuni.2011.02.006.

Guillot, L., Le Goffic, R., Bloch, S., Escriou, N., Akira, S., Chignard, M., et al. (2005). Involvement of toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus. *J Biol Chem* 280(7)**,** 5571-5580. doi: 10.1074/jbc.M410592200.

Guo, B., and Cheng, G. (2007). Modulation of the interferon antiviral response by the TBK1/IKKi adaptor protein TANK. *J Biol Chem* 282(16)**,** 11817-11826. doi: 10.1074/jbc.M700017200.

Habjan, M., Hubel, P., Lacerda, L., Benda, C., Holze, C., Eberl, C.H., et al. (2013). Sequestration by IFIT1 impairs translation of 2'O-unmethylated capped RNA. *PLoS Pathog* 9(10)**,** e1003663. doi: 10.1371/journal.ppat.1003663.

Hacker, H., Redecke, V., Blagoev, B., Kratchmarova, I., Hsu, L.C., Wang, G.G., et al. (2006). Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. *Nature* 439(7073)**,** 204-207. doi: 10.1038/nature04369.

Hagag, I.T., Mansour, S.M., Zhang, Z., Ali, A.A., Ismaiel el, B.M., Salama, A.A., et al. (2015). Pathogenicity of highly pathogenic avian influenza virus H5N1 in naturally infected poultry in Egypt. *PLoS One* 10(5)**,** e0120061. doi: 10.1371/journal.pone.0120061.

Haider, N., Sturm-Ramirez, K., Khan, S.U., Rahman, M.Z., Sarkar, S., Poh, M.K., et al. (2017). Unusually high mortality in waterfowl caused by highly pathogenic avian influenza A(H5N1) in Bangladesh. *Transbound Emerg Dis* 64(1)**,** 144-156. doi: 10.1111/tbed.12354.

Haller, O., Staeheli, P., Schwemmle, M., and Kochs, G. (2015). Mx GTPases: dynamin-like antiviral machines of innate immunity. *Trends Microbiol* 23(3)**,** 154-163. doi: 10.1016/j.tim.2014.12.003.

Hatesuer, B., Hoang, H.T., Riese, P., Trittel, S., Gerhauser, I., Elbahesh, H., et al. (2017). Deletion of Irf3 and Irf7 genes in mice results in altered interferon pathway activation and granulocyte-dominated inflammatory responses to influenza A infection. *J Innate Immun* 9(2)**,** 145-161. doi: 10.1159/000450705.

Hayashi, T., Watanabe, C., Suzuki, Y., Tanikawa, T., Uchida, Y., and Saito, T. (2014). Chicken MDA5 senses short double-stranded RNA with implications for antiviral response against avian influenza viruses in chicken. *J Innate Immun* 6(1)**,** 58-71. doi: 10.1159/000351583.

He, H., Genovese, K.J., Swaggerty, C.L., MacKinnon, K.M., and Kogut, M.H. (2012). Co-stimulation with TLR3 and TLR21 ligands synergistically up-regulates Th1-cytokine IFN-γ and regulatory cytokine IL-10 expression in chicken monocytes. *Developmental & Comparative Immunology* 36(4)**,** 756-760. doi: https://doi.org/10.1016/j.dci.2011.11.006.

Hemmi, H., Kaisho, T., Takeuchi, O., Sato, S., Sanjo, H., Hoshino, K., et al. (2002). Small anti-viral compounds activate immune cells via the TLR7 MyD88–dependent signaling pathway. *Nature Immunology* 3(2)**,** 196-200. doi: 10.1038/ni758.

Hiscott, J. (2007). Triggering the innate antiviral response through IRF-3 activation. *J Biol Chem* 282(21)**,** 15325-15329. doi: 10.1074/jbc.R700002200.

Holm, C.K., Jensen, S.B., Jakobsen, M.R., Cheshenko, N., Horan, K.A., Moeller, H.B., et al. (2012). Virus-cell fusion as a trigger of innate immunity dependent on the adaptor STING. *Nat Immunol* 13(8)**,** 737-743. doi: 10.1038/ni.2350.

Holm, C.K., Rahbek, S.H., Gad, H.H., Bak, R.O., Jakobsen, M.R., Jiang, Z., et al. (2016). Influenza A virus targets a cGAS-independent STING pathway that controls enveloped RNA viruses. *Nat Commun* 7**,** 10680. doi: 10.1038/ncomms10680.

Honda, K., and Taniguchi, T. (2006). IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. *Nat Rev Immunol* 6(9)**,** 644-658. doi: 10.1038/nri1900.

Hopkins, P.A., and Sriskandan, S. (2005). Mammalian Toll-like receptors: to immunity and beyond. *Clin Exp Immunol* 140(3)**,** 395-407. doi: 10.1111/j.1365-2249.2005.02801.x.

Horisberger, M.A., Staeheli, P., and Haller, O. (1983). Interferon induces a unique protein in mouse cells bearing a gene for resistance to influenza virus. *Proc Natl Acad Sci U S A* 80(7)**,** 1910-1914. doi: 10.1073/pnas.80.7.1910.

Hornung, V., Ellegast, J., Kim, S., Brzozka, K., Jung, A., Kato, H., et al. (2006). 5'-Triphosphate RNA is the ligand for RIG-I. *Science* 314(5801)**,** 994-997. doi: 10.1126/science.1132505.

Hou, F., Sun, L., Zheng, H., Skaug, B., Jiang, Q.X., and Chen, Z.J. (2011). MAVS forms functional prion-like aggregates to activate and propagate antiviral innate immune response. *Cell* 146(3)**,** 448-461. doi: 10.1016/j.cell.2011.06.041.

Hovanessian, A.G. (1989). The double stranded RNA-activated protein kinase induced by interferon: dsRNA-PK. *J Interferon Res* 9(6)**,** 641-647. doi: 10.1089/jir.1989.9.641.

Hron, T., Pajer, P., Paces, J., Bartunek, P., and Elleder, D. (2015). Hidden genes in birds. *Genome Biol* 16(1)**,** 164. doi: 10.1186/s13059-015-0724-z.

Hu, J., Zhao, K., Liu, X., Wang, X., Chen, Z., and Liu, X. (2013). Two highly pathogenic avian influenza H5N1 viruses of clade 2.3.2.1 with similar genetic background but with different pathogenicity in mice and ducks. *Transboundary and Emerging Diseases* 60(2)**,** 127-139. doi: 10.1111/j.1865-1682.2012.01325.x.

Hua, K., Li, Y., Chen, H., Ni, J., Bi, D., Luo, R., et al. (2018). Functional characterization of duck TBK1 in IFN-beta induction. *Cytokine* 111(1)**,** 325-333. doi: 10.1016/j.cyto.2018.09.007.

Huang, B., Qi, Z.T., Xu, Z., and Nie, P. (2010). Global characterization of interferon regulatory factor (IRF) genes in vertebrates: glimpse of the diversification in evolution. *BMC Immunol* 11(1)**,** 22. doi: 10.1186/1471-2172-11-22.

Huang, Y., Li, Y., Burt, D.W., Chen, H., Zhang, Y., Qian, W., et al. (2013). The duck genome and transcriptome provide insight into an avian influenza virus reservoir species. *Nat Genet* 45(7)**,** 776-783. doi: 10.1038/ng.2657.

Huo, H., Zhao, L., Wang, D., Chen, X., and Chen, H. (2019). LGP2 plays a critical role in MDA5-mediated antiviral activity against duck enteritis virus. *Mol Immunol* 116**,** 160-166. doi: 10.1016/j.molimm.2019.10.006.

Ibsen, M.S., Gad, H.H., Andersen, L.L., Hornung, V., Julkunen, I., Sarkar, S.N., et al. (2015). Structural and functional analysis reveals that human OASL binds dsRNA to enhance RIG-I signaling. *Nucleic Acids Res* 43(10)**,** 5236-5248. doi: 10.1093/nar/gkv389.

Iqbal, M., Philbin, V.J., and Smith, A.L. (2005). Expression patterns of chicken Toll-like receptor mRNA in tissues, immune cell subsets and cell lines. *Veterinary Immunology and Immunopathology* 104(1)**,** 117-127. doi: https://doi.org/10.1016/j.vetimm.2004.11.003.

Ishikawa, H., Ma, Z., and Barber, G.N. (2009). STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity. *Nature* 461(7265)**,** 788-792. doi: 10.1038/nature08476.

Ito, T., Wang, Y.H., and Liu, Y.J. (2005). Plasmacytoid dendritic cell precursors/type I interferon-producing cells sense viral infection by Toll-like receptor (TLR) 7 and TLR9. *Springer Semin Immunopathol* 26(3)**,** 221-229. doi: 10.1007/s00281-004-0180-4.

Jacobs, J.L., and Coyne, C.B. (2013). Mechanisms of MAVS regulation at the mitochondrial membrane. *J Mol Biol* 425(24)**,** 5009-5019. doi: 10.1016/j.jmb.2013.10.007.

James, L.C., Keeble, A.H., Khan, Z., Rhodes, D.A., and Trowsdale, J. (2007). Structural basis for PRYSPRY-mediated tripartite motif (TRIM) protein function. *Proc Natl Acad Sci U S A* 104(15)**,** 6200-6205. doi: 10.1073/pnas.0609174104.

Jang, H.J., Lee, H.J., Kang, K.S., Song, K.D., Kim, T.H., Song, C.S., et al. (2015). Molecular responses to the influenza A virus in chicken trachea-derived cells. *Poult Sci* 94(6)**,** 1190-1201. doi: 10.3382/ps/pev033.

Jiang, H., Yang, H., and Kapczynski, D.R. (2011). Chicken interferon alpha pretreatment reduces virus replication of pandemic H1N1 and H5N9 avian influenza viruses in lung cell cultures from different avian species. *Virol J* 8(1)**,** 447. doi: 10.1186/1743-422X-8-447.

Jiao, P.R., Wei, L.M., Cheng, Y.Q., Yuan, R.Y., Han, F., Liang, J., et al. (2012). Molecular cloning, characterization, and expression analysis of the Muscovy duck Toll-like receptor 3 (MdTLR3) gene. *Poult Sci* 91(10)**,** 2475-2481. doi: 10.3382/ps.2012-02394.

Jiao, P.R., Wei, L.M., Song, Y.F., Cui, J., Zhang, S., Han, F., et al. (2015). Molecular cloning and immune responsive expression of LGP2 gene, a pivotal member of the RLR gene family from Muscovy duck Cairina moschata. *Poult Sci* 94(6)**,** 1170-1176. doi: 10.3382/ps/pev082.

Jourdain, E., Gunnarsson, G., Wahlgren, J., Latorre-Margalef, N., Brojer, C., Sahlin, S., et al. (2010). Influenza virus in a natural host, the mallard: experimental infection data. *PLoS One* 5(1)**,** e8935. doi: 10.1371/journal.pone.0008935.

Justesen, J., Hartmann, R., and Kjeldgaard, N.O. (2000). Gene structure and function of the 2'-5'-oligoadenylate synthetase family. *Cell Mol Life Sci* 57(11)**,** 1593-1612. doi: 10.1007/pl00000644.

Kaiser, A., Willer, T., Sid, H., Petersen, H., Baumgärtner, W., Steinberg, P., et al. (2016). Susceptibility of primary chicken intestinal epithelial cells for low pathogenic avian influenza virus and velogenic viscerotropic Newcastle disease virus. *Virus Research* 225**,** 50-63. doi: https://doi.org/10.1016/j.virusres.2016.09.001.

Kandasamy, M., Suryawanshi, A., Tundup, S., Perez, J.T., Schmolke, M., Manicassamy, S., et al. (2016). RIG-I Signaling Is Critical for Efficient Polyfunctional T Cell Responses during Influenza Virus Infection. *PLoS Pathogens* 12(1553-7374 (Electronic)).

Kang, H.M., Choi, J.G., Kim, K.I., Kim, B.S., Batchuluun, D., Erdene-Ochir, T.O., et al. (2013). Pathogenicity in domestic ducks and mice of highly pathogenic H5N1 clade 2.3.2.1 influenza viruses recently circulating in Eastern Asia. *Vet Microbiol* 167(3-4)**,** 327-333. doi: 10.1016/j.vetmic.2013.09.013.

Kannaki, T.R., Verma, P.C., Reddy, M.R., and Shanmugam, M. (2018). Molecular characterization of duck (Anas platyrhynchos) Toll-like receptors, mRNA expressions profile in day-old duckling's tissues and cytokine response to in vitro TLR agonsists stimulation. *Indian Journal of Animal Research* 52(6)**,** 851-857. doi: UNSP B-329010.18805/ijar.B-3290.

Karpala, A.J., Morris, K.R., Broadway, M.M., McWaters, P.G.D., O'Neil, T.E., Goossens, K.E., et al. (2008). Molecular cloning, expression, and characterization of chicken IFN -λ. *Journal of Interferon & Cytokine Research* 28(6)**,** 341-350. doi: 10.1089/jir.2007.0117.

Karpala, A.J., Stewart, C., McKay, J., Lowenthal, J.W., and Bean, A.G. (2011). Characterization of chicken MDA5 activity: regulation of IFN-beta in the absence of RIG-I functionality. *J Immunol* 186(9)**,** 5397-5405. doi: 10.4049/jimmunol.1003712.

Kato, H., Sato, S., Yoneyama, M., Yamamoto, M., Uematsu, S., Matsui, K., et al. (2005). Cell type-specific involvement of RIG-I in antiviral response. *Immunity* 23(1)**,** 19-28. doi: 10.1016/j.immuni.2005.04.010.

Kato, H., Takeuchi, O., Mikamo-Satoh, E., Hirai, R., Kawai, T., Matsushita, K., et al. (2008). Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-inducible gene-I and melanoma differentiation-associated gene 5. *J Exp Med* 205(7)**,** 1601-1610. doi: 10.1084/jem.20080091.

Kawai, T., and Akira, S. (2010). The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nature Immunology* 11(5)**,** 373-384. doi: 10.1038/ni.1863.

Kawai, T., Takahashi, K., Sato, S., Coban, C., Kumar, H., Kato, H., et al. (2005). IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. *Nat Immunol* 6(10)**,** 981-988. doi: 10.1038/ni1243.

Keestra, A.M., de Zoete, M.R., Bouwman, L.I., Vaezirad, M.M., and van Putten, J.P.M. (2013). Unique features of chicken Toll-like receptors. *Developmental & Comparative Immunology* 41(3)**,** 316-323. doi: https://doi.org/10.1016/j.dci.2013.04.009.

Khaitov, M.R., Laza-Stanca, V., Edwards, M.R., Walton, R.P., Rohde, G., Contoli, M., et al. (2009). Respiratory virus induction of alpha-, beta- and lambda-interferons in bronchial epithelial cells and peripheral blood mononuclear cells. *Allergy* 64(3)**,** 375-386. doi: 10.1111/j.1398-9995.2008.01826.x.

Kida, H., Yanagawa, R., and Matsuoka, Y. (1980). Duck influenza lacking evidence of disease signs and immune response. *Infect Immun* 30(2)**,** 547-553.

Kim, J.-K., Negovetich, N.J., Forrest, H.L., and Webster, R.G. (2009). Ducks: the "Trojan horses" of H5N1 influenza. *Influenza and other respiratory viruses* 3(4)**,** 121-128. doi: 10.1111/j.1750-2659.2009.00084.x.

Kim, T.H., and Zhou, H. (2015). Functional analysis of chicken IRF7 in response to dsRNA analog poly(I:C) by integrating overexpression and knockdown. *PLoS One* 10(7)**,** e0133450. doi: 10.1371/journal.pone.0133450.

Kim, T.H., and Zhou, H. (2018).). Overexpression of chicken IRF7 increased viral replication and programmed cell death to the avian influenza virus infection through TGF-Beta/FoxO signaling axis in DF-1. *Front Genet* 9(September)**,** 415. doi: 10.3389/fgene.2018.00415.

Ko, J.H., Asano, A., Kon, Y., Watanabe, T., and Agui, T. (2004). Characterization of the chicken PKR: polymorphism of the gene and antiviral activity against vesicular stomatitis virus. *Jpn J Vet Res* 51(3-4)**,** 123-133.

Ko, J.H., Jin, H.K., Asano, A., Takada, A., Ninomiya, A., Kida, H., et al. (2002). Polymorphisms and the differential antiviral activity of the chicken Mx gene. *Genome Res* 12(4)**,** 595-601. doi: 10.1101/gr.210702.

Koerner, I., Kochs, G., Kalinke, U., Weiss, S., and Staeheli, P. (2007). Protective role of beta interferon in host defense against influenza A virus. *Journal of Virology* 81(4)**,** 2025. doi: 10.1128/JVI.01718-06.

Kogut, M.H., Iqbal, M., He, H., Philbin, V., Kaiser, P., and Smith, A. (2005). Expression and function of Toll-like receptors in chicken heterophils. *Developmental & Comparative Immunology* 29(9)**,** 791-807. doi: https://doi.org/10.1016/j.dci.2005.02.002.

Kotenko, S.V., Gallagher, G., Baurin, V.V., Lewis-Antes, A., Shen, M., Shah, N.K., et al. (2003). IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. *Nat Immunol* 4(1)**,** 69-77. doi: 10.1038/ni875.

Kuchipudi, S.V., Dunham, S.P., Nelli, R., White, G.A., Coward, V.J., Slomka, M.J., et al. (2012). Rapid death of duck cells infected with influenza: a potential mechanism for host resistance to H5N1. *Immunol Cell Biol* 90(1)**,** 116-123. doi: 10.1038/icb.2011.17.

Kuchipudi, S.V., Nelli, R., White, G.A., Bain, M., Chang, K.C., and Dunham, S. (2009). Differences in influenza virus receptors in chickens and ducks: Implications for interspecies transmission. *J Mol Genet Med* 3(1)**,** 143-151. doi: 10.4172/1747-0862.1000026.

Kumar, A., Vijayakumar, P., Gandhale, P.N., Ranaware, P.B., Kumar, H., Kulkarni, D.D., et al. (2017). Genome-wide gene expression pattern underlying differential host response to

high or low pathogenic H5N1 avian influenza virus in ducks. *Acta Virol* 61(1)**,** 66-76. doi: 10.4149/av_2017_01_66.

Kuriakose, T., Zheng, M., Neale, G., and Kanneganti, T.D. (2018).). IRF1 Is a transcriptional regulator of ZBP1 promoting NLRP3 inflammasome activation and cell death during influenza virus infection. *J Immunol* 200(4)**,** 1489-1495. doi: 10.4049/jimmunol.1701538.

Kwon, Y.K., Thomas, C., and Swayne, D.E. (2010). Variability in pathobiology of South Korean H5N1 high-pathogenicity avian influenza virus infection for 5 species of migratory waterfowl. *Veterinary Pathology* 47(3)**,** 495-506. doi: 10.1177/0300985809359602.

Le Goffic, R., Balloy, V., Lagranderie, M., Alexopoulou, L., Escriou, N., Flavell, R., et al. (2006). Detrimental contribution of the Toll-like receptor (TLR)3 to influenza A virus-induced acute pneumonia. *PLoS Pathog* 2(6)**,** e53. doi: 10.1371/journal.ppat.0020053.

Li, H., Zhai, Y., Fan, Y., Chen, H., Zhang, A., Jin, H., et al. (2016a). Molecular cloning and functional characterization of duck mitochondrial antiviral-signaling protein (MAVS). *Dev Comp Immunol* 56**,** 1-6. doi: 10.1016/j.dci.2015.11.004.

Li, L., Feng, W., Cheng, Z., Yang, J., Bi, J., Wang, X., et al. (2019). TRIM62-mediated restriction of avian leukosis virus subgroup J replication is dependent on the SPRY domain. *Poult Sci* 98(11)**,** 6019-6025. doi: 10.3382/ps/pez408.

Li, N., Hong, T., Li, R., Wang, Y., Guo, M., Cao, Z., et al. (2016b). Cherry Valley ducks mitochondrial antiviral-aignaling protein-mediated signaling pathway and antiviral activity research. *Front Immunol* 7(SEP)**,** 377. doi: 10.3389/fimmu.2016.00377.

Li, R., Lin, J., Hou, X., Han, S., Weng, H., Xu, T., et al. (2018). Characterization and roles of Cherry Valley duck NLRP3 in innate immunity during avian pathogenic *Escherichia coli* infection. *Front Immunol* 9(OCT)**,** 2300. doi: 10.3389/fimmu.2018.02300.

Li, S., Min, J.-Y., Krug, R.M., and Sen, G.C. (2006). Binding of the influenza A virus NS1 protein to PKR mediates the inhibition of its activation by either PACT or double-stranded RNA. *Virology* 349(1)**,** 13-21. doi: https://doi.org/10.1016/j.virol.2006.01.005.

Lin, H., Jiang, M., Liu, L., Yang, Z., Ma, Z., Liu, S., et al. (2019). The long noncoding RNA Lnczc3h7a promotes a TRIM25-mediated RIG-I antiviral innate immune response. *Nat Immunol* 20(7)**,** 812-823. doi: 10.1038/s41590-019-0379-0.

Lindenmann, J. (1962). Resistance of mice to mouse-adapted influenza A virus. *Virology* 16**,** 203-204. doi: 10.1016/0042-6822(62)90297-0.

Liniger, M., Summerfield, A., Zimmer, G., McCullough, K.C., and Ruggli, N. (2012). Chicken cells sense influenza A virus infection through MDA5 and CARDIF signaling involving LGP2. *J Virol* 86(2)**,** 705-717. doi: 10.1128/JVI.00742-11.

Liu, G., Lu, Y., Thulasi Raman, S.N., Xu, F., Wu, Q., Li, Z., et al. (2018a). Nuclear-resident RIG-I senses viral replication inducing antiviral immunity. *Nat Commun* 9(1)**,** 3199. doi: 10.1038/s41467-018-05745-w.

Liu, G., Park, H.-S., Pyo, H.-M., Liu, Q., and Zhou, Y. (2015a). Influenza A Virus Panhandle Structure Is Directly Involved in RIG-I Activation and Interferon Induction. *Journal of Virology* 89(11)**,** 6067. doi: 10.1128/JVI.00232-15.

Liu, G., and Zhou, Y. (2019). Cytoplasm and beyond: dynamic innate immune sensing of influenza A virus by RIG-I. *Journal of Virology* 93(8)**,** e02299-02218. doi: 10.1128/JVI.02299-18.

Liu, S., Cai, X., Wu, J., Cong, Q., Chen, X., Li, T., et al. (2015b). Phosphorylation of innate immune adaptor proteins MAVS, STING, and TRIF induces IRF3 activation. *Science* 347(6227)**,** aaa2630. doi: 10.1126/science.aaa2630.

Liu, W.J., Yang, Y.T., Huang, Y.M., Zhou, D.R., Xu, D.N., Cao, N., et al. (2018b). Identification of goose PKR Gene: structure, expression profiling, and antiviral activity against Newcastle disease virus. *J Interferon Cytokine Res* 38(8)**,** 333-340. doi: 10.1089/jir.2018.0025.

Liu, Y., Cheng, Y., Shan, W., Ma, J., Wang, H., Sun, J., et al. (2018c). Chicken interferon regulatory factor 1 (IRF1) involved in antiviral innate immunity via regulating IFN-beta production. *Dev Comp Immunol* 88(June)**,** 77-82. doi: 10.1016/j.dci.2018.07.003.

Liu, Y.J. (2005). IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annu Rev Immunol* 23(3)**,** 275-306. doi: 10.1146/annurev.immunol.23.021704.115633.

Londt, B.Z., Nunez, A., Banks, J., Alexander, D.J., Russell, C., Richard-Londt, A.C., et al. (2010). The effect of age on the pathogenesis of a highly pathogenic avian influenza (HPAI) H5N1 virus in Pekin ducks (Anas platyrhynchos) infected experimentally. *Influenza Other Respir Viruses* 4(1)**,** 17-25. doi: 10.1111/j.1750-2659.2009.00116.x.

Loo, Y.-M., and Gale, M., Jr. (2011). Immune signaling by RIG-I-like receptors. *Immunity* 34(5)**,** 680-692. doi: 10.1016/j.immuni.2011.05.003.

Lu, C., Xing, Y., Cai, H., Shi, Y., Liu, J., and Huang, Y. (2019). Identification and analysis of long non-coding RNAs in response to H5N1 influenza viruses in duck (Anas platyrhynchos). *BMC Genomics* 20(1)**,** 36. doi: 10.1186/s12864-018-5422-2.

Lund, J.M., Alexopoulou, L., Sato, A., Karow, M., Adams, N.C., Gale, N.W., et al. (2004). Recognition of single-stranded RNA viruses by Toll-like receptor 7. *Proc Natl Acad Sci U S A* 101(15)**,** 5598-5603. doi: 10.1073/pnas.0400937101.

Ma, W., Brenner, D., Wang, Z., Dauber, B., Ehrhardt, C., Högner, K., et al. (2010). The NS segment of an H5N1 highly pathogenic avian influenza virus (HPAIV) is sufficient to alter replication efficiency, cell tropism, and host range of an H7N1 HPAIV. *Journal of virology* 84(4)**,** 2122-2133. doi: 10.1128/JVI.01668-09.

MacDonald, M.R., Xia, J., Smith, A.L., and Magor, K.E. (2008). The duck toll like receptor 7: genomic organization, expression and function. *Mol Immunol* 45(7)**,** 2055-2061. doi: 10.1016/j.molimm.2007.10.018.

Malkinson, M., Perk, K., and Weisman, Y. (1981). Reovirus infection of young Muscovy ducks (*Cairina moschata*). *Avian Pathol* 10(4)**,** 433-440. doi: 10.1080/03079458108418493.

Malur, M., Gale, M., Jr., and Krug, R.M. (2012). LGP2 downregulates interferon production during infection with seasonal human influenza A viruses that activate interferon regulatory factor 3. *J Virol* 86(19)**,** 10733-10738. doi: 10.1128/JVI.00510-12.

Meng, S., Yang, L., Xu, C., Qin, Z., Xu, H., Wang, Y., et al. (2011). Recombinant chicken interferon-alpha inhibits H9N2 avian influenza virus replication in vivo by oral administration. *J Interferon Cytokine Res* 31(7)**,** 533-538. doi: 10.1089/jir.2010.0123.

Meyerson, N.R., Zhou, L., Guo, Y.R., Zhao, C., Tao, Y.J., Krug, R.M., et al. (2017). Nuclear TRIM25 specifically targets influenza virus ribonucleoproteins to block the onset of RNA chain elongation. *Cell Host Microbe* 22(5)**,** 627-638 e627. doi: 10.1016/j.chom.2017.10.003.

Miranzo-Navarro, D., and Magor, K.E. (2014). Activation of duck RIG-I by TRIM25 is independent of anchored ubiquitin. *PLoS One* 9(1)**,** e86968. doi: 10.1371/journal.pone.0086968.

Muroi, M., and Tanamoto, K. (2008). TRAF6 distinctively mediates MyD88- and IRAK-1-induced activation of NF-kappaB. *J Leukoc Biol* 83(3)**,** 702-707. doi: 10.1189/jlb.0907629.

Nanduri, S., Carpick, B.W., Yang, Y., Williams, B.R., and Qin, J. (1998). Structure of the double-stranded RNA-binding domain of the protein kinase PKR reveals the molecular basis of its dsRNA-mediated activation. *EMBO J* 17(18)**,** 5458-5465. doi: 10.1093/emboj/17.18.5458.

Nerren, J.R., He, H., Genovese, K., and Kogut, M.H. (2010). Expression of the avian-specific toll-like receptor 15 in chicken heterophils is mediated by gram-negative and gram-positive bacteria, but not TLR agonists. *Veterinary immunology and immunopathology* 136(1-2)**,** 151-156. doi: 10.1016/j.vetimm.2010.02.017.

Okamoto, M., Tsukamoto, H., Kouwaki, T., Seya, T., and Oshiumi, H. (2017). Recognition of viral RNA by pattern recognition receptors in the induction of innate immunity and excessive inflammation during respiratory viral infections. *Viral Immunol* 30(6)**,** 408-420. doi: 10.1089/vim.2016.0178.

Olsen, B., Munster, V.J., Wallensten, A., Waldenstrom, J., Osterhaus, A.D., and Fouchier, R.A. (2006). Global patterns of influenza a virus in wild birds. *Science* 312(5772)**,** 384-388. doi: 10.1126/science.1122438.

Onoguchi, K., Yoneyama, M., Takemura, A., Akira, S., Taniguchi, T., Namiki, H., et al. (2007). Viral infections activate types I and III interferon genes through a common mechanism. *J Biol Chem* 282(10)**,** 7576-7581. doi: 10.1074/jbc.M608618200.

Opitz, B., Rejaibi, A., Dauber, B., Eckhard, J., Vinzing, M., Schmeck, B., et al. (2007). IFNβ induction by influenza A virus is mediated by RIG-I which is regulated by the viral NS1 protein. *Cellular Microbiology* 9(4)**,** 930-938. doi: 10.1111/j.1462-5822.2006.00841.x.

Oven, I., Resman Rus, K., Dušanić, D., Benčina, D., Keeler, C.L., and Narat, M. (2013). Diacylated lipopeptide from Mycoplasma synoviae mediates TLR15 induced innate immune responses. *Veterinary Research* 44(1)**,** 99. doi: 10.1186/1297-9716-44-99.

Pan, C., Zhao, H., Shen, L., and Sheng, J. (2011). Molecular characterization and expression pattern of tripartite motif protein 39 in *Gallus gallus* with a complete PRY/SPRY domain. *International journal of molecular sciences* 12(6)**,** 3797-3809. doi: 10.3390/ijms12063797.

Pang, I.K., Pillai, P.S., and Iwasaki, A. (2013). Efficient influenza A virus replication in the respiratory tract requires signals from TLR7 and RIG-I. *Proceedings of the National Academy of Sciences***,** 201303275. doi: 10.1073/pnas.1303275110.

Pantin-Jackwood, M., Swayne, D.E., Smith, D., and Shepherd, E. (2013). Effect of species, breed and route of virus inoculation on the pathogenicity of H5N1 highly pathogenic influenza (HPAI) viruses in domestic ducks. *Vet Res* 44(1)**,** 62. doi: 10.1186/1297-9716-44-62.

Pantin-Jackwood, M.J., Smith, D.M., Wasilenko, J.L., Cagle, C., Shepherd, E., Sarmento, L., et al. (2012). Effect of age on the pathogenesis and innate immune responses in Pekin ducks infected with different H5N1 highly pathogenic avian influenza viruses. *Virus Research* 167(2)**,** 196-206. doi: https://doi.org/10.1016/j.virusres.2012.04.015.

Peisley, A., Wu, B., Xu, H., Chen, Z.J., and Hur, S. (2014). Structural basis for ubiquitin-mediated antiviral signal activation by RIG-I. *Nature* 509(7498)**,** 110-114. doi: 10.1038/nature13140.

Philbin, V.J., Iqbal, M., Boyd, Y., Goodchild, M.J., Beal, R.K., Bumstead, N., et al. (2005). Identification and characterization of a functional, alternatively spliced Toll-like receptor 7 (TLR7) and genomic disruption of TLR8 in chickens. *Immunology* 114(4)**,** 507-521. doi: 10.1111/j.1365-2567.2005.02125.x.

Phuong do, Q., Dung, N.T., Jorgensen, P.H., Handberg, K.J., Vinh, N.T., and Christensen, J.P. (2011). Susceptibility of Muscovy (*Cairina Moschata*) and mallard ducks (*Anas Platyrhynchos*) to experimental infections by different genotypes of H5N1 avian influenza viruses. *Vet Microbiol* 148(2-4)**,** 168-174. doi: 10.1016/j.vetmic.2010.09.007.

Pichlmair, A., Schulz, O., Tan, C.P., Näslund, T.I., Liljeström, P., Weber, F., et al. (2006). RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. *Science (New York, N.Y.)* 314(5801)**,** 997-1001. doi: 10.1126/science.1132998.

Pippig, D.A., Hellmuth, J.C., Cui, S., Kirchhofer, A., Lammens, K., Lammens, A., et al. (2009). The regulatory domain of the RIG-I family ATPase LGP2 senses double-stranded RNA. *Nucleic Acids Res* 37(6)**,** 2014-2025. doi: 10.1093/nar/gkp059.

Pirhonen, J., Sareneva, T., Julkunen, I., and Matikainen, S. (2001). Virus infection induces proteolytic processing of IL-18 in human macrophages via caspase-1 and caspase-3 activation. *Eur J Immunol* 31(3)**,** 726-733. doi: 10.1002/1521-4141(200103)31:3<726::aid-immu726>3.0.co;2-5.

Pothlichet, J., Meunier, I., Davis, B.K., Ting, J.P., Skamene, E., von Messling, V., et al. (2013). Type I IFN triggers RIG-I/TLR3/NLRP3-dependent inflammasome activation in

influenza A virus infected cells. *PLoS Pathog* 9(4), e1003256. doi: 10.1371/journal.ppat.1003256.

Prabhu, N., Ho, A.W., Wong, K.H., Hutchinson, P.E., Chua, Y.L., Kandasamy, M., et al. (2013). Gamma interferon regulates contraction of the influenza virus-specific CD8 T cell response and limits the size of the memory population. *J Virol* 87(23), 12510-12522. doi: 10.1128/JVI.01776-13.

Prakash, A., and Levy, D.E. (2006). Regulation of IRF7 through cell type-specific protein stability. *Biochem Biophys Res Commun* 342(1), 50-56. doi: 10.1016/j.bbrc.2006.01.122.

Qian, W., Wei, X., Li, Y., Guo, K., Zou, Z., Zhou, H., et al. (2018). Duck interferon regulatory factor 1 acts as a positive regulator in duck innate antiviral response. *Dev Comp Immunol* 78, 1-13. doi: 10.1016/j.dci.2017.09.004.

Qiu, Y., Shen, Y., Li, X., Ding, C., and Ma, Z. (2008). Molecular cloning and functional characterization of a novel isoform of chicken myeloid differentiation factor 88 (MyD88). *Developmental and Comparative Immunology* 32(12), 1522-1530. doi: 10.1016/j.dci.2008.05.016.

Rajsbaum, R., Albrecht, R.A., Wang, M.K., Maharaj, N.P., Versteeg, G.A., Nistal-Villán, E., et al. (2012). Species-Specific Inhibition of RIG-I Ubiquitination and IFN Induction by the Influenza A Virus NS1 Protein. *PLOS Pathogens* 8(11), e1003059. doi: 10.1371/journal.ppat.1003059.

Ran, J.S., Jin, J., Zhang, X.X., Wang, Y., Ren, P., Li, J.J., et al. (2018). Molecular Characterization, Expression and Functional Analysis of Chicken STING. *Int J Mol Sci* 19(12), 1-19. doi: 10.3390/ijms19123706.

Ranaware, P.B., Mishra, A., Vijayakumar, P., Gandhale, P.N., Kumar, H., Kulkarni, D.D., et al. (2016). Genome wide host gene expression analysis in chicken lungs infected with avian influenza viruses. *PLoS One* 11(4), e0153671. doi: 10.1371/journal.pone.0153671.

Reuter, A., Soubies, S., Härtle, S., Schusser, B., Kaspers, B., Staeheli, P., et al. (2014). Antiviral activity of lambda interferon in chickens. *Journal of Virology* 88(5), 2835. doi: 10.1128/JVI.02764-13.

Rohde, F., Schusser, B., Hron, T., Farkasova, H., Plachy, J., Hartle, S., et al. (2018). Characterization of chicken tumor necrosis factor-alpha, a long missed cytokine in birds. *Front Immunol* 9(APR), 605. doi: 10.3389/fimmu.2018.00605.

Rong, E., Hu, J., Yang, C., Chen, H., Wang, Z., Liu, X., et al. (2018a). Broad-spectrum antiviral functions of duck interferon-induced protein with tetratricopeptide repeats (AvIFIT). *Dev Comp Immunol* 84**,** 71-81. doi: 10.1016/j.dci.2018.02.007.

Rong, E., Wang, X., Chen, H., Yang, C., Hu, J., Liu, W., et al. (2018b). Molecular mechanisms for the adaptive switching between the OAS/RNase L and OASL/RIG-I pathways in birds and mammals. *Front Immunol* 9(1398)**,** 1398. doi: 10.3389/fimmu.2018.01398.

Rothenfusser, S., Goutagny, N., DiPerna, G., Gong, M., Monks, B.G., Schoenemeyer, A., et al. (2005). The RNA helicase Lgp2 inhibits TLR-independent sensing of viral replication by retinoic acid-inducible gene-I. *J Immunol* 175(8)**,** 5260-5268. doi: 10.4049/jimmunol.175.8.5260.

Ruby, T., Bed'Hom, B., Wittzell, H., Morin, V., Oudin, A., and Zoorob, R. (2005). Characterisation of a cluster of TRIM-B30.2 genes in the chicken MHC B locus. *Immunogenetics* 57(1-2)**,** 116-128. doi: 10.1007/s00251-005-0770-x.

Runstadler, J.A., Happ, G.M., Slemons, R.D., Sheng, Z.M., Gundlach, N., Petrula, M., et al. (2007). Using RRT-PCR analysis and virus isolation to determine the prevalence of avian influenza virus infections in ducks at Minto Flats State Game Refuge, Alaska, during August 2005. *Arch Virol* 152(10)**,** 1901-1910. doi: 10.1007/s00705-007-0994-1.

Saito, L.B., Diaz-Satizabal, L., Evseev, D., Fleming-Canepa, X., Mao, S., Webster, R.G., et al. (2018). IFN and cytokine responses in ducks to genetically similar H5N1 influenza A viruses of varying pathogenicity. *J Gen Virol* 99(4)**,** 464-474. doi: 10.1099/jgv.0.001015.

Santhakumar, D., Rohaim, M., Hussein, H.A., Hawes, P., Ferreira, H.L., Behboudi, S., et al. (2018). Chicken interferon-induced protein with tetratricopeptide repeats 5 antagonizes replication of RNA viruses. *Sci Rep* 8(1)**,** 6794. doi: 10.1038/s41598-018-24905-y.

Santhakumar, D., Rubbenstroth, D., Martinez-Sobrido, L., and Munir, M. (2017). Avian interferons and their antiviral effectors. *Front Immunol* 8(JAN)**,** 49. doi: 10.3389/fimmu.2017.00049.

Sardiello, M., Cairo, S., Fontanella, B., Ballabio, A., and Meroni, G. (2008). Genomic analysis of the TRIM family reveals two groups of genes with distinct evolutionary properties. *BMC Evol Biol* 8(1)**,** 225. doi: 10.1186/1471-2148-8-225.

Sarkar, S.N., Bandyopadhyay, S., Ghosh, A., and Sen, G.C. (1999a). Enzymatic characteristics of recombinant medium isozyme of 2'-5' oligoadenylate synthetase. *J Biol Chem* 274(3)**,** 1848-1855. doi: 10.1074/jbc.274.3.1848.

Sarkar, S.N., Ghosh, A., Wang, H.W., Sung, S.S., and Sen, G.C. (1999b). The nature of the catalytic domain of 2'-5'-oligoadenylate synthetases. *J Biol Chem* 274(36)**,** 25535-25542. doi: 10.1074/jbc.274.36.25535.

Sato, M., Hata, N., Asagiri, M., Nakaya, T., Taniguchi, T., and Tanaka, N. (1998). Positive feedback regulation of type I IFN genes by the IFN-inducible transcription factor IRF-7. *FEBS Lett* 441(1)**,** 106-110. doi: 10.1016/s0014-5793(98)01514-2.

Sato, S., Sugiyama, M., Yamamoto, M., Watanabe, Y., Kawai, T., Takeda, K., et al. (2003). Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF) associates with TNF receptor-associated factor 6 and TANK-binding kinase 1, and activates two distinct transcription factors, NF-kappa B and IFN-regulatory factor-3, in the Toll-like receptor signaling. *J Immunol* 171(8)**,** 4304-4310. doi: 10.4049/jimmunol.171.8.4304.

Satoh, T., Kato, H., Kumagai, Y., Yoneyama, M., Sato, S., Matsushita, K., et al. (2010). LGP2 is a positive regulator of RIG-I- and MDA5-mediated antiviral responses. *Proceedings of the National Academy of Sciences of the United States of America* 107(4)**,** 1512-1517. doi: 10.1073/pnas.0912986107.

Sawyer, S.L., Wu, L.I., Emerman, M., and Malik, H.S. (2005). Positive selection of primate TRIM5alpha identifies a critical species-specific retroviral restriction domain. *Proc Natl Acad Sci U S A* 102(8)**,** 2832-2837. doi: 10.1073/pnas.0409853102.

Schmidt, A., Schwerd, T., Hamm, W., Hellmuth, J.C., Cui, S., Wenzel, M., et al. (2009). 5'-triphosphate RNA requires base-paired structures to activate antiviral signaling via RIG-I. *Proceedings of the National Academy of Sciences of the United States of America* 106(29)**,** 12067-12072. doi: 10.1073/pnas.0900971106.

Schroder, K., Hertzog, P.J., Ravasi, T., and Hume, D.A. (2004). Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol* 75(2)**,** 163-189. doi: 10.1189/jlb.0603252.

Schusser, B., Reuter, A., von der Malsburg, A., Penski, N., Weigend, S., Kaspers, B., et al. (2011). Mx is dispensable for interferon-mediated resistance of chicken cells against influenza A virus. *J Virol* 85(16)**,** 8307-8315. doi: 10.1128/JVI.00535-11.

Seo, S.U., Kwon, H.J., Song, J.H., Byun, Y.H., Seong, B.L., Kawai, T., et al. (2010). MyD88 signaling is indispensable for primary influenza A virus infection but dispensable for secondary infection. *J Virol* 84(24)**,** 12713-12722. doi: 10.1128/JVI.01675-10.

Shiina, T., Briles, W.E., Goto, R.M., Hosomichi, K., Yanagiya, K., Shimizu, S., et al. (2007). Extended gene map reveals tripartite motif, C-type lectin, and Ig superfamily type genes within a subregion of the chicken MHC-B affecting infectious disease. *J Immunol* 178(11)**,** 7162-7172. doi: 10.4049/jimmunol.178.11.7162.

Shinya, K., Okamura, T., Sueta, S., Kasai, N., Tanaka, M., Ginting, T.E., et al. (2011). Toll-like receptor pre-stimulation protects mice against lethal infection with highly pathogenic influenza viruses. *Virology Journal* 8(1)**,** 97. doi: 10.1186/1743-422X-8-97.

Silverman, R.H., and Weiss, S.R. (2014). Viral phosphodiesterases that antagonize double-stranded RNA signaling to RNase L by degrading 2-5A. *J Interferon Cytokine Res* 34(6)**,** 455-463. doi: 10.1089/jir.2014.0007.

Smietanka, K., Minta, Z., Wyrostek, K., Jóźwiak, M., Olszewska, M., Domańska-Blicharz, A.K., et al. (2011). Susceptibility of pigeons to clade 1 and 2.2 high pathogenicity avian influenza H5N1 virus. *Avian diseases* 55(1)**,** 106-112. doi: 10.1637/9514-090110-ResNote.1.

Smith, J., Smith, N., Yu, L., Paton, I.R., Gutowska, M.W., Forrest, H.L., et al. (2015). A comparative analysis of host responses to avian influenza infection in ducks and chickens highlights a role for the interferon-induced transmembrane proteins in viral resistance. *BMC Genomics* 16(1)**,** 574. doi: 10.1186/s12864-015-1778-8.

Smith, S.E., Gibson, M.S., Wash, R.S., Ferrara, F., Wright, E., Temperton, N., et al. (2013). Chicken interferon-inducible transmembrane protein 3 restricts influenza viruses and lyssaviruses in vitro. *J Virol* 87(23)**,** 12957-12966. doi: 10.1128/JVI.01443-13.

Sokawa, J., Shimizu, N., and Sokawa, Y. (1984). "Presence of (2•OE-5•OE)Oligoadenylate Synthetase in Avian Erythrocytes".).

Sommereyns, C., Paul, S., Staeheli, P., and Michiels, T. (2008). IFN-lambda (IFN-lambda) is expressed in a tissue-dependent fashion and primarily acts on epithelial cells in vivo. *PLoS Pathog* 4(3)**,** e1000017. doi: 10.1371/journal.ppat.1000017.

Sparrer, K.M.J., Gableske, S., Zurenski, M.A., Parker, Z.M., Full, F., Baumgart, G.J., et al. (2017). TRIM23 mediates virus-induced autophagy via activation of TBK1. *Nat Microbiol* 2(11)**,** 1543-1557. doi: 10.1038/s41564-017-0017-2.

St. Paul, M., Mallick, A.I., Read, L.R., Villanueva, A.I., Parvizi, P., Abdul-Careem, M.F., et al. (2012). Prophylactic treatment with Toll-like receptor ligands enhances host immunity to avian influenza virus in chickens. *Vaccine* 30(30)**,** 4524-4531. doi: https://doi.org/10.1016/j.vaccine.2012.04.033.

Staeheli, P., Grob, R., Meier, E., Sutcliffe, J.G., and Haller, O. (1988). Influenza virus-susceptible mice carry Mx genes with a large deletion or a nonsense mutation. *Mol Cell Biol* 8(10)**,** 4518-4523. doi: 10.1128/mcb.8.10.4518.

Stewart, C.R., Bagnaud-Baule, A., Karpala, A.J., Lowther, S., Mohr, P.G., Wise, T.G., et al. (2012). Toll-like receptor 7 ligands inhibit influenza A infection in chickens. *J Interferon Cytokine Res* 32(1)**,** 46-51. doi: 10.1089/jir.2011.0036.

Stremlau, M., Owens, C.M., Perron, M.J., Kiessling, M., Autissier, P., and Sodroski, J. (2004). The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. *Nature* 427(6977)**,** 848-853. doi: 10.1038/nature02343.

Sturm-Ramirez, K.M., Ellis, T., Bousfield, B., Bissett, L., Dyrting, K., Rehg, J.E., et al. (2004). Reemerging H5N1 influenza viruses in Hong Kong in 2002 are highly pathogenic to ducks. *Journal of virology* 78(9)**,** 4892-4901. doi: 10.1128/jvi.78.9.4892-4901.2004.

Tag-El-Din-Hassan, H.T., Morimatsu, M., and Agui, T. (2018). Functional analysis of duck, goose, and ostrich 2'-5'-oligoadenylate synthetase. *Infect Genet Evol* 62**,** 220-232. doi: 10.1016/j.meegid.2018.04.036.

Tag-El-Din-Hassan, H.T., Sasaki, N., Moritoh, K., Torigoe, D., Maeda, A., and Agui, T. (2012). The chicken 2 '-5 ' oligoadenylate synthetase A inhibits the replication of West Nile Virus. *Japanese Journal of Veterinary Research* 60(2-3)**,** 95-103.

Tanaka, Y., and Chen, Z.J. (2012). STING specifies IRF3 phosphorylation by TBK1 in the cytosolic DNA signaling pathway. *Sci Signal* 5(214)**,** ra20. doi: 10.1126/scisignal.2002521.

Taubenberger, J.K., and Kash, J.C. (2010). Influenza virus evolution, host adaptation, and pandemic formation. *Cell host & microbe* 7(6)**,** 440-451. doi: 10.1016/j.chom.2010.05.009.

te Velthuis, A.J.W., Long, J.C., Bauer, D.L.V., Fan, R.L.Y., Yen, H.-L., Sharps, J., et al. (2018). Mini viral RNAs act as innate immune agonists during influenza virus infection. *Nature Microbiology* 3(11)**,** 1234-1242. doi: 10.1038/s41564-018-0240-5.

Teijaro, J.R., Walsh, K.B., Rice, S., Rosen, H., and Oldstone, M.B. (2014). Mapping the innate signaling cascade essential for cytokine storm during influenza virus infection. *Proc Natl Acad Sci U S A* 111(10)**,** 3799-3804. doi: 10.1073/pnas.1400593111.

Temperley, N.D., Berlin, S., Paton, I.R., Griffin, D.K., and Burt, D.W. (2008). Evolution of the chicken Toll-like receptor gene family: a story of gene gain and gene loss. *BMC Genomics* 9**,** 62. doi: 10.1186/1471-2164-9-62.

Thomas, P.G., Dash, P., Aldridge, J.R., Jr., Ellebedy, A.H., Reynolds, C., Funk, A.J., et al. (2009). The intracellular sensor NLRP3 mediates key innate and healing responses to influenza A virus via the regulation of caspase-1. *Immunity* 30(4)**,** 566-575. doi: 10.1016/j.immuni.2009.02.006.

Uhlen, M., Fagerberg, L., Hallstrom, B.M., Lindskog, C., Oksvold, P., Mardinoglu, A., et al. (2015). Proteomics. Tissue-based map of the human proteome. *Science* 347(6220)**,** 1260419. doi: 10.1126/science.1260419.

van der Veen, A.G., Maillard, P.V., Schmidt, J.M., Lee, S.A., Deddouche-Grass, S., Borg, A., et al. (2018). The RIG-I-like receptor LGP2 inhibits Dicer-dependent processing of long double-stranded RNA and blocks RNA interference in mammalian cells. *EMBO J* 37(4). doi: 10.15252/embj.201797479.

van Gent, M., Sparrer, K.M.J., and Gack, M.U. (2018). TRIM Proteins and Their Roles in Antiviral Host Defenses. *Annu Rev Virol* 5(1)**,** 385-405. doi: 10.1146/annurev-virology-092917-043323.

Vanderven, H.A., Petkau, K., Ryan-Jean, K.E., Aldridge, J.R., Jr., Webster, R.G., and Magor, K.E. (2012). Avian influenza rapidly induces antiviral genes in duck lung and intestine. *Mol Immunol* 51(3-4)**,** 316-324. doi: 10.1016/j.molimm.2012.03.034.

Vidana, B., Dolz, R., Busquets, N., Ramis, A., Sanchez, R., Rivas, R., et al. (2018). Transmission and immunopathology of the avian influenza virus A/Anhui/1/2013 (H7N9) human isolate in three commonly commercialized avian species. *Zoonoses Public Health* 65(3)**,** 312-321. doi: 10.1111/zph.12393.

Wang, B., Chen, Y., Mu, C., Su, Y., Liu, R., Huang, Z., et al. (2015). Identification and expression analysis of the interferon-induced protein with tetratricopeptide repeats 5 (IFIT5) gene in duck (Anas platyrhynchos domesticus). *PLoS One* 10(3)**,** e0121065. doi: 10.1371/journal.pone.0121065.

Wang, X., Hinson, E.R., and Cresswell, P. (2007). The interferon-inducible protein viperin inhibits influenza virus release by perturbing lipid rafts. *Cell Host Microbe* 2(2)**,** 96-105. doi: 10.1016/j.chom.2007.06.009.

Wang, X.X., Lu, C., Rong, E.G., Hu, J.X., Xing, Y.L., Liu, Z.Y., et al. (2019). Identification of novel genes associated with duck OASL in response to influenza A virus. *Journal of Integrative Agriculture* 18(7)**,** 1451-1459. doi: 10.1016/S2095-3119(19)62685-7.

Wang, Y., Yin, Y., Lan, X., Ye, F., Tian, K., Zhao, X., et al. (2017). Molecular characterization, expression of chicken TBK1 gene and its effect on IRF3 signaling pathway. *PLoS One* 12(5)**,** e0177608. doi: 10.1371/journal.pone.0177608.

Weber, M., Sediri, H., Felgenhauer, U., Binzen, I., Bänfer, S., Jacob, R., et al. (2015). Influenza virus adaptation PB2-627K modulates nucleocapsid inhibition by the pathogen sensor RIG-I. *Cell host & microbe* 17(3)**,** 309-319. doi: 10.1016/j.chom.2015.01.005.

Webster, R.G., Bean, W.J., Gorman, O.T., Chambers, T.M., and Kawaoka, Y. (1992). Evolution and ecology of influenza A viruses. *Microbiol Rev* 56(1)**,** 152-179.

Wei, L., Cui, J., Song, Y., Zhang, S., Han, F., Yuan, R., et al. (2014). Duck MDA5 functions in innate immunity against H5N1 highly pathogenic avian influenza virus infections. *Vet Res* 45(1)**,** 66. doi: 10.1186/1297-9716-45-66.

Wei, L., Song, Y., Cui, J., Qu, N., Wang, N., Ouyang, G., et al. (2016a). Cloning, characterization, and expression analysis of LGP2 cDNA from goose, Anser cygnoides. *Poult Sci* 95(10)**,** 2290-2296. doi: 10.3382/ps/pew162.

Wei, X., Qian, W., Sizhu, S., Li, Y., Guo, K., Jin, M., et al. (2018). Negative regulation of interferon-beta production by alternative splicing of tumor necrosis factor receptor-associated factor 3 in ducks. *Front Immunol* 9(MAR)**,** 409. doi: 10.3389/fimmu.2018.00409.

Wei, X., Qian, W., Sizhu, S., Shi, L., Jin, M., and Zhou, H. (2016b). Molecular cloning and functional analysis of the duck TIR domain-containing adaptor inducing IFN-beta (TRIF) gene. *Dev Comp Immunol* 65**,** 369-376. doi: 10.1016/j.dci.2016.08.006.

Weiss, I.D., Wald, O., Wald, H., Beider, K., Abraham, M., Galun, E., et al. (2010). IFN-gamma treatment at early stages of influenza virus infection protects mice from death in a NK cell-dependent manner. *J Interferon Cytokine Res* 30(6)**,** 439-449. doi: 10.1089/jir.2009.0084.

Wheaton, S., Lambourne, M.D., Sarson, A.J., Brisbin, J.T., Mayameei, A., and Sharif, S. (2007). Molecular cloning and expression analysis of chicken MyD88 and TRIF genes. *DNA Seq* 18(6)**,** 480-486. doi: 10.1080/10425170701295856.

Wozniakowski, G., Samorek-Salamonowicz, E., and Gawel, A. (2014). Occurrence of reovirus infection in Muscovy ducks (Cairina moschata) in south western Poland. *Pol J Vet Sci* 17(2)**,** 299-305. doi: 10.2478/pjvs-2014-0041.

Wu, B., and Hur, S. (2015a). How RIG-I like receptors activate MAVS. *Curr Opin Virol* 12**,** 91-98. doi: 10.1016/j.coviro.2015.04.004.

Wu, B., Peisley, A., Tetrault, D., Li, Z., Egelman, E.H., Magor, K.E., et al. (2014). Molecular imprinting as a signal-activation mechanism of the viral RNA sensor RIG-I. *Mol Cell* 55(4)**,** 511-523. doi: 10.1016/j.molcel.2014.06.010.

Wu, S., Zhang, J., Huang, J., Li, W., Liu, Z., He, Z., et al. (2019). Immune-related gene expression in ducks infected with waterfowl-origin H5N6 highly pathogenic avian influenza viruses. *Front Microbiol* 10(February)**,** 1782. doi: 10.3389/fmicb.2019.01782.

Wu, W., Wang, X., Zhang, W., Tian, L., Booth, J.L., Duggan, E.S., et al. (2018). RIG-I signaling via MAVS is dispensable for survival in lethal influenza infection in vivo. *Mediators Inflamm* 2018**,** 6808934. doi: 10.1155/2018/6808934.

Wu, W., Zhang, W., Duggan, E.S., Booth, J.L., Zou, M.H., and Metcalf, J.P. (2015b). RIG-I and TLR3 are both required for maximum interferon induction by influenza virus in human lung alveolar epithelial cells. *Virology* 482**,** 181-188. doi: 10.1016/j.virol.2015.03.048.

Xiao, Y., Reeves, M.B., Caulfield, A.F., Evseev, D., and Magor, K.E. (2018). The core promoter controls basal and inducible expression of duck retinoic acid inducible gene-I (RIG-I). *Mol Immunol* 103(September)**,** 156-165. doi: 10.1016/j.molimm.2018.09.002.

Xu, L., Yu, D., Fan, Y., Liu, Y.-P., and Yao, Y.-G. (2019). Evolutionary selection on MDA5 and LGP2 in the chicken preserves antiviral competence in the absence of RIG-I. *Journal of Genetics and Genomics* 46(10)**,** 499-503. doi: https://doi.org/10.1016/j.jgg.2019.10.001.

Xu, L., Yu, D., Fan, Y., Peng, L., Wu, Y., and Yao, Y.G. (2016). Loss of RIG-I leads to a functional replacement with MDA5 in the Chinese tree shrew. *Proc Natl Acad Sci U S A* 113(39)**,** 10950-10955. doi: 10.1073/pnas.1604939113.

Yamamoto, M., Sato, S., Hemmi, H., Hoshino, K., Kaisho, T., Sanjo, H., et al. (2003). Role of Adaptor TRIF in the MyD88-Independent Toll-Like Receptor Signaling Pathway. *Science* 301(5633)**,** 640. doi: 10.1126/science.1087262.

Yamamoto, Y., Nakamura, K., Yamada, M., and Mase, M. (2012). Limited susceptibility of pigeons experimentally inoculated with H5N1 highly pathogenic avian influenza viruses. *The Journal of veterinary medical science* 74(2)**,** 205-208. doi: 10.1292/jvms.11-0312.

Yang, H.L., Feng, Z.Q., Zeng, S.Q., Li, S.M., Zhu, Q., and Liu, Y.P. (2015). Molecular cloning and expression analysis of TRAF3 in chicken. *Genet Mol Res* 14(2)**,** 4408-4419. doi: 10.4238/2015.April.30.14.

Yang, Y., Huang, Y., Yu, Y., Zhou, S., Wang, S., Yang, M., et al. (2016). Negative regulation of the innate antiviral immune response by TRIM62 from orange spotted grouper. *Fish Shellfish Immunol* 57**,** 68-78. doi: 10.1016/j.fsi.2016.08.035.

Yao, Q., Fischer, K.P., Arnesen, K., Tyrrell, D.L., and Gutfreund, K.S. (2014). Molecular cloning, expression and characterization of Pekin duck interferon-λ. *Gene* 548(1)**,** 29-38. doi: https://doi.org/10.1016/j.gene.2014.06.066.

Ye, J., Yu, M., Zhang, K., Liu, J., Wang, Q., Tao, P., et al. (2015). Tissue-specific expression pattern and histological distribution of NLRP3 in Chinese yellow chicken. *Vet Res Commun* 39(3)**,** 171-177. doi: 10.1007/s11259-015-9641-6.

Yoneyama, M., Kikuchi, M., Matsumoto, K., Imaizumi, T., Miyagishi, M., Taira, K., et al. (2005). Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. *J Immunol* 175(5)**,** 2851-2858. doi: 10.4049/jimmunol.175.5.2851.

Yoneyama, M., Kikuchi, M., Natsukawa, T., Shinobu, N., Imaizumi, T., Miyagishi, M., et al. (2004). The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat Immunol* 5(7)**,** 730-737. doi: 10.1038/ni1087.

Yuk, S.S., Lee, D.H., Park, J.K., Tseren-Ochir, E.O., Kwon, J.H., Noh, J.Y., et al. (2016). Pre-immune state induced by chicken interferon gamma inhibits the replication of H1N1

human and H9N2 avian influenza viruses in chicken embryo fibroblasts. *Virol J* 13(1)**,** 71. doi: 10.1186/s12985-016-0527-1.

Zhang, B., Liu, X., Chen, W., and Chen, L. (2013). IFIT5 potentiates anti-viral response through enhancing innate immune signaling pathways. *Acta Biochim Biophys Sin (Shanghai)* 45(10)**,** 867-874. doi: 10.1093/abbs/gmt088.

Zhang, M., Song, K., Li, C., Chen, Z., Ding, C., and Liu, G. (2015a). Molecular cloning of Peking duck Toll-like receptor 3 (duTLR3) gene and its responses to reovirus infection. *Virol J* 12(1)**,** 207. doi: 10.1186/s12985-015-0434-x.

Zhang, W., Chen, S., Mahalingam, S., Wang, M., and Cheng, A. (2017). An updated review of avian-origin Tembusu virus: a newly emerging avian Flavivirus. *J Gen Virol* 98(10)**,** 2413-2420. doi: 10.1099/jgv.0.000908.

Zhang, Z., Jia, Y., Almeida, P., Mank, J.E., van Tuinen, M., Wang, Q., et al. (2018). Whole-genome resequencing reveals signatures of selection and timing of duck domestication. *GigaScience* 7(4). doi: 10.1093/gigascience/giy027.

Zhang, Z., Zou, T., Hu, X., and Jin, H. (2015b). Type III interferon gene expression in response to influenza virus infection in chicken and duck embryonic fibroblasts. *Mol Immunol* 68(2 Pt C)**,** 657-662. doi: 10.1016/j.molimm.2015.10.013.

Zhong, B., Yang, Y., Li, S., Wang, Y.Y., Li, Y., Diao, F., et al. (2008). The adaptor protein MITA links virus-sensing receptors to IRF3 transcription factor activation. *Immunity* 29(4)**,** 538-550. doi: 10.1016/j.immuni.2008.09.003.

Zhong, Z., Ji, Y., Fu, Y., Liu, B., and Zhu, Q. (2015). Molecular characterization and expression analysis of the duck viperin gene. *Gene* 570(1)**,** 100-107. doi: 10.1016/j.gene.2015.06.003.

Zhou, X., Michal, J.J., Zhang, L., Ding, B., Lunney, J.K., Liu, B., et al. (2013). Interferon induced IFIT family genes in host antiviral defense. *Int J Biol Sci* 9(2)**,** 200-208. doi: 10.7150/ijbs.5613.

Zhu, J., Zhang, Y., Ghosh, A., Cuevas, R.A., Forero, A., Dhar, J., et al. (2014). Antiviral activity of human OASL protein is mediated by enhancing signaling of the RIG-I RNA sensor. *Immunity* 40(6)**,** 936-948. doi: 10.1016/j.immuni.2014.05.007.

Zou, J., Chang, M., Nie, P., and Secombes, C.J. (2009). Origin and evolution of the RIG-I like RNA helicase gene family. *BMC Evol Biol* 9(1)**,** 85. doi: 10.1186/1471-2148-9-85.

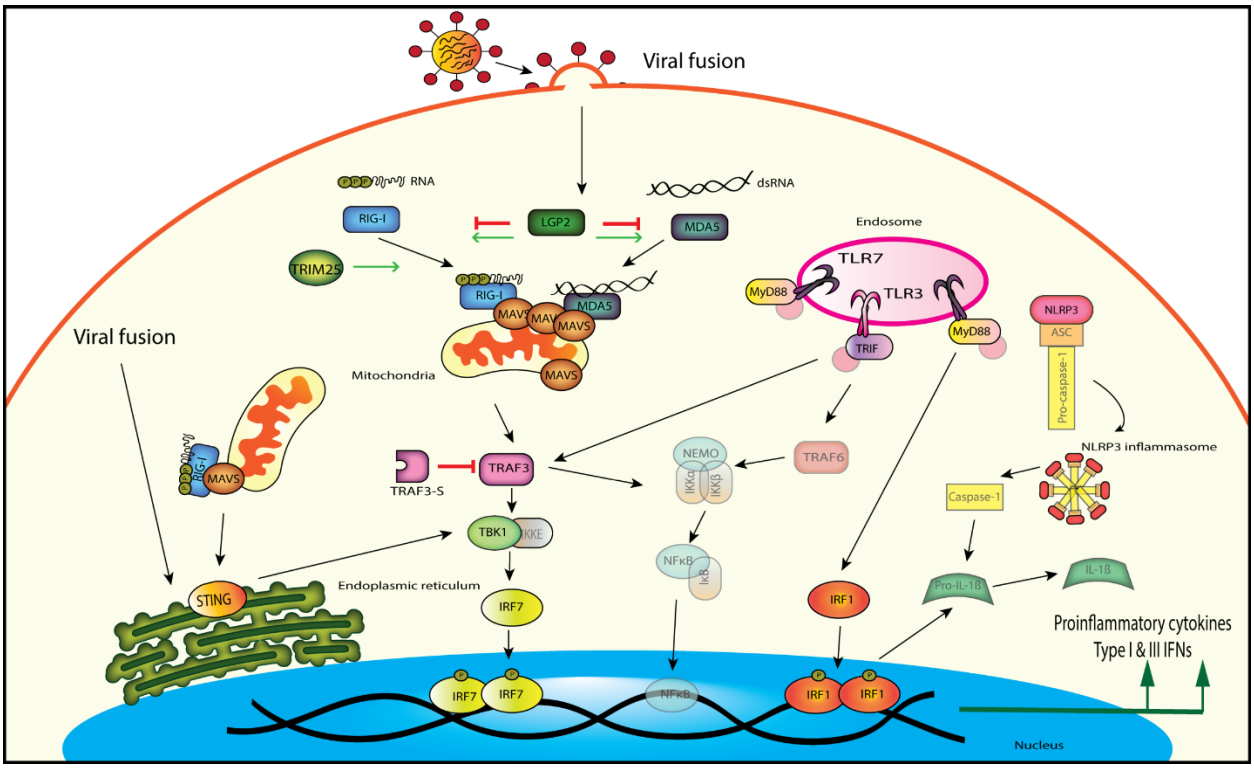


Figure 1.1. Influenza A virus is detected by several different innate immune signaling proteins in the cell. RIG-I and MDA5 both can bind to viral RNA and signal downstream through MAVS, TBK1, and IRF7. Viral fusion can also be detected by the endoplasmic reticulum localized STING, which then signals through TBK1 to induce interferon production. TLRs located in the endosome can recognize viral RNA and signal through different adaptor proteins to induce proinflammatory cytokines and IFNs. TLR3 uses TRIF as an adaptor protein to amplify signaling through TRAF3 or TRAF6, while TLR7 uses the adaptor MyD88 to signal through TRAF3. TLR signaling through MyD88 can also activate the NLRP3 inflammasome, which increases pro-inflammatory cytokine production through IRF1.

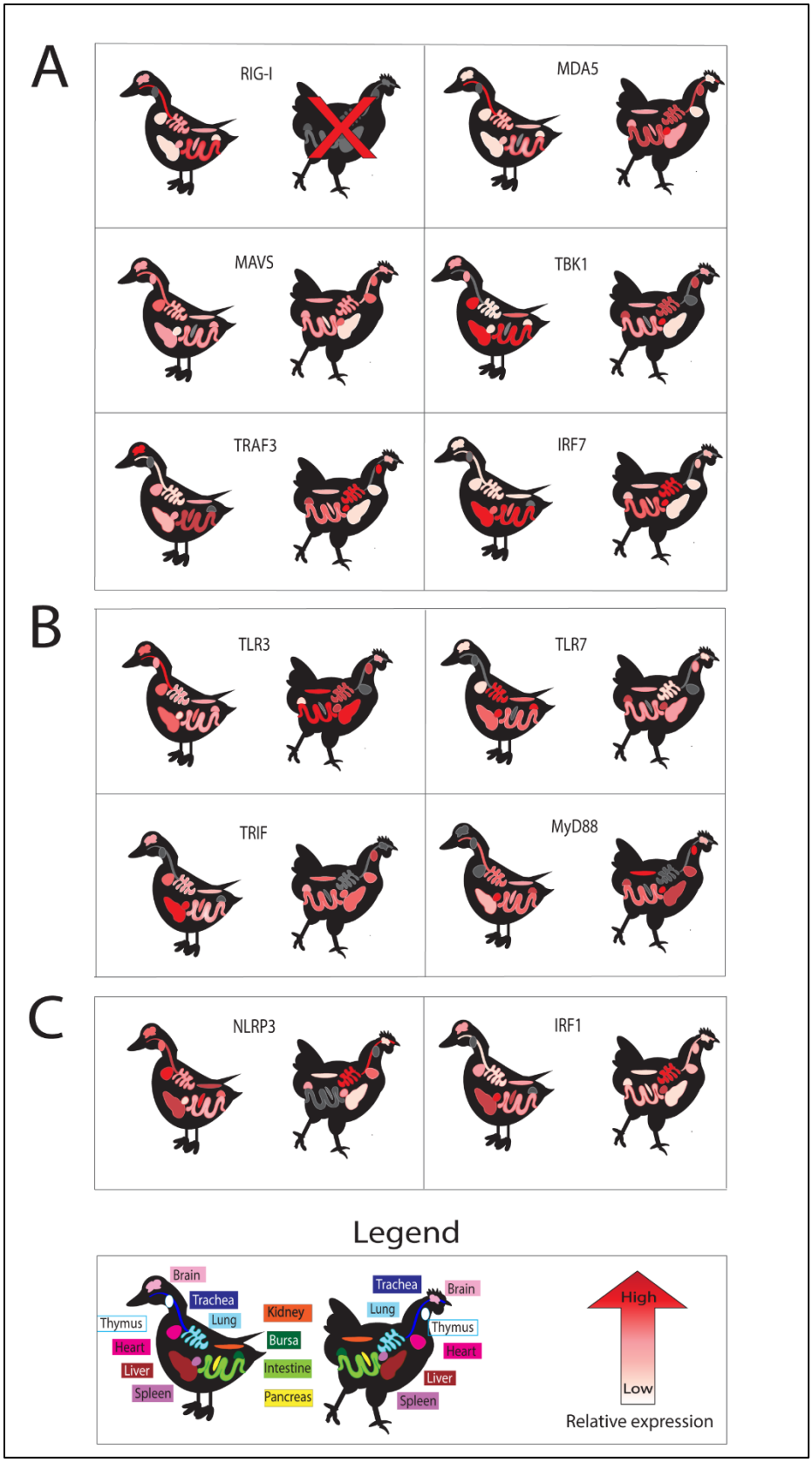


Figure 1.2. Basal tissue expression of genes in uninfected ducks compared to those in

chickens. Tissue expression is shown for components of RLR (A), TLR (B), and NLRP3 inflammasome (C) signaling pathways. We show relative expression of each gene studied in those tissues. High relative basal gene expression is denoted by red, while lower expression is indicated by pinks and whites. Gray coloring indicates no data available for the gene in the indicated tissue. All data was extracted from individual studies in this review, and color scales are relative for data from individual studies. All data is for mallard duck, except RIG-I and MDA5, which are Muscovy duck. Data obtained for chicken MDA5, MAVS, and IRF1 were obtained from the chicken atlas (http://biogps.org/), and averages for each tissue in adult chickens were used to estimate relative expression.

CHAPTER 2

TRIM protein family evolution in direct and indirect restriction of Influenza A virus

This chapter outlines the current understanding of the evolution of TRIM genes, and highlights TRIM genes involved in indirect pathway modulation of anti-viral signaling pathways implicated in restriction of influenza A virus (IAV) in the cell as well as direct antiviral activity of TRIM proteins which target IAV. A comprehensive review of genes involved in IAV restriction in ducks compared to chickens and humans can be found in Chapter 1, while this chapter focuses on TRIM protein restriction of influenza A virus through augmentation and restriction, specifically.

2.1 Introduction

TRIM proteins are named for their conserved N-terminal tripartite motif. This motif consists of a RING domain, one or two B-box domains, and a coiled-coil domain (Ozato et al., 2008; Meroni, 2012). The RING domain usually acts as an E3 ubiquitin ligase domain and catalyzes the reaction of adding ubiquitin to substrate proteins (Ardley et al., 2005; Metzger et al., 2014). RING domains also can allow some TRIM proteins to form higher-order structures or oligomerize. The RING domain of TRIM proteins is a zinc-binding domain, and a canonical RING structure is Cys-X_2-Cys-$X_{(9-39)}$-Cys-$X_{(1-3)}$-His/Cys-$X_{(2-3)}$-Cys/His-$X_{(4-48)}$-Cys-X_2-Cys (Borden et al., 1996). The conserved cysteine and histidine residues are located in the fold of the domain and aid in forming a cross-brace structure through their interactions with two zinc atoms (Massiah, 2019). TRIM RING domains are classified as RING-H2 or RING-C2 depending on the residue located at the fifth conserved site.

B-box domains are related to RING domains in that they are also zinc-binding domains with conserved cystine and histidine residues. B-box domains in TRIM proteins consist of either a tandem B-box type I followed by a B-box type II domain, or a singular B-box domain (Usually B-box Type II) (Massiah, 2019). The B-box type I consensus sequence is Cys-X_2-Cys-$X_{(6-17)}$-Cys-X_2-C-$X_{(4-8)}$-Cys--$X_{(2-3)}$-Cys/His-$X_{(3-4)}$-His-$X_{(5-10)}$-His [C5(C/H)H2] while the B-box type 2 consensus sequence is Cys-$X_{(2-4)}$-His-$X_{(7-10)}$-Asp/$Cys_{(4-7)}$-Cys-X_2-Cys-$X_{(3-6)}$-His-$X_{(2-5)}$-His

[CHC(D/C)C2H2]. B-box type I structure more closely resembles the fold structure seen in RING domains (Meroni, 2012). B-box domain function is less well understood than other domains, however RINGless TRIM proteins such as TRIM16 and TRIM29 can both confer E3 ubiquitin ligase activity, and it is suggested this activity comes from their B-box domains (Bell et al., 2012; Xing et al., 2016). B-box domains also aid in oligomerization and heterodimerization of TRIM proteins to increase E3 ubiquitin ligase activity of RING domains (Wallenhammar et al., 2017; Dickson et al., 2018).

Coiled-coil domains are often associated with higher order assembly and oligomerization of TRIM proteins. In both TRIM5α and TRIM25, the coiled-coil domain forms anti-parallel hairpin dimers (Sanchez et al., 2014). TRIM32 also oligomerizes using its coiled-coil domain (Koliopoulos et al., 2016). This self-association of TRIM proteins through their various domains has been suggested as necessary for these proteins to have catalytic activity.

TRIM proteins have variable C-terminal domains which further break them down into subfamilies. The C-terminal domains of TRIM proteins are thought to offer substrate specificity to these proteins and are what aid this incredibly diverse family of proteins to have so many different target substrates, and cellular functions. TRIM proteins are broken down into 11 subfamilies, depending on the variable C-terminal domain present (Short et al., 2006; Ozato et al., 2008; Watanabe et al., 2017). With this designation, there are 11 subfamilies of TRIM proteins, denoted as C-I to C-XI (Figure 2.1).

In the following sections we will outline when these different C-terminal domains first appeared in eukaryotic evolution, as defined by Marín and colleagues (Marín, 2012), as well as document general function and associations of these subfamily members. We will then provide a comprehensive review of TRIM proteins that augment antiviral signaling pathways in the cell, and TRIM proteins that directly restrict IAV. These sections summarize research done on mammalian TRIM proteins, unless otherwise noted. Finally, we summarize what is known about antiviral TRIM proteins in invertebrates, and lower vertebrates.

2.2 TRIM genes in eukaryotes

The following sections details TRIM37, the hypothesized ancestral TRIM, which appears to be present in all eukaryotes.

2.2.1 C-VIII

The C-VIII subfamily is composed of one member, TRIM37. The defining characteristic of the C-VIII subfamily is the merpin and TRAF homology (MATH) domain.

TRIM genes initially were considered to be metazoan specific, with vast expansions of the gene family in vertebrates (Sardiello et al., 2008). However, a study by Marín (2012) found TRIM37 homologs in almost all branches of eukaryotes, including in fungi, plants and various classes of protists. This study suggested that TRIM genes and TNF-receptor associated factor (TRAF) genes may share distant ancestry. TRAF proteins share similar domain architecture to TRIM37, although instead of the classical RING-B-box-CC-MATH domains seen in TRIM37, TRAF proteins have a RING domain followed by 1 to 7 TRAF specific cystine rich regions, and finally a C-terminal MATH domain (Park, 2018). The TRAF-specific cysteine-rich regions have an analogous zinc-binding cross-bracing architecture to that seen in TRIM B-box domains (Marín, 2012). It was suggested that TRAF genes arose in metazoans and that TRIM37-like genes are ancestral to both all TRIM genes and TRAF genes, however, the analysis performed only looked at MATH domain ancestry between these groups. As the MATH domains were approximately 100 AA in size, the bootstrap values between groups were too low to make conclusions of ancestry. Additionally, TRAF genes have now been identified in plants, suggesting that TRAF genes could be ancestral to modern TRIM genes (Qi et al., 2022).

2.3 TRIM genes in animals

The following sections detail the TRIM proteins which arose early in metazoan evolution and have members present in all animals.

2.3.1 C-I

The C-I subfamily is defined by its C-terminal subgroup one signature (COS), fibronectin, type III (FN3) and pre-SPRY splA kinase and ryanodine receptor (PRYSPRY) domains. The members of this subfamily include: TRIM1/MID2, TRIM9, TRIM18/MID1, TRIM36, TRIM46, TRIM67, TRIM76/CMYA5, FSD1, FSD2, FSD1L.

The C-I subfamily of TRIM proteins have diverse functions. For example, TRIM9, TRIM46 and TRIM67 are considered brain-specific TRIM proteins, involved with various aspects of neuronal maintenance and development (Berti et al., 2002; Tanji et al., 2010; van Beuningen et al., 2015; Boyer et al., 2018). TRIM1 and TRIM18, however, function in cell

migration, epithelial-mesenchymal transition and cytokinesis (Zanchetta et al., 2019; Qiao et al., 2020).

2.3.2 C-II

The C-II subfamily is defined by its C-terminal COS and acid domains. The members of this subfamily include TRIM54, TRIM55, TRIM63. These genes were initially termed muscle RING fingers (MuRFs). As the name describes, these genes are highly upregulated in muscle cells and are involved in skeletal muscle development (Foletta et al., 2011a; Perera et al., 2012; Rom et al., 2016) and protein homeostasis in muscle cells (Spencer et al., 2000; Foletta et al., 2011b).

2.3.3 C-VII

The C-VII subfamily is defined by its C-terminal filamin domain (FIL) and multiple NCL-1, HT2A and Lin-41 repeats (NHL) domains. The members of this subfamily include TRIM2, TRIM3, TRIM32, TRIM71 and NHLRC1. The NHL domains are associated with RNA-binding activity (Goyani et al., 2021). Both TRIM32 (Hammell et al., 2009) and TRIM72 (Maller Schulman et al., 2008; Chang et al., 2012) are RNA-binding proteins (RBP) that can bind RNA using their NHL domains (Loedige et al., 2013), and thus help regulate transcriptional activity in the cell.

2.3.4 C-X

The C-X subfamily member, TRIM45 is defined by its C-terminal FIL domain. TRIM45 acts as a tumor suppressor (Zhang et al., 2017; Peng et al., 2020) and transcriptional suppressor (Wang et al., 2004).

2.4 TRIM genes in bilaterians

This section details the TRIM proteins which first arose in the bilaterian group of animals.

2.4.1 C-VI

The C-VI subfamily is defined by its C-terminal plant homeo domain (PHD) and bromodomain (BROMO). The members of this subfamily include TRIM24, TRIM28, TRIM33 and TRIM66. The members of the subfamily are known to modify transcriptional activity (Liang et al., 2011; Agarwal et al., 2021) and it is thought that the C-terminal PHD and BROMO domains help target these proteins to specific histones to aid this function (Chen et al., 2019).

2.4.2 C-IX

The C-IX subfamily is defined by its C-terminal ADP ribosylation factor-like (ARF) domain. TRIM23 is the lone member of this subfamily. The ARF domain of TRIM23 has GTP-ase activity, that is activated upon non-classical K27-linked self-ubiquitination of the ARF domain (Hatakeyama, 2017; Sparrer et al., 2017). TRIM23 is associated with autophagic processes in the cell.

2.5 TRIM genes in vertebrates

The following sections detail the TRIM proteins which arose in vertebrate evolution and have rapidly expanded since then.

2.5.1 C-III

The C-III subfamily is defined by its C-terminal COS and FN3 domains. The sole member of this subfamily is TRIM43. TRIM43 is a centrosomal protein in mammals which was identified as a restriction factor of herpes virus but remains largely understudied (Full et al., 2019).

2.5.2 C-IV

The C-IV subfamily is defined by its C-terminal PRYSPRY domain and is one of the subfamilies of TRIM genes which has gone through the most species-specific expansions. In mammals, the members of this subfamily include (but are not limited to): TRIM1, TRIM1L, TRIM4, TRIM5, TRIM6, TRIM7, TRIM10, TRIM11, TRIM12, TRIM14, TRIM15, TRIM 16, TRIM16L, TRIM17, TRIM21, TRIM22, TRIM25, TRIM26, TRIM27, TRIM34, TRIM35, TRIM38, TRIM39, TRIM41, TRIM43, TRIM47, TRIM48, TRIM49, TRIM50, TRIM51, TRIM53, TRIM58, TRIM60, TRIM62, TRIM64, TRIM65, TRIM68, TRIM69, TRIM72, TRIM75, TRIM77 and many other TRIM-like genes. PRYSPRY domains bind substrate proteins (James et al., 2007; D'Cruz et al., 2013) and are also known to bind RNA (Liu et al., 2016; Choudhury et al., 2017). Many of the C-IV subfamily genes are associated with the MHC in humans (Meyer et al., 2003; Jia et al., 2021), chickens (Ruby et al., 2005; Shiina et al., 2007; Kaufman, 2022), ducks (Blaine et al., 2015) and fish (Boudinot et al., 2011). Notably, many proteins in the C-IV family are interferon inducible and associated with antiviral function (Ozato et al., 2008; Rajsbaum et al., 2008; Sardiello et al., 2008; Carthagena et al., 2009).

2.5.3 C-XI

The C-XI subfamily is defined by its C-terminal transmembrane (TM) domain. The members of this subfamily include TRIM13 and TRIM59. TRIM13 localizes to the endoplasmic reticulum and causes degradation of STING (Narayan et al., 2014; Huang et al., 2017; Li et al., 2022). TRIM59 regulates autophagic processes in the cell and the dysregulation of TRIM59 is implicated in many cancers, indicating TRIM59 may regulate cell cycle progression (Valiyeva et al., 2011).

2.5.4 C-V

The C-V subfamily is composed of TRIM proteins with an undefined C-terminal region. The members of this subfamily include TRIM8, TRIM19/PML, TRIM20/PYRIN, TRIM29, TRIM31, TRIM40, TRIM44, TRIM52, TRIM56, TRIM61, TRIM73, TRIM74 and RNF207. The C-terminal architecture in this group is incredibly diverse, suggesting this group is less related than other subfamilies of TRIM protein. TRIM19 (also known as promyelocytic leukemia or PML) for example, contains a C-terminal domain of unknown function (DUF) which contains an exonuclease III fold and allows TRIM19 to localize to the nucleus (Condemine et al., 2007), while TRIM29 does not have a structurally defined C-terminal domain, yet this area of the protein is known to bind with MAVS (Xing et al., 2018). Additionally, TRIM44 is a unique TRIM as it contains an N-terminal ubiquitin protease domain (UBP) and acts as a deubiquitinase (Hatakeyama, 2017).

2.6 TRIM proteins that augment anti-viral signaling pathways

2.6.1 Innate signaling

In this section we summarize the current knowledge of TRIM protein augmentation of intracellular antiviral signaling pathways. As this thesis is centered on innate immune responses, we focused on RLR, TLR and cGAS pathways. Although RLR and TLR receptors can detect IAV viral RNA directly to activate signaling pathways, cGAS and DNA sensing is still relevant in anti-IAV signaling as these pathways detect viral infection through DNA damage. This section also will not go into detail on pathway component signaling mechanisms, as that was summarized for these pathways in Chapter 1. All pathways and interactions are summarized in Figure 2.2.

Throughout this section, we describe ubiquitination by TRIM proteins, which act as E3 ubiquitin ligases. E3 ubiquitin ligases target substrate proteins and recruit E2 ubiquitin-

conjugating enzymes to attach ubiquitin or polyubiquitin to substrate proteins (Ardley et al., 2005; Yang et al., 2021). Ubiquitin is a highly conserved polypeptide made up of 76 amino acids. Polyubiquitin chains use differing linkages to convey certain messages and outcomes for target proteins. The polyubiquitin chains utilize inner lysine (K) residues or the first methionine (M) to form linkages, and 8 main types have been described: M1, K6, K11, K27, K29, K33, K48 and K63 (Swatek et al., 2016; Tracz et al., 2021). K48 and K63-linked chains are the best described with K48-linked ubiquitin targeting substrate proteins for proteasomal degradation (Hershko et al., 1998; Finley, 2009) and K63-linked ubiquitin activating or stabilizing substrate proteins (Chen et al., 2009; Nathan et al., 2013). The noncanonical ubiquitin linkages (M1, K6, K11, K27, K29 and K33) have a variety of functional roles from activation, degradation and transcriptional regulation (Tracz et al., 2021).

2.6.1.1 TRIM protein interactions with RLRs and MAVs

Two important RNA virus sensing pattern recognition receptors (PRRs) are retinoic acid-inducible gene I (RIG-I) and melanoma differentiation-associated protein 5 (MDA5), members of the RIG-I-like receptor (RLR) family. Both RIG-I and MDA5 sense viral RNA in the cytoplasm (As discussed in detail in Chapter 1) and interact with MAVS through their CARD domains to activate downstream antiviral signaling (Wu et al., 2015). Many TRIM members have been shown to augment this signaling pathway. Perhaps the best known is TRIM25, which stabilizes RIG-I through K63-linked ubiquitination of the CARD domains and increases RIG-I interactions with the signaling adapter MAVS, to increase IFN-β expression (Gack et al., 2007; Miranzo-Navarro et al., 2014). However, recently works have been published suggesting that RNF135/RIPLET, a TRIM-like protein, which also stabilizes RIG-I through K63-linked ubiquitination is essential for RIG-I activation, while TRIM25 is redundant (Hayman et al., 2019). TRIM4 also positively regulates RIG-I signaling through K63-linked ubiquitination of RIG-I (Yan et al., 2014). TRIM65 activates MDA5 through K63-linked ubiquitination of MDA5 (Lang et al., 2017; Meng et al., 2017; Kato et al., 2021). TRIM38 increases signaling downstream of RIG-I and MDA5 through E3 SUMO ligase activity (Hu et al., 2017). TRIM38 SUMOylates both MDA5 and RIG-I, preventing K48-linked ubiquitination and thus preventing early degradation of RIG-I during infection. TRIM13 conversely, positively regulates RIG-I and negatively regulates MDA5 activation through ubiquitination (Versteeg et al., 2013; Narayan et al., 2014), likely functioning to fine tune responses to the specific viral threats. Every pathway

needs modulators which eventually turn off signaling. TRIM40 negatively regulates both MDA5 and RIG-I through K48 and K7-linked polyubiquitination leading to degradation of MDA5 and RIG-I, respectively (Zhao et al., 2017).

MAVS forms non-pathogenic prion-like aggregates to increase antiviral signaling and help in the docking of signaling effecter proteins (Hou et al., 2011). Many TRIM proteins have been documented as affecting MAVS aggregation or recruitment of adapter proteins to MAVS to augment downstream signaling. TRIM31 promotes MAVS aggregation through K68-linked polyubiquitination of MAVS in mice (Liu et al., 2017a). TRIM21 also promotes IFN signaling downstream of MAVS through K27-linked polyubiquitination of MAVS, which results in increased recruitment of TBK1 to the MAVS signaling complex (Xue et al., 2018). TRIM14 appears to perform a similar role to that of TRIM21 in that it forms a complex with MAVS, catalyzes the addition of K63-linked ubiquitin chains to MAVS which helps recruit NEMO to the MAVS signaling complex (Zhou et al., 2014). TRIM25 promotes MAVS degradation through K48-linked proteasomal ubiquitination (Castanier et al., 2012). This degradation strengthens the downstream signaling via the release of the activated signaling component TBK1. TRIM44 reduces K48-linked polyubiquitination of MAVS thus preventing its degradation and increasing virus induced IFN and NF-kB signaling downstream of MAVS (Yang et al., 2013). TRIM29 induces MAVS degradation through K11-linked ubiquitination (Xing et al., 2018). ECSIT (evolutionarily conserved signaling intermediate in Toll pathways) is a protein that helps bridge the contact between both RIG-I, MDA5 and MAVS, increasing downstream antiviral signaling through IRF3 and NF-κB (Lei et al., 2015). TRIM59 associates with ECSIT and inhibits downstream activation of both IRF3 and NF-κB signaling (Kondo et al., 2012).

2.6.1.2 TRIM protein interactions with TLR adapters

As described in Chapter 1, TLR4 is located on the surface of the cell and can detect virus and signal downstream through TRIF while TLR3 detects viral RNA in the endosome and also signals through TRIF. TRIM56 acts as a scaffolding protein and is critical in the stabilization of TRIF downstream of TLR3 and increases downstream antiviral signaling (Shen et al., 2012). TRIM38 is stimulated by high levels of IFN in mice and negatively regulates TRIF during TLR3 signaling through K48-linked ubiquitination of TRIF which leads to proteasomal degradation (Hu et al., 2015). TRIM8 disrupts the interactions between TRIF and TBK1 through K6 and K33-linked ubiquitination, resulting in decreased downstream signaling (Ye et al., 2017).

TRIM32 helps in the degradation of TRIF through selective autophagy, as TRIM32 forms a complex with the noncanonical autophagic protein TAXBP1 (Yang et al., 2017).

2.6.1.3 TRIM protein interactions with DNA sensing pathways

Cyclic GMP–AMP synthase (cGAS) detects long DNA from pathogens, dimerizes to form the messenger 2′3′-Cyclic GMP-AMP (cGAMP) and activates the endoplasmic reticulum associated protein stimulator of interferon genes (STING) (Liu et al., 2017b; Yu et al., 2021a). This then recruits and activates IκB kinase-ε (IKKε) and TANK-binding kinase-1 (TBK1). TRIM14 stabilizes cGAS through recruitment of ubiquitin-specific protease 14 (USP14), which cleaves polyubiquitin chains off cGAS (Chen et al., 2016). TRIM41 also binds cGAS and activates downstream IFN signaling through monoubiquitylation of cGAS (Liu et al., 2018). TRIM38 SUMOlyates both cGAS and STING (Hu et al., 2016). The SUMOlyation of cGAS prevents its polyubiquitination and degradation, while the SUMOlyation of STING promotes its stabilization and activation. TRIM56 and TRIM32 both also activate STING signaling. TRIM56 induces STING dimerization through K63-linked polyubiquitination, which recruits TBK1 and induces IFN production (Tsuchida et al., 2010). TRIM32 also catalyzes K63-linked polyubiquitination of STING, similar to TRIM56 (Zhang et al., 2012).

DNA sensing pathways are also inhibited by TRIM genes. DEAD-box helicase 1 (DDX41) senses cytosolic DNA and activates interferon signaling through STING (Zhang et al., 2011). TRIM21 inhibits this pathway through K48-linked ubiquitination of the DEAD domain of DDX41 (Zhang et al., 2013b). Similarly, TRIM29 and TRIM30α target STING for K48-linked ubiquitination and degradation (Wang et al., 2015b; Xing et al., 2017).

2.6.1.4 TRIM interactions with NF-κB and IFN signaling adapters

Nuclear factor-kappa-B (NF-κB) essential modulator (NEMO) is involved in both IRF3 and NF-κB activation leading to an antiviral response (Fang et al., 2017a; Fang et al., 2017b). Viral induction of NF-κB and IRF3 through NEMO is activated by non-classical K27-linked polyubiquitination by the singular member of the C-XI subfamily, TRIM23 (Sparrer et al., 2017). TRIM41 also increases IRF3 and NF-κB pathway activation through K63-linked ubiquitination of BCL10, which increases NEMO recruitment (Yu et al., 2021b). NEMO is also involved in the cGAS-STING DNA sensing pathway (Fang et al., 2017b). TRIM32 and TRIM56 are activated by DNA in the cytosol and synthesize ubiquitin chains to activate NEMO (Fang et al., 2017b). Of the proteins which negatively regulate NEMO, the RINGless TRIM29, directly

targets and ubiquitinates NEMO for degradation (Xing et al., 2018), while TRIM40 aids in the degradation of NEMO utilizing the process of neddylation (Noguchi et al., 2011). TRIM68 targets TRK-fused gene (TFG) for lysosomal degradation (Wynne et al., 2014). TRK-TGF targets both NEMO and TANK and stabilizes the signaling complex (Miranda et al., 2006), thus TRIM68 indirectly inhibits both NEMO and TANK. Cytoplasmic TRIM26 is normally associated with NEMO (Ran et al., 2016). Upon viral infection, TRIM26 autoubiquitinates, causing TRIM26 to associate with TBK1. This association helps recruit NEMO to the TBK1-MAVS signaling complex resulting in phosphorylation of the protein GSK3β (Qin et al., 2016). GSK3β then enhances TBK1 oligomerization and autophosphorylation (Lei et al., 2010), which enhances IRF3 activity downstream. TRIM11 also interacts with the TBK1 signaling complex (Lee et al., 2013). Overexpression of TRIM11 decreases IFN-β promoter activity when cells were stimulated with RIG-I, MAVS or TBK1, while knockdown of TRIM11 increased the IFN-β promoter activity of these stimulated cells. The non-canonical IKKε interacts with TBK1 to phosphorylate IRF3 to activate antiviral signaling (Fitzgerald et al., 2003). TRIM27 was identified as an inhibitor of IFN signaling through interactions with IKKε and TBK1 (Zha et al., 2006).

Along with its role in stabilizing RIG-I and increasing signaling downstream of RIG-I, TRIM25 also appears to help increase NF-κB signaling downstream of TRAF6 in the MDA5-MAVS signaling pathway (Lee et al., 2015). In addition to increasing antiviral signaling downstream of NEMO, TRIM23 also increases NF-κB signaling through forming a complex with TRAF6 (Poole et al., 2009). TRIM37 and TRIM38 both negatively regulates TRAF6 signaling (Zhao et al., 2012; Zhao et al., 2021b). Downstream of TRAF6 signaling, the C-I subfamily members TRIM9 and TRIM67 both bind the protein β-transducin repeat-containing protein (β-TrCP) (Shi et al., 2014; Fan et al., 2022). This binding prevents β-TrCP from binding its substrates IκBα and p100, thus preventing NF-κB activation. IκBα is an inhibitor of NF-κB. IKKα and IKKβ phosphorylate IκBα preventing it from inhibiting NF-κB activation. TRIM27 interacts with IKKα and IKKβ to inhibit NF-κB activation (Zha et al., 2006). TRIM39 also inhibits NF-κB through binding and ubiquitin-independent stabilization of Cactus interactor (Cactin), a nuclear inhibitor of NF-κB (Suzuki et al., 2016).

TRIM59 prevents the phosphorylation and dimerization of IRF7 and IRF3, thus preventing the translocation of these transcription factors to the nucleus. It is unsure how

TRIM59 is preventing this phosphorylation, but suggested TRIM59 is interacting with a phosphorylase upstream of IRF3 and IRF7. TRIM21 interacts directly with IRF3 using its C-terminal PRYSPRY domain, causing the ubiquitination and subsequence degradation of IRF3 in the proteasome (Higgs et al., 2008). TRIM26 also binds and catalyzes K48-linked ubiquitination of IRF3, although TRIM26 binds specifically to phosphorylated IRF3 in the nucleus (Wang et al., 2015a). TRIM28 binds specifically to IRF7 and not IRF3 using its RBCC motif (Liang et al., 2011). TRIM28 acts as a SUMO E3 ligase, catalyzing the addition of SUMO to IRF7, and negatively regulating IRF7.

2.7 TRIM proteins that directly restrict Influenza A virus

This section details TRIM proteins that directly target IAV viral proteins in the cell. A summary of these TRIM proteins and their targets can be found in figure 2.3.

2.7.1 TRIM14

TRIM14 is a RINGless TRIM which is a member of the C-IV subfamily. TRIM14 is a type-I interferon inducible gene (Rajsbaum et al., 2008; Carthagena et al., 2009) which can directly target viruses such as Hepatitis C (Wang et al., 2016a) and Hepatitis B (Tan et al., 2018) virus. TRIM14 targets proteins from both these viruses using its C-terminal PRYSPRY domain. TRIM14 also directly targets the IAV nucleoprotein (NP) for degradation (Wu et al., 2019). NP is part of the viral ribonucleoprotein (vRNP) complex, and it is critical for viral transcription and replication (Herz et al., 1981; Jackson et al., 1982). TRIM14 targets NP using its PRYSPRY domain, causing NP to undergo K48-linked ubiquitination resulting in proteasomal degradation of NP (Wu et al., 2019). As TRIM14 is RINGless it is unknown if the catalyzing of polyubiquitin chains to NP is from the TRIM14 B-box domain, or if another protein is acting as the E3 ubiquitin ligase. Interestingly, deletion of the PRYSPRY domain also allowed TRIM14 to bind NP, however this binding increased vRNP formation and NP accumulation in HeLa cells. When human TRIM14 was expressed in transgenic mice, the overexpression of human TRIM14 markedly reduced H5N2 infection and increased survival in these mice (Nenasheva et al., 2021). Interestingly, the overexpression of human TRIM14 in these mice also coincided with less transcription of the proinflammatory cytokines IL-6, IL-1β and TNFα in the lungs of mice infected with H5N2, suggesting human TRIM14 could down regulate inflammatory responses in mice.

2.7.2 TRIM22

TRIM22 is an IFN-stimulated gene (Le Goffic et al., 2010) which belongs to a locus of expanded TRIM genes which first appeared in Eutherians (Hattlmann et al., 2012). This locus is located on chromosome 11 in humans and contains TRIM34, TRIM6, TRIM5 and TRIM22 (Sawyer et al., 2007). TRIM5 and TRIM22 have undergone many changes during mammalian evolution, with these genes expanding and deleting in different lineages. For examples, cows have an expansion of TRIM5 genes, but no TRIM22, while dogs have no TRIM5 gene. Yangochiroptera bats have expansions of both TRIM5 and TRIM22 in their lineages (Fernandes et al., 2022). The diversification of TRIM22 in different mammalian species is perhaps a result of the broad antiviral specificity of this protein. TRIM22 can inhibit HIV particle production through the targeting of the HIV Gag protein (Barr et al., 2008). TRIM22 ubiquitinates the hepatitis C viral (HCV) protein NS5A, thus restricting HCV replication (Yang et al., 2016). TRIM22 also inhibits hepatitis B virus by targeting the viral core promoter (Gao et al., 2009).

Notably, TRIM22 also inhibits IAV. TRIM22 was identified as a host factor which interacted with IAV virus in human bronchial epithelial cells (Shapira et al., 2009). When TRIM22 was knocked out of the human lung epithelial cell line A549, IAV replication was significantly enhanced (Di Pietro et al., 2013). Likewise, overexpression of TRIM22 in MDCK drastically reduced IAV titre by over 100 times. The mechanism of this viral restriction was due to TRIM22 interacting with the IAV NP and catalyzing attachment of both mono- and polyubiquitin to NP, causing it to be degraded by the proteasome.

2.7.3 TRIM25

Along with multiple augmentation steps in the RIG-I signaling pathway, TRIM25 can also directly restrict IAV in mammalian cells. Initially identified as an RBP in a screening of HeLa cells searching for cellular proteins which bound mRNA (Castello et al., 2012), further studies found that TRIM25 bound a diverse repertoire of coding and non-coding cellular RNA using its C-terminal PRYSPRY domain (Choudhury et al., 2017). TRIM25 binds the IAV vRNP complex and inhibits the viral polymerase complex from viral mRNA chain elongation (Meyerson et al., 2017). Interestingly, gibbon TRIM25 was more effective at binding and inhibiting viral mRNA chain elongation than human TRIM25. As gibbons are not a natural host of IAV, it seems that human adapted IAV may have evolved a partial escape mechanism for this nuclear vRNP binding.

2.7.4 TRIM41

TRIM41 was initially recognized as an anti-viral TRIM protein through a host-IAV protein interaction screen that suggested TRIM41 interacted with the IAV NP (Wang et al., 2017). TRIM41 is not upregulated by either IFN or IAV infection in A549 cells, however overexpression of TRIM41 did reduce IAV virus titres, but not Sendai or vaccinia virus (Patil et al., 2018). TRIM41 also catalyzed the addition of ubiquitin chains to IAV NP, causing NP to be degraded by the proteasome. TRIM41 is also able to restrict other viruses. TRIM41 inhibits the N protein of Vesicular stomatitis virus (VSV) through catalyzing the addition of ubiquitin to the N protein (Patil et al., 2020). Additionally, a screen of 38 human TRIM proteins searching for anti-HBV TRIM proteins identified TRIM41 as inhibitory to HBV replication (Zhang et al., 2013a).

2.7.5 TRIM32

TRIM32 has many cellular functions but was initially identified as a potential antiviral mediator in a screening for proteins which bound the HIV transcriptional activator tat (Fridell et al., 1995). TRIM32 directly restricts IAV through targeting of the PB1 protein (Fu et al., 2015). TRIM32 translocates into the nucleus with PB1, and then using the RING domain polyubiquitinates PB1 to target it for proteasomal degradation. Unlike many other TRIM proteins which target substrate through their C-terminal domains, TRIM32 appears to associate with PB1 through its coiled-coil domain.

2.7.6 TRIM35

Much like TRIM25, TRIM35 is implicated in restricting IAV by both immune signaling pathway modulation and directly targeting IAV proteins. As mentioned in the previous sections, TRIM35 helps to catalyze the addition of K63-linked polyubiquitin to TRAF3 to increase downstream antiviral signaling (Sun et al., 2020). The IAV protein PB2 suppresses this activation of TRAF3 by inhibiting the ubiquitination of TRAF3. TRIM35 counteracts this suppression of TRAF3 ubiquitination by mediating K48-linked ubiquitination of PB2. TRIM35 interacts with PB2 using its C-terminal PRYSPRY domain, and aids in PB2 ubiquitination using its RING domain.

2.7.7 TRIM56

TRIM56 is an RNA-binding TRIM protein (Williams et al., 2019). TRIM56 inhibits many viruses through binding of viral RNA such as Zika virus (Yang et al., 2019), Dengue virus,

yellow fever virus (Liu et al., 2014) and bovine viral diarrhea virus (Wang et al., 2011). TRIM56 also restricts both IAV and influenza B virus (IBV) but in the same experiment did not restrict Sendai or human metapneumovirus (Liu et al., 2016). TRIM56 restricted both IAV and IBV by binding viral RNA in the nucleus using its C-terminal PRYSPRY domain. This restriction was independent of E3 ubiquitin ligase activity, and instead it seemed that the binding of TRIM56 to viral RNA inhibited viral synthesis.

2.8 Antiviral TRIM proteins characterized in non-mammalian animals

2.8.1 Antiviral TRIM proteins characterized in invertebrates

Relatively few invertebrate TRIM proteins have been formally identified, and only two orthologs to vertebrate TRIM proteins have been studied for antiviral function. In whiteleg shrimp (*Litopenaeus vannamei*) an ortholog to mammalian TRIM32 (named lvTRIM32) was identified as a restriction factor during white spot syndrome virus (WSSV) infection (Wang et al., 2020). lvTRIM32 expression was upregulated in whiteleg shrimp haemocytes when cells were infected with WSSV or stimulated by other immunomodulatory substances, and the knockdown of lvTRIM32 caused increased mortality in haemocytes infected with WSSV. Similarly, giant tiger prawn (*Penaeus monodon*) TRIM32 (pmTRIM32) overexpression increased survival of haemocytes (Peng et al., 2021). Peng and colleagues demonstrated that pmTRIM32 bound and restricted WSSV through ubiquitination of the viral envelope protein. As mammalian TRIM32 also directly targets viral proteins (Fu et al., 2015), it appears the mechanism of restriction employed by TRIM32 is highly evolutionarily conserved.

Two TRIM9 orthologs have been identified in whiteleg shrimp, named lvTRIM9 and lvTRIM9-1. lvTRIM9 interacts with the NF-κB pathway in an analogous manner to that of human TRIM9. Human TRIM9 interacts with β-TrCP which prevents NF-κB activation (Shi et al., 2014). lvTRIM9 also interacts with β-TrCP, however in shrimp β-TrCP inhibits NF-κB activation, thus in shrimp the interaction between lvTRIM and β-TrCP increased NF-κB signaling (Sun et al., 2019). lvTRIM9 overexpression can activate NF-κB in human cells (Sun et al., 2022), however unlike lvTRIM9, lvTRIM9-1 enhances WSSV replication, as when lvTRIM9-1 is knocked-down in haemocytes, WSSV replication is decreased.

TRIM50-like was identified as an antiviral restriction factor in giant tiger prawn (pmTRIM50) and is functionally analogous to pmTRIM32 (Zhao et al., 2021a). Like

pmTRIM32, pmTRIM50 targets the WSSV envelope protein and aids in ubiquitination and subsequent degradation of this viral target.

2.8.2 Antiviral TRIM proteins characterized in fish

Teleost fish have a lineage specific expansion of TRIM genes. Expansions of TRIM genes hypothesized to be orthologous to TRIM35 in humans were named the htlrTRIMs while TRIM genes presumed to be orthologous to human TRIM39 were named bloodthirsty TRIMs (btyTRIM) (van der Aa et al., 2009; Boudinot et al., 2011). A teleost specific expansion without clear orthology to human TRIM genes were named the fish novel TRIMs (finTRIMs). Out of the estimated 208 TRIM genes thought to be present in zebrafish, at least 84 of those are finTRIM, 39 are btyTRIM genes and 37 are htlrTRIMs (van der Aa et al., 2009). This is compared to the 66 TRIM genes documented in pufferfish (Boudinot et al., 2011) where much less TRIM expansion has happened. These expansions are likely due to the whole genome duplications that have occurred in teleost fish (Glasauer et al., 2014).

TRIM39 from the orange spotted grouper (*Epinephelus coioides*) was recently characterized as being upregulated by and having antiviral function against red-spotted grouper nervous necrosis virus (RGNNV) (Wang et al., 2016b). A mechanism for this restriction was not investigated, however the RING domain of ecTRIM39 was necessary for antiviral activity.

Like mammal and shrimp TRIM32, TRIM32 orthologs in fish can also restrict viruses. TRIM32 from the common carp (*Cyprinus carpio*) is upregulated in response to spring viremia carp virus (SVCV) (Wang et al., 2016c). Overexpression of carp TRIM32 significantly decreased SVCV in the immortalized carp cell line epithelioma papulosum cyprinid (EPC), however a mechanism for this restriction is still unknown. TRIM32 has also been functionally characterized in orange spotted grouper (Yu et al., 2017). Overexpression of grouper TRIM32 inhibits growth of the DNA-virus Singapore grouper iridovirus (SGIV) and the RNA-virus RGNNV. It is unknown if this restriction is due to direct antiviral effects, however in grouper TRIM32 overexpression also significantly increased the interferon response.

Only one of the finTRIM genes in teleost fish has been formally characterized for antiviral activity. Overexpression of finTRIM83 (ftr83) drastically reduced both infectious hematopoietic necrosis virus 32-87 (IHNV) and viral hemorrhagic septicemia virus 07-71 (VHSV) and SVCV in EPC cells. The authors suggest this inhibition is due to ftr83 upregulating IFN signaling pathways when overexpressed. Like many other members of the C-IV subfamily,

ftr83 relies on both its RING and PRYSPRY domain for both signaling induction and antiviral effect.

2.8.3 Antiviral TRIM proteins characterized in Diapsids

TRIM proteins are not well studied in birds or reptiles (Diapsida) in either general cellular function or immune function, although more work has been done on investigating antiviral TRIM proteins in birds than in reptiles.

Perhaps the most studied of the avian TRIM proteins is TRIM25. TRIM25 has been amplified from chicken (Feng et al., 2015), duck (Miranzo-Navarro et al., 2014; Kaikai et al., 2021), and goose (Wei et al., 2016). In goslings, TRIM25 is upregulated by IFN-γ, IFN-λ, IFN-α and poly (I:C). Unlike chicken and duck TRIM25, goose TRIM25 appears to be missing the N-terminal RING domain. TRIM25 in ducks is upregulated by both HPAI and LPAI IAV (Huang et al., 2013; Fleming-Canepa et al., 2019; Campbell et al., 2021). TRIM25 is also upregulated by duck Tembusu virus (DTMUV) in duck embryonic fibroblasts (Kaikai et al., 2021). Duck TRIM25 has been functionally characterized in two different studies. The first investigated the ubiquitination of RIG-I by duck TRIM25 and compared this to the mechanism employed by human TRIM25 (Discussed in detail in chapter 1, section 1.2.4) (Miranzo-Navarro et al., 2014). The second study demonstrated that overexpression of duck TRIM25 reduced DTMUV viral RNA, and knockdown of TRIM25 in DEF increased overall DTMUV viral RNA (Kaikai et al., 2021). In chickens, TRIM25 is upregulated in response to avian leukosis virus (ALV) (Zhou et al., 2020) and infectious bursal disease (Zhou et al., 2020). Interestingly, chicken breeds that were classified as resistant to a HPAI H5N8 IAV strain did not upregulate TRIM25 in lung during infection, while chickens classified as susceptible to H5N8 upregulated TRIM25 in the lungs (Perlas et al., 2021). To our knowledge no studies have attempted to characterize anti-IAV activity of TRIM25 in any bird species, despite the well-known function of TRIM25 as an anti-IAV mediator in mammals.

TRIM62 in chicken is upregulated by the retrovirus Reticuloendotheliosis Virus (REV) in chicken embryonic fibroblasts (CEF) (Li et al., 2020). Knockdowns of TRIM62 in CEF cells increase viral replication, while overexpression decreased REV replication in DEF cells. TRIM62 also restricts avian leukosis virus (ALV) in chicken cells (Li et al., 2019). In both instances of retroviral restriction, TRIM62 appears to use its PRYSPRY domain to exert this

effect (Li et al., 2019; Li et al., 2020), although exact mechanisms for viral inhibition is not known.

The singular TRIM protein that has been functionally characterized in reptiles is a TRIM39 ortholog amplified form Chinese softshell turtle (*Pelodiscus sinensis*) named psTRIM39 (Shi et al., 2019). The authors found that psTRIM39 was upregulated in response to iridovirus and Gram-negative bacterium. Overexpression of psTRIM39 activated NF-κB signaling in fat headed minnow cells, but no tests were done on reptilian cells to analyze pathway augmentation, likely because reptilian cell lines are not available.

Perhaps unsurprisingly, TRIM32 has also been characterized as an antiviral protein in ducks. TRIM32 is significantly upregulated in the lungs of ducks infected with highly pathogenic H6N2 IAV (Wu et al., 2020) and TRIM32 overexpression in duck embryonic fibroblasts also inhibits H6N2. Additionally, duck TRIM32 overexpression increased IFN-β activity and increased transcript level of Mx, IRF7 and IFN-β. Like human TRIM32 (Zhang et al., 2012), duck TRIM32 directly interacts with STING to increase IFN signaling (Wu et al., 2020). Duck TRIM32 overexpression also restricts duck Tembusu virus replication in DEF cells (Li et al., 2021).

2.9 Conclusions

While many of the human or mouse TRIM genes have been functionally characterized, TRIM genes are largely understudied in other animals. Most of the TRIM genes studied in non-mammalian vertebrates or invertebrates have direct orthologs in mammals that have anti-viral function or are implicated in immune signaling processes in mammals (Table 2.1), ignoring the rest of the TRIM repertoire in these species. Additionally, almost all the non-human or mice TRIM proteins studied are from animals with agricultural significance, and wild natural reservoir hosts of important zoonotic viruses are largely ignored.

Bats (Chiroptera) are a notable group of mammals which act as the reservoir to diverse RNA viruses (Van Brussel et al., 2022). While studies have investigated global immune responses of bats to viruses to determine how bats are so resistant to damage from replicating virus (Banerjee et al., 2020; Irving et al., 2021), there has been little research into the TRIM gene repertoire of bats. Bats are the suspected reservoir for many zoonotic viruses such as Ebola (Hayman et al., 2012; Koch et al., 2020), Nipah virus (Reynes et al., 2005; Rahman et al., 2010),

Marburg virus (Towner et al., 2009), diverse corona viruses (Huynh et al., 2012; Latinne et al., 2020; Ravelomanantsoa et al., 2020) and many others (Letko et al., 2020; Van Brussel et al., 2022). The study we highlighted previously in this chapter documented bats have lineage specific duplications of TRIM22 and TRIM5 (Fernandes et al., 2022). More research into the evolution and diversification of TRIM genes in bats could potentially identify more Chiropteran specific TRIM gene adaptations and duplications, as well as identify species-specific TRIM genes in the Chiropterans.

Reptiles are also often a source of nutrition for arthropods which also feed on humans such as mosquitos, ticks and sandflies (Cupp et al., 2004). Moreso, reptiles have been considered reservoir hosts for viruses such as western and eastern equine encephalitis in garter snakes (Burton Althea et al., 1966; Bingham et al., 2012), Crimean-Congo hemorrhagic fever in tortoises (Kar et al., 2020), and Chikungunya virus in snakes and toads (Bosco-Lauth et al., 2018). Reptiles present a unique opportunity to study reservoir host responses as this incredibly large and diverse group (with approximately 11,000 extant species) that has remained largely unchanged in their biology, ecology and morphology since their emergence approximately 310 million years ago (Tucker et al., 1982; Lepetz et al., 2009). We only found one study on a TRIM gene in reptiles. As reptiles arose in earlier in vertebrate evolution than birds, their TRIM protein function and diversification would offer unique insight into TRIM protein evolution and immune function in vertebrates.

Wild birds also present a large and diverse class of vertebrates that act as the reservoir host to many zoonotic viral pathogens (Reed et al., 2003; Nabi et al., 2021). As discussed in the previous chapter, ducks and other waterfowl are the primordial host and reservoir of IAV (Webster et al., 1992; Olsen et al., 2006; Kim et al., 2009). Wild birds also host both delta- and gamma-coronaviruses with zoonotic potential (Wille et al., 2020). Migratory birds of various species act as the reservoir host to West Nile virus (Rappole et al., 2000; Peterson et al., 2003; Taieb et al., 2020). While some TRIM proteins have been identified in birds as antiviral effectors (As outlined in section 2.7.3), the entire TRIM repertoire has only been documented in chickens (Sardiello et al., 2008) and has not been updated since 2008. Additionally, TRIM protein function has only been studied in chickens, ducks and geese, leaving many bird species unstudied.

Finally, while it is true that many of the TRIM genes that have antiviral function in

mammals could have orthologous function in lower vertebrates, it is also true that some TRIM genes in mammals that do not have documented antiviral function could have orthologs that do have antiviral function in lower vertebrates, and that function was lost during evolution. By categorizing the TRIM gene repertoire in lower vertebrates and assessing immune function of these proteins we can gain further insight into TRIM gene evolution, but also uncover novel anti-viral TRIM proteins.

2.10 Aims and rationale for this thesis work

The tripartite motif (TRIM) family of proteins provide protection against influenza A virus (IAV) infection either by direct restriction of the virus or through modification of innate immune pathways (Hatakeyama et al, 2017; van Tol et al, 2017; Rajsbaum et al, 2014). TRIM family evolution also suggests that these genes have responded to selective pressures from viruses by evolving species-specific pathogen restriction mechanisms (Song et al, 2005). I am investigating the role of TRIM proteins in the resistance to IAV in its natural host and reservoir, the duck.

Aim 1: To compare differential expression in spleens, lungs and intestines of ducks infected with VN1203 or BC500

There are significant differences in basal expression of duck innate receptors and downstream effectors between tissues (Campbell et al., 2020), it is still unknown how different tissues contribute to resistance to IAV and yet control damage from IAV infection despite high viral load. In Aim 1, I investigated changes in differential expression in ducks infected with either a HPAI H5N1 strain (VN1203) or LPAI H5N2 strain (BC500) of IAV, focusing specifically on tissues involved in IAV replication. LPAI IAV replicates in intestines of infected ducks while VN1203 preferentially replicates in the lung (Bingham et al., 2009; Daoust et al., 2011; Vidana et al., 2018). Previous studies with BC500 and VN1203 demonstrated that VN1203 was more severe, and could even be lethal to ducks, while BC500 did not cause severe symptoms or disease, yet could still replicate to high titres (Vanderven et al., 2012). Other research groups have investigated the transcriptional responses in ducks to IAV, however these groups used later time points (5 days post infection) (Kumar et al., 2017), only sampled lungs of ducks infected with HPAI (Huang et al., 2013), or sampled more than tissue but did not compare

responses between the tissues (Smith et al, 2015). I investigated the differences and similarities between these two IAV strains of differing severities to try to determine what responses were protective and which responses were specific to the strain of virus or tissue sampled. By comparing transcriptional responses to HPAI and LPAI infection in ducks, we can learn what genes are globally protective and which genes are important for tissue specific responses.

Aim 2: The characterization and relative expression of duck TRIM and TRIM-like genes

TRIM protein family members have been formally characterized in both mammals and fish (Boudinot et al., 2011; van Gent et al., 2018; Venuto et al., 2019). The only study attempting to summarize an avian TRIM repertoire was performed by Sardiello and colleagues in 2008 (Sardiello et al., 2008). This study found 37 TRIM genes in chickens. There has never been a formal study characterizing the TRIM protein family in ducks or comparing the TRIM family repertoire between any bird species. As more sequencing data has become publicly available since 2008, and sequencing techniques have decreased in cost and increased in accuracy, it is likely that more TRIM genes will be able to be identified in both duck and chicken. In Aim 2, I utilized the publicly available sequence read archive (SRA) on NCBI to assemble a *de novo* transcriptome of sequences derived from domestic Pekin duck (*Anas platyrhynchos*). I interrogated the transcriptome to determine how many TRIM or TRIM-like genes were present in the duck, and documented phylogenies for duck TRIM proteins, chicken and duck TRIM proteins and the C-IV TRIM subfamily for mammals, birds and reptiles. Additionally, I analyzed relative tissue expression of TRIM genes in ducks. In this study I document not only the duck TRIM repertoire and what tissues these TRIM genes have predominant expression in, but also categorize MHC-linked TRIM genes in higher vertebrates using phylogenetics. By examining the TRIM gene repertoire in ducks, we can gain insight into the evolution and expansion of TRIM genes in vertebrates.

Aim 3: To analyze differential expression of duck TRIM and TRIM-like genes during IAV infection

Approximately half of the human TRIM gene repertoire is upregulated in response to IFNs or innate pathway stimulation (Carthagena et al., 2009). It was not known how many of the 57 duck TRIM or TRIM-like genes were upregulated in response to infection. However, as the

duck shares a long co-evolutionary history with IAV (Webster et al, 1992), it is likely that many of the TRIM proteins in ducks change expression in response to virus and are antiviral. In Aim 2, I investigate differential expression of the TRIM gene repertoire in response to HPAI (VN1203) and LPAI (BC500) viruses in the lung, spleen and intestine of infected ducks. I compare and contrast the differential expression of the duck TRIM gene repertoire to find candidate genes to study for antiviral function. While many TRIM proteins have documented antiviral function in humans, very few have been studied for antiviral ability in the duck. I used the results from my differential analyses to determine if overexpression of duck TRIM19.1, TRIM19.2, TRIM27.1, TRIM27.2, TRIM25, TRIM32 and diaTRIM58 were able to restrict IAV replication in either chicken DF-1 cells or duck embryonic fibroblasts. The results of these experiments will not only give candidate genes to functionally characterize in the duck for antiviral activity but will provide insight into the evolution of antiviral ability of TRIM genes in vertebrates.

Aim 4: To determine how TRIM27L is augmenting the RIG-I signaling pathway

Due to coevolution between host and pathogen, many host proteins can develop species specific antiviral ability, such as the case with TRIM5α, which can restrict HIV in monkeys but not humans (Stremlau et al., 2004; Song et al., 2005). TRIM27L is an interesting example of a TRIM protein which appears to have been lost in a specific group, as it appears to be missing in Galliformes but is present in ducks, other birds and reptiles (Blaine et al., 2015). TRIM27L increases IFN-β promoter activity in chicken cells downstream of active RIG-I (Blaine et al., 2015), supporting that TRIM27L is involved in antiviral signaling pathways in birds. Chickens also appear to be missing RIG-I but have functional downstream components of the signaling pathway which can trigger antiviral responses in cells once active (Barber et al., 2010). It is unknown why Galliformes, which are more susceptible to influenza A virus, would have lost TRIM27L, a protein that appears to regulate cellular antiviral signaling. The fourth Aim of this thesis details what part of the RIG-I signaling pathway TRIM27L is augmenting in both duck and chicken cells. Additionally, I created domain and mutant constructs to determine what specific domains TRIM27L was using for RIG-I pathway augmentation. The results of these experiments will help us understand how TRIM27L regulates innate immune pathways in response to IFN-β signaling and how TRIM27L regulation of the RIG-I pathway differs between differs between species. The results of these experiments will help us understand how TRIM27L

regulates innate immune pathways in response to infection and how it contributes to IAV resistance in the duck.

Significance of proposed research:

TRIM protein biology and function in antiviral immunity is an understudied area of immunological research. Many of these proteins are known to be differentially expressed during viral infection, but most have not been functionally characterized. By increasing our knowledge of the regulation and function of TRIM proteins in influenza infection in ducks we can gain a better understanding of both TRIM protein biology and function in host pathogen interactions in the reservoir host.

2.11 References

Agarwal, N., Rinaldetti, S., Cheikh, B.B., Zhou, Q., Hass, E.P., Jones, R.T., et al. (2021). TRIM28 is a transcriptional activator of the mutant TERT promoter in human bladder cancer. *Proc Natl Acad Sci U S A* 118(38). doi: 10.1073/pnas.2102423118.

Ardley, H.C., and Robinson, P.A. (2005). E3 ubiquitin ligases. *Essays Biochem* 41(1)**,** 15-30. doi: 10.1042/EB0410015.

Banerjee, A., Baker, M.L., Kulcsar, K., Misra, V., Plowright, R., and Mossman, K. (2020). Novel insights into immune systems of bats. *Frontiers in Immunology* 11.

Barber, M.R., Aldridge, J.R., Jr., Webster, R.G., and Magor, K.E. (2010). Association of RIG-I with innate immunity of ducks to influenza. *Proc Natl Acad Sci U S A* 107(13)**,** 5913-5918. doi: 10.1073/pnas.1001755107.

Barber, M.R.W., Aldridge, J.R., Jr., Fleming-Canepa, X., Wang, Y.-D., Webster, R.G., and Magor, K.E. (2013). Identification of avian RIG-I responsive genes during influenza infection. *Molecular immunology* 54(1)**,** 89-97. doi: 10.1016/j.molimm.2012.10.038.

Barr, S.D., Smiley, J.R., and Bushman, F.D. (2008). The interferon response inhibits HIV particle production by induction of TRIM22. *PLOS Pathogens* 4(2)**,** e1000007. doi: 10.1371/journal.ppat.1000007.

Bell, J.L., Malyukova, A., Holien, J.K., Koach, J., Parker, M.W., Kavallaris, M., et al. (2012). TRIM16 acts as an E3 ubiquitin ligase and can heterodimerize with other TRIM family members. *PLoS One* 7(5)**,** e37470. doi: 10.1371/journal.pone.0037470.

Berti, C., Messali, S., Ballabio, A., Reymond, A., and Meroni, G. (2002). TRIM9 is specifically expressed in the embryonic and adult nervous system. *Mech Dev* 113(2)**,** 159-162. doi: 10.1016/s0925-4773(02)00013-8.

Bingham, A.M., Graham, S.P., Burkett-Cadena, N.D., White, G.S., Hassan, H.K., and Unnasch, T.R. (2012). Detection of eastern equine encephalomyelitis virus RNA in North American snakes. *Am J Trop Med Hyg* 87(6)**,** 1140-1144. doi: 10.4269/ajtmh.2012.12-0257.

Bingham, J., Green, D.J., Lowther, S., Klippel, J., Burggraaf, S., Anderson, D.E., et al. (2009). Infection studies with two highly pathogenic avian influenza strains (Vietnamese and Indonesian) in Pekin ducks (Anas platyrhynchos), with particular reference to clinical disease, tissue tropism and viral shedding. *Avian Pathol* 38(4)**,** 267-278. doi: 10.1080/03079450903055371.

Blaine, A.H., Miranzo-Navarro, D., Campbell, L.K., Aldridge, J.R., Jr., Webster, R.G., and Magor, K.E. (2015). Duck TRIM27-L enhances MAVS signaling and is absent in chickens and turkeys. *Mol Immunol* 67(2 Pt B)**,** 607-615. doi: 10.1016/j.molimm.2015.07.011.

Borden, K.L., and Freemont, P.S. (1996). The RING finger domain: a recent example of a sequence-structure family. *Curr Opin Struct Biol* 6(3)**,** 395-401. doi: 10.1016/s0959-440x(96)80060-1.

Bosco-Lauth, A.M., Hartwig, A.E., and Bowen, R.A. (2018). Reptiles and amphibians as potential reservoir hosts of Chikungunya virus. *Am J Trop Med Hyg* 98(3)**,** 841-844. doi: 10.4269/ajtmh.17-0730.

Boudinot, P., van der Aa, L.M., Jouneau, L., Du Pasquier, L., Pontarotti, P., Briolat, V., et al. (2011). Origin and evolution of TRIM proteins: new insights from the complete TRIM repertoire of zebrafish and pufferfish. *PloS one* 6(7)**,** e22022-e22022. doi: 10.1371/journal.pone.0022022.

Boyer, N.P., Monkiewicz, C., Menon, S., Moy, S.S., and Gupton, S.L. (2018). Mammalian TRIM67 functions in brain development and behavior. *eNeuro* 5(3). doi: 10.1523/eneuro.0186-18.2018.

Burton Althea, N., McLintock, J., and Rempel, J.G. (1966). Western equine encephalitis virus in Saskatchewan garter snakes and leopard frogs. *Science* 154(3752)**,** 1029-1031. doi: 10.1126/science.154.3752.1029.

Campbell, L.K., Fleming-Canepa, X., Webster, R.G., and Magor, K.E. (2021). Tissue specific transcriptome changes upon influenza A virus replication in the duck. *Frontiers in Immunology* 12(4653). doi: 10.3389/fimmu.2021.786205.

Campbell, L.K., and Magor, K.E. (2020). Pattern recognition receptor signaling and innate responses to influenza A viruses in the mallard duck, compared to humans and chickens. *Frontiers in Cellular and Infection Microbiology* 10(209). doi: 10.3389/fcimb.2020.00209.

Carthagena, L., Bergamaschi, A., Luna, J.M., David, A., Uchil, P.D., Margottin-Goguet, F., et al. (2009). Human TRIM gene expression in response to interferons. *PLoS One* 4(3)**,** e4894. doi: 10.1371/journal.pone.0004894.

Castanier, C., Zemirli, N., Portier, A., Garcin, D., Bidère, N., Vazquez, A., et al. (2012). MAVS ubiquitination by the E3 ligase TRIM25 and degradation by the proteasome is involved in type I interferon production after activation of the antiviral RIG-I-like receptors. *BMC Biol* 10**,** 44. doi: 10.1186/1741-7007-10-44.

Castello, A., Fischer B Fau - Eichelbaum, K., Eichelbaum K Fau - Horos, R., Horos R Fau - Beckmann, B.M., Beckmann Bm Fau - Strein, C., Strein C Fau - Davey, N.E., et al. (2012) Insights into RNA biology from an atlas of mammalian mRNA-binding proteins. *Cell*. doi: 10.1016/j.cell.2012.04.031. Epub 2012 May 31. PMID: 22658674.

Chang, H.-M., Martinez, N.J., Thornton, J.E., Hagan, J.P., Nguyen, K.D., and Gregory, R.I. (2012). TRIM71 cooperates with microRNAs to repress Cdkn1a expression and promote embryonic stem cell proliferation. *Nature Communications* 3(1)**,** 923. doi: 10.1038/ncomms1909.

Chen, J., Wang, Z., Guo, X., Li, F., Wei, Q., Chen, X., et al. (2019). TRIM66 reads unmodified H3R2K4 and H3K56ac to respond to DNA damage in embryonic stem cells. *Nat Commun* 10(1)**,** 4273. doi: 10.1038/s41467-019-12126-4.

Chen, M., Meng, Q., Qin, Y., Liang, P., Tan, P., He, L., et al. (2016). TRIM14 Inhibits cGAS degradation mediated by selective autophagy receptor p62 to promote innate immune responses. *Mol Cell* 64(1)**,** 105-119. doi: 10.1016/j.molcel.2016.08.025.

Chen, Z.J., and Sun, L.J. (2009). Nonproteolytic functions of ubiquitin in cell signaling. *Mol Cell* 33(3)**,** 275-286. doi: 10.1016/j.molcel.2009.01.014.

Choudhury, N.R., Heikel, G., Trubitsyna, M., Kubik, P., Nowak, J.S., Webb, S., et al. (2017). RNA-binding activity of TRIM25 is mediated by its PRY/SPRY domain and is required for ubiquitination. *BMC Biol* 15(1)**,** 105. doi: 10.1186/s12915-017-0444-9.

Condemine, W., Takahashi, Y., Le Bras, M., and de Thé, H. (2007). A nucleolar targeting signal in PML-I addresses PML to nucleolar caps in stressed or senescent cells. *J Cell Sci* 120(Pt 18)**,** 3219-3227. doi: 10.1242/jcs.007492.

Cupp, E.W., Zhang, D., Yue, X., Cupp, M.S., Guyer, C., Sprenger, T.R., et al. (2004). Identification of reptilian and amphibian blood meals from mosquitoes in an eastern equine encephalomyelitis virus focus in central Alabama. *Am J Trop Med Hyg* 71(3)**,** 272-276.

D'Cruz, A.A., Babon, J.J., Norton, R.S., Nicola, N.A., and Nicholson, S.E. (2013). Structure and function of the SPRY/B30.2 domain proteins involved in innate immunity. *Protein Sci* 22(1)**,** 1-10. doi: 10.1002/pro.2185.

Daoust, P.Y., Kibenge, F.S., Fouchier, R.A., van de Bildt, M.W., van Riel, D., and Kuiken, T. (2011). Replication of low pathogenic avian influenza virus in naturally infected Mallard ducks (Anas platyrhynchos) causes no morphologic lesions. *J Wildl Dis* 47(2)**,** 401-409. doi: 10.7589/0090-3558-47.2.401.

Di Pietro, A., Kajaste-Rudnitski, A., Oteiza, A., Nicora, L., Towers, G.J., Mechti, N., et al. (2013). TRIM22 inhibits influenza A virus infection by targeting the viral nucleoprotein for degradation. *J Virol* 87(8)**,** 4523-4533. doi: 10.1128/JVI.02548-12.

Dickson, C., Fletcher, A.J., Vaysburd, M., Yang, J.-C., Mallery, D.L., Zeng, J., et al. (2018). Intracellular antibody signalling is regulated by phosphorylation of the Fc receptor TRIM21. *eLife* 7**,** e32660. doi: 10.7554/eLife.32660.

Fan, W., Liu, X., Zhang, J., Qin, L., Du, J., Li, X., et al. (2022). TRIM67 suppresses TNFalpha-triggered NF-kB activation by competitively binding Beta-TrCP to IkBa. *Frontiers in Immunology* 13.

Fang, R., Jiang, Q., Zhou, X., Wang, C., Guan, Y., Tao, J., et al. (2017a). MAVS activates TBK1 and IKKε through TRAFs in NEMO dependent and independent manner. *PLOS Pathogens* 13(11)**,** e1006720. doi: 10.1371/journal.ppat.1006720.

Fang, R., Wang, C., Jiang, Q., Lv, M., Gao, P., Yu, X., et al. (2017b). NEMO-IKKβ are essential for IRF3 and NF-κB activation in the cGAS-STING pathway. *J Immunol* 199(9)**,** 3222-3233. doi: 10.4049/jimmunol.1700699.

Feng, Z.Q., Cheng, Y., Yang, H.L., Zhu, Q., Yu, D., and Liu, Y.P. (2015). Molecular characterization, tissue distribution and expression analysis of TRIM25 in Gallus gallus domesticus. *Gene* 561(1)**,** 138-147. doi: 10.1016/j.gene.2015.02.025.

Fernandes, A.P., Águeda-Pinto, A., Pinheiro, A., Rebelo, H., and Esteves, P.J. (2022). Evolution of TRIM5 and TRIM22 in bats reveals a complex duplication process. *Viruses* 14(2). doi: 10.3390/v14020345.

Finley, D. (2009). Recognition and processing of ubiquitin-protein conjugates by the proteasome. *Annu Rev Biochem* 78**,** 477-513. doi: 10.1146/annurev.biochem.78.081507.101607.

Fitzgerald, K.A., McWhirter, S.M., Faia, K.L., Rowe, D.C., Latz, E., Golenbock, D.T., et al. (2003). IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. *Nat Immunol* 4(5)**,** 491-496. doi: 10.1038/ni921.

Fleming-Canepa, X., Aldridge, J.R., Jr., Canniff, L., Kobewka, M., Jax, E., Webster, R.G., et al. (2019). Duck innate immune responses to high and low pathogenicity H5 avian influenza viruses. *Vet Microbiol* 228**,** 101-111. doi: 10.1016/j.vetmic.2018.11.018.

Foletta, V.C., White, L.J., Larsen, A.E., Leger, B., and Russell, A.P. (2011a). The role and regulation of MAFbx/atrogin-1 and MuRF1 in skeletal muscle atrophy. *Pflugers Arch* 461(3)**,** 325-335. doi: 10.1007/s00424-010-0919-9.

Foletta, V.C., White, L.J., Larsen, A.E., Léger, B., and Russell, A.P. (2011b). The role and regulation of MAFbx/atrogin-1 and MuRF1 in skeletal muscle atrophy. *Pflügers Archiv - European Journal of Physiology* 461(3)**,** 325-335. doi: 10.1007/s00424-010-0919-9.

Fridell, R.A., Harding, L.S., Bogerd, H.P., and Cullen, B.R. (1995). Identification of a novel human zinc finger protein that specifically interacts with the activation domain of lentiviral Tat proteins. *Virology* 209(2)**,** 347-357. doi: 10.1006/viro.1995.1266.

Fu, B., Wang, L., Ding, H., Schwamborn, J.C., Li, S., and Dorf, M.E. (2015). TRIM32 senses and restricts influenza A virus by ubiquitination of PB1 polymerase. *PLoS Pathog* 11(6)**,** e1004960. doi: 10.1371/journal.ppat.1004960.

Full, F., van Gent, M., Sparrer, K.M.J., Chiang, C., Zurenski, M.A., Scherer, M., et al. (2019). Centrosomal protein TRIM43 restricts herpesvirus infection by regulating nuclear lamina integrity. *Nat Microbiol* 4(1)**,** 164-176. doi: 10.1038/s41564-018-0285-5.

Gack, M.U., Shin, Y.C., Joo, C.H., Urano, T., Liang, C., Sun, L., et al. (2007). TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity. *Nature* 446(7138)**,** 916-920. doi: 10.1038/nature05732.

Gao, B., Duan, Z., Xu, W., and Xiong, S. (2009). Tripartite motif-containing 22 inhibits the activity of hepatitis B virus core promoter, which is dependent on nuclear-located RING domain. *Hepatology* 50(2)**,** 424-433. doi: https://doi.org/10.1002/hep.23011.

Glasauer, S.M., and Neuhauss, S.C. (2014). Whole-genome duplication in teleost fishes and its evolutionary consequences. *Mol Genet Genomics* 289(6)**,** 1045-1060. doi: 10.1007/s00438-014-0889-2.

Goyani, S., Roy, M., and Singh, R. (2021). TRIM-NHL as RNA Binding Ubiquitin E3 Ligase (RBUL): Implication in development and disease pathogenesis. *Biochim Biophys Acta Mol Basis Dis* 1867(7)**,** 166066. doi: 10.1016/j.bbadis.2020.166066.

Hammell, C.M., Lubin, I., Boag, P.R., Blackwell, T.K., and Ambros, V. (2009). nhl-2 modulates microRNA activity in *Caenorhabditis elegans*. *Cell* 136(5)**,** 926-938. doi: 10.1016/j.cell.2009.01.053.

Hatakeyama, S. (2017). TRIM family proteins: Roles in autophagy, immunity, and carcinogenesis. *Trends Biochem Sci* 42(4)**,** 297-311. doi: 10.1016/j.tibs.2017.01.002.

Hattlmann, C.J., Kelly, J.N., and Barr, S.D. (2012). TRIM22: A diverse and dynamic antiviral protein. *Mol Biol Int* 2012**,** 153415. doi: 10.1155/2012/153415.

Hayman, D.T., Yu, M., Crameri, G., Wang, L.F., Suu-Ire, R., Wood, J.L., et al. (2012). Ebola virus antibodies in fruit bats, Ghana, West Africa. *Emerg Infect Dis* 18(7)**,** 1207-1209. doi: 10.3201/eid1807.111654.

Hayman, T.J., Hsu, A.C., Kolesnik, T.B., Dagley, L.F., Willemsen, J., Tate, M.D., et al. (2019). RIPLET, and not TRIM25, is required for endogenous RIG-I-dependent antiviral responses. *Immunology & Cell Biology* 97(9)**,** 840-852. doi: https://doi.org/10.1111/imcb.12284.

Hershko, A., and Ciechanover, A. (1998). The ubiquitin system. *Annu Rev Biochem* 67**,** 425-479. doi: 10.1146/annurev.biochem.67.1.425.

Herz, C., Stavnezer, E., Krug, R., and Gurney, T., Jr. (1981). Influenza virus, an RNA virus, synthesizes its messenger RNA in the nucleus of infected cells. *Cell* 26(3 Pt 1)**,** 391-400. doi: 10.1016/0092-8674(81)90208-7.

Higgs, R., Gabhann, J.N., Larbi, N.B., Breen, E.P., Fitzgerald, K.A., and Jefferies, C.A. (2008). The E3 ubiquitin ligase Ro52 negatively regulates IFN-β production post-pathogen recognition by polyubiquitin-mediated degradation of IRF3. *The Journal of Immunology* 181(3)**,** 1780. doi: 10.4049/jimmunol.181.3.1780.

Hou, F., Sun, L., Zheng, H., Skaug, B., Jiang, Q.X., and Chen, Z.J. (2011). MAVS forms functional prion-like aggregates to activate and propagate antiviral innate immune response. *Cell* 146(3)**,** 448-461. doi: 10.1016/j.cell.2011.06.041.

Hu, M.M., Liao, C.Y., Yang, Q., Xie, X.Q., and Shu, H.B. (2017). Innate immunity to RNA virus is regulated by temporal and reversible sumoylation of RIG-I and MDA5. *J Exp Med* 214(4)**,** 973-989. doi: 10.1084/jem.20161015.

Hu, M.M., Xie, X.Q., Yang, Q., Liao, C.Y., Ye, W., Lin, H., et al. (2015). TRIM38 negatively regulates TLR3/4-mediated innate immune and inflammatory responses by two sequential and distinct mechanisms. *J Immunol* 195(9)**,** 4415-4425. doi: 10.4049/jimmunol.1500859.

Hu, M.M., Yang, Q., Xie, X.Q., Liao, C.Y., Lin, H., Liu, T.T., et al. (2016). Sumoylation promotes the stability of the DNA sensor cGAS and the adaptor STING to regulate the kinetics of response to DNA virus. *Immunity* 45(3)**,** 555-569. doi: 10.1016/j.immuni.2016.08.014.

Huang, B., and Baek, S.H. (2017). TRIM13 potentiates toll-Like receptor 2-mediated nuclear factor κB activation via K29-linked polyubiquitination of tumor necrosis factor receptor-associated factor 6. *Mol Pharmacol* 91(4)**,** 307-316. doi: 10.1124/mol.116.106716.

Huang, Y., Li, Y., Burt, D.W., Chen, H., Zhang, Y., Qian, W., et al. (2013). The duck genome and transcriptome provide insight into an avian influenza virus reservoir species. *Nat Genet* 45(7)**,** 776-783. doi: 10.1038/ng.2657.

Huynh, J., Li, S., Yount, B., Smith, A., Sturges, L., Olsen, J.C., et al. (2012). Evidence supporting a zoonotic origin of human coronavirus strain NL63. *J Virol* 86(23)**,** 12816-12825. doi: 10.1128/jvi.00906-12.

Irving, A.T., Ahn, M., Goh, G., Anderson, D.E., and Wang, L.-F. (2021). Lessons from the host defences of bats, a unique viral reservoir. *Nature* 589(7842)**,** 363-370. doi: 10.1038/s41586-020-03128-0.

Jackson, D.A., Caton, A.J., McCready, S.J., and Cook, P.R. (1982). Influenza virus RNA is synthesized at fixed sites in the nucleus. *Nature* 296(5855)**,** 366-368. doi: 10.1038/296366a0.

James, L.C., Keeble, A.H., Khan, Z., Rhodes, D.A., and Trowsdale, J. (2007). Structural basis for PRYSPRY-mediated tripartite motif (TRIM) protein function. *Proceedings of the National Academy of Sciences* 104(15)**,** 6200. doi: 10.1073/pnas.0609174104.

Jia, X., Zhao, C., and Zhao, W. (2021). Emerging roles of MHC class I region-encoded E3 ubiquitin ligases in innate immunity. *Frontiers in Immunology* 12. doi: 10.3389/fimmu.2021.687102.

Kaikai, H., Zhao, D., Liu, Y., Liu, Q., Huang, X., Yang, J., et al. (2021). The E3 ubiquitin ligase TRIM25 inhibits Tembusu virus replication in vitro. *Front Vet Sci* 8**,** 722113. doi: 10.3389/fvets.2021.722113.

Kar, S., Rodriguez, S.E., Akyildiz, G., Cajimat, M.N.B., Bircan, R., Mears, M.C., et al. (2020). Crimean-Congo hemorrhagic fever virus in tortoises and Hyalomma aegyptium ticks in East Thrace, Turkey: potential of a cryptic transmission cycle. *Parasites & Vectors* 13(1)**,** 201. doi: 10.1186/s13071-020-04074-6.

Kato, K., Ahmad, S., Zhu, Z., Young, J.M., Mu, X., Park, S., et al. (2021). Structural analysis of RIG-I-like receptors reveals ancient rules of engagement between diverse RNA helicases and TRIM ubiquitin ligases. *Mol Cell* 81(3)**,** 599-613.e598. doi: 10.1016/j.molcel.2020.11.047.

Kaufman, J. (2022). Innate immune genes of the chicken MHC and related regions. *Immunogenetics* 74(1)**,** 167-177. doi: 10.1007/s00251-021-01229-2.

Kim, J.-K., Negovetich, N.J., Forrest, H.L., and Webster, R.G. (2009). Ducks: the "Trojan horses" of H5N1 influenza. *Influenza and other respiratory viruses* 3(4)**,** 121-128. doi: 10.1111/j.1750-2659.2009.00084.x.

Koch, L.K., Cunze, S., Kochmann, J., and Klimpel, S. (2020). Bats as putative Zaire ebolavirus reservoir hosts and their habitat suitability in Africa. *Scientific Reports* 10(1)**,** 14268. doi: 10.1038/s41598-020-71226-0.

Koliopoulos, M.G., Esposito, D., Christodoulou, E., Taylor, I.A., and Rittinger, K. (2016). Functional role of TRIM E3 ligase oligomerization and regulation of catalytic activity. *The EMBO Journal* 35(11)**,** 1204-1218. doi: https://doi.org/10.15252/embj.201593741.

Kondo, T., Watanabe, M., and Hatakeyama, S. (2012). TRIM59 interacts with ECSIT and negatively regulates NF-κB and IRF-3/7-mediated signal pathways. *Biochemical and Biophysical Research Communications* 422(3)**,** 501-507. doi: https://doi.org/10.1016/j.bbrc.2012.05.028.

Lang, X., Tang, T., Jin, T., Ding, C., Zhou, R., and Jiang, W. (2017). TRIM65-catalized ubiquitination is essential for MDA5-mediated antiviral innate immunity. *J Exp Med* 214(2)**,** 459-473. doi: 10.1084/jem.20160592.

Latinne, A., Hu, B., Olival, K.J., Zhu, G., Zhang, L., Li, H., et al. (2020). Origin and cross-species transmission of bat coronaviruses in China. *Nature Communications* 11(1)**,** 4235. doi: 10.1038/s41467-020-17687-3.

Le Goffic, R., Bouguyon, E., Chevalier, C., Vidic, J., Da Costa, B., Leymarie, O., et al. (2010). Influenza A virus protein PB1-F2 exacerbates IFN-β expression of human respiratory epithelial cells. *The Journal of Immunology* 185(8)**,** 4812. doi: 10.4049/jimmunol.0903952.

Lee, N.R., Kim, H.I., Choi, M.S., Yi, C.M., and Inn, K.S. (2015). Regulation of MDA5-MAVS antiviral signaling axis by TRIM25 through TRAF6-mediated NF-κB activation. *Mol Cells* 38(9)**,** 759-764. doi: 10.14348/molcells.2015.0047.

Lee, Y., Song, B., Park, C., and Kwon, K.S. (2013). TRIM11 negatively regulates IFNβ production and antiviral activity by targeting TBK1. *PLoS One* 8(5)**,** e63255. doi: 10.1371/journal.pone.0063255.

Lei, C.Q., Zhang, Y., Li, M., Jiang, L.Q., Zhong, B., Kim, Y.H., et al. (2015). ECSIT bridges RIG-I-like receptors to VISA in signaling events of innate antiviral responses. *J Innate Immun* 7(2)**,** 153-164. doi: 10.1159/000365971.

Lei, C.Q., Zhong, B., Zhang, Y., Zhang, J., Wang, S., and Shu, H.B. (2010). Glycogen synthase kinase 3β regulates IRF3 transcription factor-mediated antiviral response via activation of the kinase TBK1. *Immunity* 33(6)**,** 878-889. doi: 10.1016/j.immuni.2010.11.021.

Lepetz, V., Massot, M., Chaine, A.S., and Clobert, J. (2009). Climate warming and the evolution of morphotypes in a reptile. *Global Change Biology* 15(2)**,** 454-466. doi: https://doi.org/10.1111/j.1365-2486.2008.01761.x.

Letko, M., Seifert, S.N., Olival, K.J., Plowright, R.K., and Munster, V.J. (2020). Bat-borne virus diversity, spillover and emergence. *Nature Reviews Microbiology* 18(8)**,** 461-471. doi: 10.1038/s41579-020-0394-z.

Li, L., Feng, W., Cheng, Z., Yang, J., Bi, J., Wang, X., et al. (2019). TRIM62-mediated restriction of avian leukosis virus subgroup J replication is dependent on the SPRY domain. *Poult Sci* 98(11)**,** 6019-6025. doi: 10.3382/ps/pez408.

Li, L., Niu, D., Yang, J., Bi, J., Zhang, L., Cheng, Z., et al. (2020). TRIM62 from chicken as a negative regulator of reticuloendotheliosis virus replication. *Frontiers in Veterinary Science* 7.

Li, T., Hu, X., Zhang, T., Song, X., Zhang, H., Dai, N., et al. (2021). Cloning, analysis, and anti-duck Tembusu virus innate immune response of Cherry Valley duck tripartite motif-containing 32. *Poult Sci* 100(5)**,** 101048. doi: 10.1016/j.psj.2021.101048.

Li, X., Yu, Z., Fang, Q., Yang, M., Huang, J., Li, Z., et al. (2022). The transmembrane endoplasmic reticulum-associated E3 ubiquitin ligase TRIM13 restrains the pathogenic-DNA-triggered inflammatory response. *Sci Adv* 8(4)**,** eabh0496. doi: 10.1126/sciadv.abh0496.

Liang, Q., Deng, H., Li, X., Wu, X., Tang, Q., Chang, T.H., et al. (2011). Tripartite motif-containing protein 28 is a small ubiquitin-related modifier E3 ligase and negative regulator of IFN regulatory factor 7. *J Immunol* 187(9)**,** 4754-4763. doi: 10.4049/jimmunol.1101704.

Liu, B., Li, N.L., Shen, Y., Bao, X., Fabrizio, T., Elbahesh, H., et al. (2016). The C-Terminal tail of TRIM56 dictates antiviral restriction of Influenza A and B Viruses by impeding viral RNA synthesis. *J Virol* 90(9)**,** 4369-4382. doi: 10.1128/JVI.03172-15.

Liu, B., Li, N.L., Wang, J., Shi, P.Y., Wang, T., Miller, M.A., et al. (2014). Overlapping and distinct molecular determinants dictating the antiviral activities of TRIM56 against flaviviruses and coronavirus. *J Virol* 88(23)**,** 13821-13835. doi: 10.1128/jvi.02505-14.

Liu, B., Zhang, M., Chu, H., Zhang, H., Wu, H., Song, G., et al. (2017a). The ubiquitin E3 ligase TRIM31 promotes aggregation and activation of the signaling adaptor MAVS through Lys63-linked polyubiquitination. *Nat Immunol* 18(2)**,** 214-224. doi: 10.1038/ni.3641.

Liu, Y., Lin, R., and Olagnier, D. (2017b). RIGulation of STING expression: at the crossroads of viral RNA and DNA sensing pathways. *Inflamm Cell Signal* 4(1)**,** e1491. doi: 10.14800/ics.1491.

Liu, Z.-S., Zhang, Z.-Y., Cai, H., Zhao, M., Mao, J., Dai, J., et al. (2018). RINCK-mediated monoubiquitination of cGAS promotes antiviral innate immune responses. *Cell & Bioscience* 8(1)**,** 35. doi: 10.1186/s13578-018-0233-3.

Loedige, I., Gaidatzis, D., Sack, R., Meister, G., and Filipowicz, W. (2013). The mammalian TRIM-NHL protein TRIM71/LIN-41 is a repressor of mRNA function. *Nucleic Acids Res* 41(1)**,** 518-532. doi: 10.1093/nar/gks1032.

Maller Schulman, B.R., Liang, X., Stahlhut, C., DelConte, C., Stefani, G., and Slack, F.J. (2008). The let-7 microRNA target gene, Mlin41/Trim71 is required for mouse embryonic survival and neural tube closure. *Cell Cycle* 7(24)**,** 3935-3942. doi: 10.4161/cc.7.24.7397.

Marín, I. (2012). Origin and diversification of TRIM ubiquitin ligases. *PLOS ONE* 7(11)**,** e50030. doi: 10.1371/journal.pone.0050030.

Massiah, M.A. (2019). *Zinc-Binding B-Box Domains with RING Folds Serve Critical Roles in the Protein Ubiquitination Pathways in Plants and Animals.* London: IntechOpen.

Meng, J., Yao, Z., He, Y., Zhang, R., Zhang, Y., Yao, X., et al. (2017). ARRDC4 regulates enterovirus 71-induced innate immune response by promoting K63 polyubiquitination of MDA5 through TRIM65. *Cell Death Dis* 8(6)**,** e2866. doi: 10.1038/cddis.2017.257.

Meroni, G. (2012). Genomics and evolution of the TRIM gene family. *Adv Exp Med Biol* 770**,** 1-9. doi: 10.1007/978-1-4614-5398-7_1.

Metzger, M.B., Pruneda, J.N., Klevit, R.E., and Weissman, A.M. (2014). RING-type E3 ligases: Master manipulators of E2 ubiquitin-conjugating enzymes and ubiquitination. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 1843(1)**,** 47-60. doi: https://doi.org/10.1016/j.bbamcr.2013.05.026.

Meyer, M., Gaudieri, S., Rhodes, D.A., and Trowsdale, J. (2003). Cluster of TRIM genes in the human MHC class I region sharing the B30.2 domain. *Tissue Antigens* 61(1)**,** 63-71. doi: 10.1034/j.1399-0039.2003.610105.x.

Meyerson, N.R., Zhou, L., Guo, Y.R., Zhao, C., Tao, Y.J., Krug, R.M., et al. (2017). Nuclear TRIM25 specifically targets influenza virus ribonucleoproteins to block the onset of RNA chain elongation. *Cell Host Microbe* 22(5)**,** 627-638 e627. doi: 10.1016/j.chom.2017.10.003.

Miranda, C., Roccato, E., Raho, G., Pagliardini, S., Pierotti, M.A., and Greco, A. (2006). The TFG protein, involved in oncogenic rearrangements, interacts with TANK and NEMO, two proteins involved in the NF-kappaB pathway. *J Cell Physiol* 208(1)**,** 154-160. doi: 10.1002/jcp.20644.

Miranzo-Navarro, D., and Magor, K.E. (2014). Activation of duck RIG-I by TRIM25 is independent of anchored ubiquitin. *PLoS One* 9(1)**,** e86968. doi: 10.1371/journal.pone.0086968.

Nabi, G., Wang, Y., Lü, L., Jiang, C., Ahmad, S., Wu, Y., et al. (2021). Bats and birds as viral reservoirs: A physiological and ecological perspective. *Sci Total Environ* 754**,** 142372. doi: 10.1016/j.scitotenv.2020.142372.

Narayan, K., Waggoner, L., Pham, S.T., Hendricks, G.L., Waggoner, S.N., Conlon, J., et al. (2014). TRIM13 Is a Negative Regulator of MDA5-Mediated Type I Interferon Production. *Journal of Virology* 88(18)**,** 10748. doi: 10.1128/JVI.02593-13.

Nathan, J.A., Kim, H.T., Ting, L., Gygi, S.P., and Goldberg, A.L. (2013). Why do cellular proteins linked to K63-polyubiquitin chains not associate with proteasomes? *Embo j* 32(4)**,** 552-565. doi: 10.1038/emboj.2012.354.

Nenasheva, V.V., Nikitenko, N.A., Stepanenko, E.A., Makarova, I.V., Andreeva, L.E., Kovaleva, G.V., et al. (2021). Human TRIM14 protects transgenic mice from influenza A viral infection without activation of other innate immunity pathways. *Genes & Immunity* 22(1)**,** 56-63. doi: 10.1038/s41435-021-00128-6.

Noguchi, K., Okumura, F., Takahashi, N., Kataoka, A., Kamiyama, T., Todo, S., et al. (2011). TRIM40 promotes neddylation of IKKγ and is downregulated in gastrointestinal cancers. *Carcinogenesis* 32(7)**,** 995-1004. doi: 10.1093/carcin/bgr068.

Olsen, B., Munster, V.J., Wallensten, A., Waldenstrom, J., Osterhaus, A.D., and Fouchier, R.A. (2006). Global patterns of influenza a virus in wild birds. *Science* 312(5772)**,** 384-388. doi: 10.1126/science.1122438.

Ozato, K., Shin, D.M., Chang, T.H., and Morse, H.C., 3rd (2008). TRIM family proteins and their emerging roles in innate immunity. *Nat Rev Immunol* 8(11)**,** 849-860. doi: 10.1038/nri2413.

Park, H.H. (2018). Structure of TRAF Family: current Uunderstanding of receptor recognition. *Frontiers in Immunology* 9.

Patil, G., Xu, L., Wu, Y., Song, K., Hao, W., Hua, F., et al. (2020). TRIM41-mediated ubiquitination of nucleoprotein Limits Vesicular Stomatitis Virus Infection. *Viruses* 12(2). doi: 10.3390/v12020131.

Patil, G., Zhao, M., Song, K., Hao, W., Bouchereau, D., Wang, L., et al. (2018). TRIM41-mediated ubiquitination of nucleoprotein limits influenza A virus infection. *J Virol* 92(16). doi: 10.1128/JVI.00905-18.

Peng, C., Zhao, C., Wang, P.F., Yan, L.L., Fan, S.G., and Qiu, L.H. (2021). Identification of a TRIM32 from Penaeus monodon is involved in autophagy and innate immunity during white spot syndrome virus infection. *Dev Comp Immunol* 123**,** 104169. doi: 10.1016/j.dci.2021.104169.

Peng, X., Wen, Y., Zha, L., Zhuang, J., Lin, L., Li, X., et al. (2020). TRIM45 suppresses the development of non-small cell lung cancer. *Curr Mol Med* 20(4)**,** 299-306. doi: 10.2174/1566524019666191017143833.

Perera, S., Mankoo, B., and Gautel, M. (2012). Developmental regulation of MURF E3 ubiquitin ligases in skeletal muscle. *J Muscle Res Cell Motil* 33(2)**,** 107-122. doi: 10.1007/s10974-012-9288-7.

Perlas, A., Argilaguet, J., Bertran, K., Sánchez-González, R., Nofrarías, M., Valle, R., et al. (2021). Dual host and pathogen RNA-seq analysis unravels chicken genes potentially involved in resistance to highly pathogenic avian influenza virus infection. *Frontiers in Immunology* 12.

Peterson, A.T., Vieglais, D.A., and Andreasen, J.K. (2003). Migratory birds modeled as critical transport agents for West Nile Virus in North America. *Vector Borne Zoonotic Dis* 3(1)**,** 27-37. doi: 10.1089/153036603765627433.

Poole, E., Groves, I., MacDonald, A., Pang, Y., Alcami, A., and Sinclair, J. (2009). Identification of TRIM23 as a cofactor involved in the regulation of NF-kappaB by human cytomegalovirus. *J Virol* 83(8)**,** 3581-3590. doi: 10.1128/jvi.02072-08.

Qi, H., Xia, F.-N., Xiao, S., and Li, J. (2022). TRAF proteins as key regulators of plant development and stress responses. *Journal of Integrative Plant Biology* 64(2)**,** 431-448. doi: https://doi.org/10.1111/jipb.13182.

Qiao, Y., Zhou, Y., Song, C., Zhang, X., and Zou, Y. (2020). MID1 and MID2 regulate cell migration and epithelial-mesenchymal transition via modulating Wnt/β-catenin signaling. *Ann Transl Med* 8(16)**,** 1021. doi: 10.21037/atm-20-5583.

Qin, Y., Liu, Q., Tian, S., Xie, W., Cui, J., and Wang, R.F. (2016). TRIM9 short isoform preferentially promotes DNA and RNA virus-induced production of type I interferon by recruiting GSK3β to TBK1. *Cell Res* 26(5)**,** 613-628. doi: 10.1038/cr.2016.27.

Rahman, S.A., Hassan, S.S., Olival, K.J., Mohamed, M., Chang, L.Y., Hassan, L., et al. (2010). Characterization of Nipah virus from naturally infected Pteropus vampyrus bats, Malaysia. *Emerg Infect Dis* 16(12)**,** 1990-1993. doi: 10.3201/eid1612.091790.

Rajsbaum, R., Stoye, J.P., and O'Garra, A. (2008). Type I interferon-dependent and -independent expression of tripartite motif proteins in immune cells. *Eur J Immunol* 38(3)**,** 619-630. doi: 10.1002/eji.200737916.

Ran, Y., Zhang, J., Liu, L.L., Pan, Z.Y., Nie, Y., Zhang, H.Y., et al. (2016). Autoubiquitination of TRIM26 links TBK1 to NEMO in RLR-mediated innate antiviral immune response. *J Mol Cell Biol* 8(1)**,** 31-43. doi: 10.1093/jmcb/mjv068.

Rappole, J.H., Derrickson, S.R., and Hubálek, Z. (2000). Migratory birds and spread of West Nile virus in the Western Hemisphere. *Emerg Infect Dis* 6(4)**,** 319-328. doi: 10.3201/eid0604.000401.

Ravelomanantsoa, N.A.F., Guth, S., Andrianiaina, A., Andry, S., Gentles, A., Ranaivoson, H.C., et al. (2020). The zoonotic potential of bat-borne coronaviruses. *Emerging Topics in Life Sciences* 4(4)**,** 365-381. doi: 10.1042/ETLS20200097.

Reed, K.D., Meece, J.K., Henkel, J.S., and Shukla, S.K. (2003). Birds, migration and emerging zoonoses: west nile virus, lyme disease, influenza A and enteropathogens. *Clin Med Res* 1(1)**,** 5-12. doi: 10.3121/cmr.1.1.5.

Reynes, J.M., Counor, D., Ong, S., Faure, C., Seng, V., Molia, S., et al. (2005). Nipah virus in Lyle's flying foxes, Cambodia. *Emerg Infect Dis* 11(7)**,** 1042-1047. doi: 10.3201/eid1107.041350.

Rom, O., and Reznick, A.Z. (2016). The role of E3 ubiquitin-ligases MuRF-1 and MAFbx in loss of skeletal muscle mass. *Free Radic Biol Med* 98**,** 218-230. doi: 10.1016/j.freeradbiomed.2015.12.031.

Ruby, T., Bed'Hom, B., Wittzell, H., Morin, V., Oudin, A., and Zoorob, R. (2005). Characterisation of a cluster of TRIM-B30.2 genes in the chicken MHC B locus. *Immunogenetics* 57(1-2)**,** 116-128. doi: 10.1007/s00251-005-0770-x.

Saito, L.B., Diaz-Satizabal, L., Evseev, D., Fleming-Canepa, X., Mao, S., Webster, R.G., et al. (2018). IFN and cytokine responses in ducks to genetically similar H5N1 influenza A viruses of varying pathogenicity. *J Gen Virol* 99(4)**,** 464-474. doi: 10.1099/jgv.0.001015.

Sanchez, J.G., Okreglicka, K., Chandrasekaran, V., Welker, J.M., Sundquist, W.I., and Pornillos, O. (2014). The tripartite motif coiled-coil is an elongated antiparallel hairpin dimer. *Proc Natl Acad Sci U S A* 111(7)**,** 2494-2499. doi: 10.1073/pnas.1318962111.

Sardiello, M., Cairo, S., Fontanella, B., Ballabio, A., and Meroni, G. (2008). Genomic analysis of the TRIM family reveals two groups of genes with distinct evolutionary properties. *BMC Evol Biol* 8(1)**,** 225. doi: 10.1186/1471-2148-8-225.

Sawyer, S.L., Emerman, M., and Malik, H.S. (2007). Discordant evolution of the adjacent antiretroviral genes TRIM22 and TRIM5 in Mammals. *PLOS Pathogens* 3(12)**,** e197. doi: 10.1371/journal.ppat.0030197.

Shapira, S.D., Gat-Viks, I., Shum, B.O., Dricot, A., de Grace, M.M., Wu, L., et al. (2009). A physical and regulatory map of host-influenza interactions reveals pathways in H1N1 infection. *Cell* 139(7)**,** 1255-1267. doi: 10.1016/j.cell.2009.12.018.

Shen, Y., Li, N.L., Wang, J., Liu, B., Lester, S., and Li, K. (2012). TRIM56 is an essential component of the TLR3 antiviral signaling pathway. *J Biol Chem* 287(43)**,** 36404-36413. doi: 10.1074/jbc.M112.397075.

Shi, M., Cho, H., Inn, K.S., Yang, A., Zhao, Z., Liang, Q., et al. (2014). Negative regulation of NF-κB activity by brain-specific TRIpartite Motif protein 9. *Nat Commun* 5**,** 4820. doi: 10.1038/ncomms5820.

Shi, Y., Hu, S., Duan, W., Ding, T., and Zhao, Z. (2019). The distinct evolutionary properties of the tripartite motif-containing protein 39 in the Chinese softshell turtle based on its structural and functional characterization. *Developmental & Comparative Immunology* 99**,** 103407. doi: https://doi.org/10.1016/j.dci.2019.103407.

Shiina, T., Briles, W.E., Goto, R.M., Hosomichi, K., Yanagiya, K., Shimizu, S., et al. (2007). Extended gene map reveals tripartite motif, C-type lectin, and Ig superfamily type genes within a subregion of the chicken MHC-B affecting infectious disease. *J Immunol* 178(11)**,** 7162-7172. doi: 10.4049/jimmunol.178.11.7162.

Short, K.M., and Cox, T.C. (2006). Subclassification of the RBCC/TRIM superfamily reveals a novel motif necessary for microtubule binding. *Journal of Biological Chemistry* 281(13)**,** 8970-8980. doi: https://doi.org/10.1074/jbc.M512755200.

Song, B., Javanbakht, H., Perron, M., Park, D.H., Stremlau, M., and Sodroski, J. (2005). Retrovirus restriction by TRIM5alpha variants from Old World and New World primates. *Journal of virology* 79(7)**,** 3930-3937. doi: 10.1128/JVI.79.7.3930-3937.2005.

Sparrer, K.M.J., Gableske, S., Zurenski, M.A., Parker, Z.M., Full, F., Baumgart, G.J., et al. (2017). TRIM23 mediates virus-induced autophagy via activation of TBK1. *Nat Microbiol* 2(11)**,** 1543-1557. doi: 10.1038/s41564-017-0017-2.

Spencer, J.A., Eliazer, S., Ilaria, R.L., Jr., Richardson, J.A., and Olson, E.N. (2000). Regulation of Microtubule Dynamics and Myogenic Differentiation by Murf, a Striated Muscle Ring-Finger Protein. *Journal of Cell Biology* 150(4)**,** 771-784. doi: 10.1083/jcb.150.4.771.

Stremlau, M., Owens, C.M., Perron, M.J., Kiessling, M., Autissier, P., and Sodroski, J. (2004). The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. *Nature* 427(6977)**,** 848-853. doi: 10.1038/nature02343.

Sun, M., Li, S., Jin, S., Li, X., Xiang, J., and Li, F. (2022). A Novel TRIM9 protein promotes NF-κB activation through interacting with LvIMD in shrimp during WSSV infection. *Frontiers in Immunology* 13.

Sun, M., Li, S., Yu, K., Xiang, J., and Li, F. (2019). An E3 ubiquitin ligase TRIM9 is involved in WSSV infection via interaction with β-TrCP. *Developmental & Comparative Immunology* 97**,** 57-63. doi: https://doi.org/10.1016/j.dci.2019.03.014.

Sun, N., Jiang, L., Ye, M., Wang, Y., Wang, G., Wan, X., et al. (2020). TRIM35 mediates protection against influenza infection by activating TRAF3 and degrading viral PB2. *Protein Cell* 11(12)**,** 894-914. doi: 10.1007/s13238-020-00734-6.

Suzuki, M., Watanabe, M., Nakamaru, Y., Takagi, D., Takahashi, H., Fukuda, S., et al. (2016). TRIM39 negatively regulates the NFκB-mediated signaling pathway through stabilization

of Cactin. *Cellular and Molecular Life Sciences* 73(5)**,** 1085-1101. doi: 10.1007/s00018-015-2040-x.

Swatek, K.N., and Komander, D. (2016). Ubiquitin modifications. *Cell Res* 26(4)**,** 399-422. doi: 10.1038/cr.2016.39.

Taieb, L., Ludwig, A., Ogden, N.H., Lindsay, R.L., Iranpour, M., Gagnon, C.A., et al. (2020). Bird species involved in West Nile virus epidemiological cycle in southern Québec. *Int J Environ Res Public Health* 17(12). doi: 10.3390/ijerph17124517.

Tan, G., Xu, F., Song, H., Yuan, Y., Xiao, Q., Ma, F., et al. (2018). Identification of TRIM14 as a Type I IFN-Stimulated gene controlling hepatitis B virus replication by targeting HBx. *Front Immunol* 9**,** 1872. doi: 10.3389/fimmu.2018.01872.

Tanji, K., Kamitani, T., Mori, F., Kakita, A., Takahashi, H., and Wakabayashi, K. (2010). TRIM9, a novel brain-specific E3 ubiquitin ligase, is repressed in the brain of Parkinson's disease and dementia with Lewy bodies. *Neurobiology of Disease* 38(2)**,** 210-218. doi: https://doi.org/10.1016/j.nbd.2010.01.007.

Towner, J.S., Amman, B.R., Sealy, T.K., Carroll, S.A., Comer, J.A., Kemp, A., et al. (2009). Isolation of genetically diverse Marburg viruses from Egyptian fruit bats. *PLoS Pathog* 5(7)**,** e1000536. doi: 10.1371/journal.ppat.1000536.

Tracz, M., and Bialek, W. (2021). Beyond K48 and K63: non-canonical protein ubiquitination. *Cellular & Molecular Biology Letters* 26(1)**,** 1. doi: 10.1186/s11658-020-00245-6.

Tsuchida, T., Zou, J., Saitoh, T., Kumar, H., Abe, T., Matsuura, Y., et al. (2010). The ubiquitin ligase TRIM56 regulates innate immune responses to intracellular double-stranded DNA. *Immunity* 33(5)**,** 765-776. doi: 10.1016/j.immuni.2010.10.013.

Tucker, M.E., and Benton, M.J. (1982). Triassic environments, climates and reptile evolution. *Palaeogeography, Palaeoclimatology, Palaeoecology* 40(4)**,** 361-379. doi: https://doi.org/10.1016/0031-0182(82)90034-7.

Valiyeva, F., Jiang, F., Elmaadawi, A., Moussa, M., Yee, S.P., Raptis, L., et al. (2011). Characterization of the oncogenic activity of the novel TRIM59 gene in mouse cancer models. *Mol Cancer Ther* 10(7)**,** 1229-1240. doi: 10.1158/1535-7163.Mct-11-0077.

van Beuningen, S.F.B., Will, L., Harterink, M., Chazeau, A., van Battum, E.Y., Frias, C.P., et al. (2015). TRIM46 controls neuronal polarity and axon specification by driving the

formation of parallel microtubule arrays. *Neuron* 88(6)**,** 1208-1226. doi: 10.1016/j.neuron.2015.11.012.

Van Brussel, K., and Holmes, E.C. (2022). Zoonotic disease and virome diversity in bats. *Current Opinion in Virology* 52**,** 192-202. doi: https://doi.org/10.1016/j.coviro.2021.12.008.

van der Aa, L.M., Levraud, J.-P., Yahmi, M., Lauret, E., Briolat, V., Herbomel, P., et al. (2009). A large new subset of TRIM genes highly diversified by duplication and positive selection in teleost fish. *BMC Biology* 7(1)**,** 7. doi: 10.1186/1741-7007-7-7.

van Gent, M., Sparrer, K.M.J., and Gack, M.U. (2018). TRIM proteins and their roles in antiviral host defenses. *Annu Rev Virol* 5(1)**,** 385-405. doi: 10.1146/annurev-virology-092917-043323.

Vanderven, H.A., Petkau, K., Ryan-Jean, K.E., Aldridge, J.R., Jr., Webster, R.G., and Magor, K.E. (2012). Avian influenza rapidly induces antiviral genes in duck lung and intestine. *Mol Immunol* 51(3-4)**,** 316-324. doi: 10.1016/j.molimm.2012.03.034.

Venuto, S., and Merla, G. (2019). E3 ubiquitin ligase TRIM proteins, cell cycle and mitosis. *Cells* 8(5). doi: 10.3390/cells8050510.

Versteeg, G.A., Rajsbaum, R., Sánchez-Aparicio, M.T., Maestre, A.M., Valdiviezo, J., Shi, M., et al. (2013). The E3-ligase TRIM family of proteins regulates signaling pathways triggered by innate immune pattern-recognition receptors. *Immunity* 38(2)**,** 384-398. doi: 10.1016/j.immuni.2012.11.013.

Vidana, B., Dolz, R., Busquets, N., Ramis, A., Sanchez, R., Rivas, R., et al. (2018). Transmission and immunopathology of the avian influenza virus A/Anhui/1/2013 (H7N9) human isolate in three commonly commercialized avian species. *Zoonoses Public Health* 65(3)**,** 312-321. doi: 10.1111/zph.12393.

Wallenhammar, A., Anandapadamanaban, M., Lemak, A., Mirabello, C., Lundström, P., Wallner, B., et al. (2017). Solution NMR structure of the TRIM21 B-box2 and identification of residues involved in its interaction with the RING domain. *PLoS One* 12(7)**,** e0181551. doi: 10.1371/journal.pone.0181551.

Wang, J., Liu, B., Wang, N., Lee, Y.M., Liu, C., and Li, K. (2011). TRIM56 is a virus- and interferon-inducible E3 ubiquitin ligase that restricts pestivirus infection. *J Virol* 85(8)**,** 3733-3745. doi: 10.1128/jvi.02546-10.

Wang, L., Fu, B., Li, W., Patil, G., Liu, L., Dorf, M.E., et al. (2017). Comparative influenza protein interactomes identify the role of plakophilin 2 in virus restriction. *Nature Communications* 8(1)**,** 13876. doi: 10.1038/ncomms13876.

Wang, L., Lu, K.C., Chen, G.L., Li, M., Zhang, C.Z., and Chen, Y.H. (2020). A Litopenaeus vannamei TRIM32 gene is involved in oxidative stress response and innate immunity. *Fish Shellfish Immunol* 107(Pt B)**,** 547-555. doi: 10.1016/j.fsi.2020.11.002.

Wang, P., Zhao, W., Zhao, K., Zhang, L., and Gao, C. (2015a). TRIM26 negatively regulates interferon-β production and antiviral response through polyubiquitination and degradation of nuclear IRF3. *PLoS Pathog* 11(3)**,** e1004726. doi: 10.1371/journal.ppat.1004726.

Wang, S., Chen, Y., Li, C., Wu, Y., Guo, L., Peng, C., et al. (2016a). TRIM14 inhibits hepatitis C virus infection by SPRY domain-dependent targeted degradation of the viral NS5A protein. *Scientific Reports* 6(1)**,** 32336. doi: 10.1038/srep32336.

Wang, W., Huang, Y., Yu, Y., Yang, Y., Xu, M., Chen, X., et al. (2016b). Fish TRIM39 regulates cell cycle progression and exerts its antiviral function against iridovirus and nodavirus. *Fish & Shellfish Immunology* 50**,** 1-10. doi: https://doi.org/10.1016/j.fsi.2016.01.016.

Wang, Y., Li, Y., Qi, X., Yuan, W., Ai, J., Zhu, C., et al. (2004). TRIM45, a novel human RBCC/TRIM protein, inhibits transcriptional activities of ElK-1 and AP-1. *Biochem Biophys Res Commun* 323(1)**,** 9-16. doi: 10.1016/j.bbrc.2004.08.048.

Wang, Y., Li, Z., Lu, Y., Hu, G., Lin, L., Zeng, L., et al. (2016c). Molecular characterization, tissue distribution and expression, and potential antiviral effects of TRIM32 in the common carp (Cyprinus carpio). *International Journal of Molecular Sciences* 17(10). doi: 10.3390/ijms17101693.

Wang, Y., Lian, Q., Yang, B., Yan, S., Zhou, H., He, L., et al. (2015b). TRIM30α is a negative-feedback regulator of the intracellular DNA and DNA virus-triggered response by targeting STING. *PLoS Pathog* 11(6)**,** e1005012. doi: 10.1371/journal.ppat.1005012.

Watanabe, M., and Hatakeyama, S. (2017). TRIM proteins and diseases. *J Biochem* 161(2)**,** 135-144. doi: 10.1093/jb/mvw087.

Webster, R.G., Bean, W.J., Gorman, O.T., Chambers, T.M., and Kawaoka, Y. (1992). Evolution and ecology of influenza A viruses. *Microbiol Rev* 56(1)**,** 152-179.

Wei, Y., Zhou, H., Wang, A., Sun, L., Wang, M., Jia, R., et al. (2016). TRIM25 identification in the Chinese Goose: Gene structure, tissue expression profiles, and antiviral immune responses in vivo and in vitro. *Biomed Res Int* 2016**,** 1403984. doi: 10.1155/2016/1403984.

Wille, M., and Holmes, E.C. (2020). Wild birds as reservoirs for diverse and abundant gamma- and deltacoronaviruses. *FEMS Microbiology Reviews* 44(5)**,** 631-644. doi: 10.1093/femsre/fuaa026.

Williams, F.P., Haubrich, K., Perez-Borrajero, C., and Hennig, J. (2019). Emerging RNA-binding roles in the TRIM family of ubiquitin ligases. *Biological Chemistry* 400(11)**,** 1443-1464. doi: doi:10.1515/hsz-2019-0158.

Wu, B., and Hur, S. (2015). How RIG-I like receptors activate MAVS. *Curr Opin Virol* 12**,** 91-98. doi: 10.1016/j.coviro.2015.04.004.

Wu, S., Zhang, J., Xue, Q., Liu, J., Huang, B., He, Z., et al. (2020). Duck TRIM32 functions in IFN-β signaling against the Infection of H5N6 highly pathogenic avian influenza virus. *Front Immunol* 11**,** 377. doi: 10.3389/fimmu.2020.00377.

Wu, X., Wang, J., Wang, S., Wu, F., Chen, Z., Li, C., et al. (2019). Inhibition of influenza A virus replication by TRIM14 via its multifaceted protein-protein interaction with NP. *Front Microbiol* 10(FEB)**,** 344. doi: 10.3389/fmicb.2019.00344.

Wynne, C., Lazzari, E., Smith, S., McCarthy, E.M., J, N.G., Kallal, L.E., et al. (2014). TRIM68 negatively regulates IFN-β production by degrading TRK fused gene, a novel driver of IFN-β downstream of anti-viral detection systems. *PLoS One* 9(7)**,** e101503. doi: 10.1371/journal.pone.0101503.

Xing, J., Weng, L., Yuan, B., Wang, Z., Jia, L., Jin, R., et al. (2016). Identification of a role for TRIM29 in the control of innate immunity in the respiratory tract. *Nat Immunol* 17(12)**,** 1373-1380. doi: 10.1038/ni.3580.

Xing, J., Zhang, A., Minze, L.J., Li, X.C., and Zhang, Z. (2018). TRIM29 negatively regulates the Type I IFN production in response to RNA virus. *J Immunol* 201(1)**,** 183-192. doi: 10.4049/jimmunol.1701569.

Xing, J., Zhang, A., Zhang, H., Wang, J., Li, X.C., Zeng, M.-S., et al. (2017). TRIM29 promotes DNA virus infections by inhibiting innate immune response. *Nature Communications* 8(1)**,** 945. doi: 10.1038/s41467-017-00101-w.

Xue, B., Li, H., Guo, M., Wang, J., Xu, Y., Zou, X., et al. (2018). TRIM21 promotes innate immune response to RNA viral infection through Lys27-linked polyubiquitination of MAVS. *J Virol* 92(14). doi: 10.1128/jvi.00321-18.

Yan, J., Li, Q., Mao, A.P., Hu, M.M., and Shu, H.B. (2014). TRIM4 modulates type I interferon induction and cellular antiviral response by targeting RIG-I for K63-linked ubiquitination. *J Mol Cell Biol* 6(2)**,** 154-163. doi: 10.1093/jmcb/mju005.

Yang, B., Wang, J., Wang, Y., Zhou, H., Wu, X., Tian, Z., et al. (2013). Novel function of TRIM44 promotes an antiviral response by stabilizing VISA. *J Immunol* 190(7)**,** 3613-3619. doi: 10.4049/jimmunol.1202507.

Yang, C., Zhao, X., Sun, D., Yang, L., Chong, C., Pan, Y., et al. (2016). Interferon alpha (IFNα)-induced TRIM22 interrupts HCV replication by ubiquitinating NS5A. *Cellular & Molecular Immunology* 13(1)**,** 94-102. doi: 10.1038/cmi.2014.131.

Yang, D., Li, N.L., Wei, D., Liu, B., Guo, F., Elbahesh, H., et al. (2019). The E3 ligase TRIM56 is a host restriction factor of Zika virus and depends on its RNA-binding activity but not miRNA regulation, for antiviral function. *PLOS Neglected Tropical Diseases* 13(6)**,** e0007537. doi: 10.1371/journal.pntd.0007537.

Yang, Q., Liu, T.T., Lin, H., Zhang, M., Wei, J., Luo, W.W., et al. (2017). TRIM32-TAX1BP1-dependent selective autophagic degradation of TRIF negatively regulates TLR3/4-mediated innate immune responses. *PLoS Pathog* 13(9)**,** e1006600. doi: 10.1371/journal.ppat.1006600.

Yang, Q., Zhao, J., Chen, D., and Wang, Y. (2021). E3 ubiquitin ligases: styles, structures and functions. *Molecular Biomedicine* 2(1)**,** 23. doi: 10.1186/s43556-021-00043-2.

Ye, W., Hu, M.M., Lei, C.Q., Zhou, Q., Lin, H., Sun, M.S., et al. (2017). TRIM8 negatively regulates TLR3/4-mediated innate immune response by blocking TRIF-TBK1 interaction. *J Immunol* 199(5)**,** 1856-1864. doi: 10.4049/jimmunol.1601647.

Yu, L., and Liu, P. (2021a). Cytosolic DNA sensing by cGAS: regulation, function, and human diseases. *Signal Transduct Target Ther* 6(1)**,** 170. doi: 10.1038/s41392-021-00554-y.

Yu, Y., Huang, X., Liu, J., Zhang, J., Hu, Y., Yang, Y., et al. (2017). Fish TRIM32 functions as a critical antiviral molecule against iridovirus and nodavirus. *Fish & Shellfish Immunology* 60**,** 33-43. doi: https://doi.org/10.1016/j.fsi.2016.11.036.

Yu, Z., Li, X., Yang, M., Huang, J., Fang, Q., Jia, J., et al. (2021b). TRIM41 is required to innate antiviral response by polyubiquitinating BCL10 and recruiting NEMO. *Signal Transduction and Targeted Therapy* 6(1)**,** 90. doi: 10.1038/s41392-021-00477-8.

Zanchetta, M.E., and Meroni, G. (2019). Emerging roles of the TRIM E3 ubiquitin ligases MID1 and MID2 in cytokinesis. *Frontiers in Physiology* 10.

Zha, J., Han, K.J., Xu, L.G., He, W., Zhou, Q., Chen, D., et al. (2006). The Ret finger protein inhibits signaling mediated by the noncanonical and canonical IkappaB kinase family members. *J Immunol* 176(2)**,** 1072-1080. doi: 10.4049/jimmunol.176.2.1072.

Zhang, J., Hu, M.M., Wang, Y.Y., and Shu, H.B. (2012). TRIM32 protein modulates type I interferon induction and cellular antiviral response by targeting MITA/STING protein for K63-linked ubiquitination. *J Biol Chem* 287(34)**,** 28646-28655. doi: 10.1074/jbc.M112.362608.

Zhang, J., Zhang, C., Cui, J., Ou, J., Han, J., Qin, Y., et al. (2017). TRIM45 functions as a tumor suppressor in the brain via its E3 ligase activity by stabilizing p53 through K63-linked ubiquitination. *Cell Death Dis* 8(5)**,** e2831. doi: 10.1038/cddis.2017.149.

Zhang, S., Guo, J.T., Wu, J.Z., and Yang, G. (2013a). Identification and characterization of multiple TRIM proteins that inhibit hepatitis B virus transcription. *PLoS One* 8(8)**,** e70001. doi: 10.1371/journal.pone.0070001.

Zhang, Z., Bao, M., Lu, N., Weng, L., Yuan, B., and Liu, Y.J. (2013b). The E3 ubiquitin ligase TRIM21 negatively regulates the innate immune response to intracellular double-stranded DNA. *Nat Immunol* 14(2)**,** 172-178. doi: 10.1038/ni.2492.

Zhang, Z., Yuan, B., Bao, M., Lu, N., Kim, T., and Liu, Y.J. (2011). The helicase DDX41 senses intracellular DNA mediated by the adaptor STING in dendritic cells. *Nat Immunol* 12(10)**,** 959-965. doi: 10.1038/ni.2091.

Zhao, C., Jia, M., Song, H., Yu, Z., Wang, W., Li, Q., et al. (2017). The E3 ubiquitin ligase TRIM40 attenuates antiviral immune responses by targeting MDA5 and RIG-I. *Cell Rep* 21(6)**,** 1613-1623. doi: 10.1016/j.celrep.2017.10.020.

Zhao, C., Peng, C., Wang, P., Yan, L., Fan, S., and Qiu, L. (2021a). Identification of a shrimp E3 ubiquitin ligase TRIM50-like involved in restricting white spot syndrome virus proliferation by its mediated autophagy and ubiquitination. *Front Immunol* 12**,** 682562. doi: 10.3389/fimmu.2021.682562.

Zhao, L., Hao, Y., Song, Z., Fan, Y., and Li, S. (2021b). TRIM37 negatively regulates inflammatory responses induced by virus infection via controlling TRAF6 ubiquitination. *Biochem Biophys Res Commun* 556**,** 87-92. doi: 10.1016/j.bbrc.2021.03.147.

Zhao, W., Wang, L., Zhang, M., Yuan, C., and Gao, C. (2012). E3 ubiquitin ligase tripartite motif 38 negatively regulates TLR-mediated immune responses by proteasomal degradation of TNF receptor-associated factor 6 in macrophages. *J Immunol* 188(6)**,** 2567-2574. doi: 10.4049/jimmunol.1103255.

Zhou, J.-r., Liu, J.-h., Li, H.-m., Zhao, Y., Cheng, Z., Hou, Y.-m., et al. (2020). Regulatory effects of chicken TRIM25 on the replication of ALV-A and the MDA5-mediated type I interferon response. *Veterinary Research* 51(1)**,** 145. doi: 10.1186/s13567-020-00870-1.

Zhou, Z., Jia, X., Xue, Q., Dou, Z., Ma, Y., Zhao, Z., et al. (2014). TRIM14 is a mitochondrial adaptor that facilitates retinoic acid-inducible gene-I-like receptor-mediated innate immune response. *Proc Natl Acad Sci U S A* 111(2)**,** E245-254. doi: 10.1073/pnas.1316941111.

Table 2.1. TRIM proteins involved in RIG-I pathway augmentation or direct antiviral activity, and antiviral orthologous TRIM proteins in lower vertebrates.

TRIM subfamily	Present in	Members in humans	Augment RIG-I pathway	Directly restrict IAV	Antiviral homologs in invertebrates	Antiviral homologs in fish	Antiviral homologs in birds/reptiles
C-I	Animals	TRIM1, TRIM9, TRIM18, TRIM36, TRIM46, TRIM67	TRIM9		TRIM9		
C-II	Animals	TRIM54, TRIM55, TRIM63					
C-III	Vertebrates	TRIM43					
C-IV	Vertebrates	TRIM1, TRIM1L, TRIM4, TRIM5, TRIM6, TRIM7, TRIM10, TRIM11, TRIM14, TRIM15, TRIM16, TRIM16L, TRIM17, TRIM21, TRIM22, TRIM25, TRIM26, TRIM27, TRIM34, TRIM35, TRIM38, TRIM39, TRIM41, TRIM43, TRIM47, TRIM48, TRIM49, TRIM50, TRIM51, TRIM53, TRIM58, TRIM60, TRIM62, TRIM64, TRIM65, TRIM68, TRIM69, TRIM72, TRIM75, TRIM77, RNF135	TRIM4, TRIM6, TRIM11, TRIM14, TRIM21, TRIM25, TRIM26, TRIM27, TRIM38, TRIM41, TRIM65, TRIM68, RNF135	TRIM14, TRIM25, TRIM22, TRIM25, TRIM35, TRIM41	TRIM50	TRIM39	TRIM25, TRIM62, TRIM39
C-V	Vertebrates	TRIM8, TRIM19, TRIM20, TRIM29, TRIM31, TRIM40, TRIM44, TRIM52, TRIM56, TRIM61, TRIM73, TRIM74, RNF207	TRIM19, TRIM29, TRIM31, TRIM40, TRIM44	TRIM56			
C-VI	Bilaterians	TRIM24, TRIM28, TRIM33, TRIM66	TRIM28				
C-VII	Animals	TRIM2, TRIM3, TRIM32, TRIM71, NHLRC1		TRIM32	TRIM32	TRIM32	TRIM32
C-VIII	Eukaryotes	TRIM37					
C-IX	Vertebrates	TRIM23	TRIM23	TRIM23			
C-X	Animals	TRIM45					
C-XI	Bilaterians	TRIM13, TRIM59	TRIM13, TRIM59				

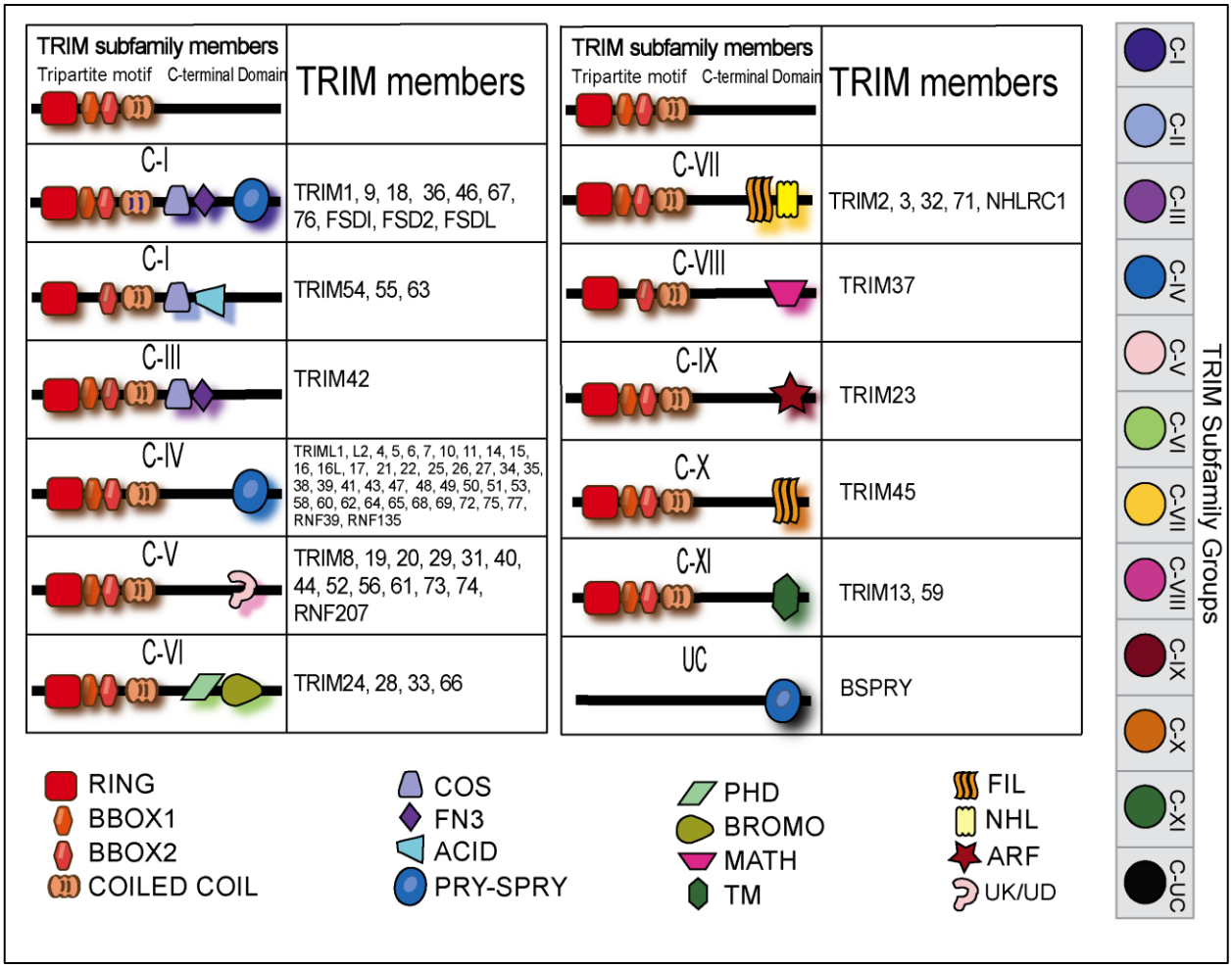


Figure 2.1. TRIM protein subfamilies based on variable C-terminal domain. RING: Really interesting new gene. COS: C-terminal subgroup one signature. FN3: Fibronectin, type III. PHD: Plant Homeo Domain. BROMO: Bromodomian. MATH: meprin and TRAF homology domain. TM: Transmembrane domain. FIL: Filamin domain. NHL: NCL-1, HT2A and Lin-41 repeats. ARF: ADP ribosylation factor-like. UC: Uncharacterized.

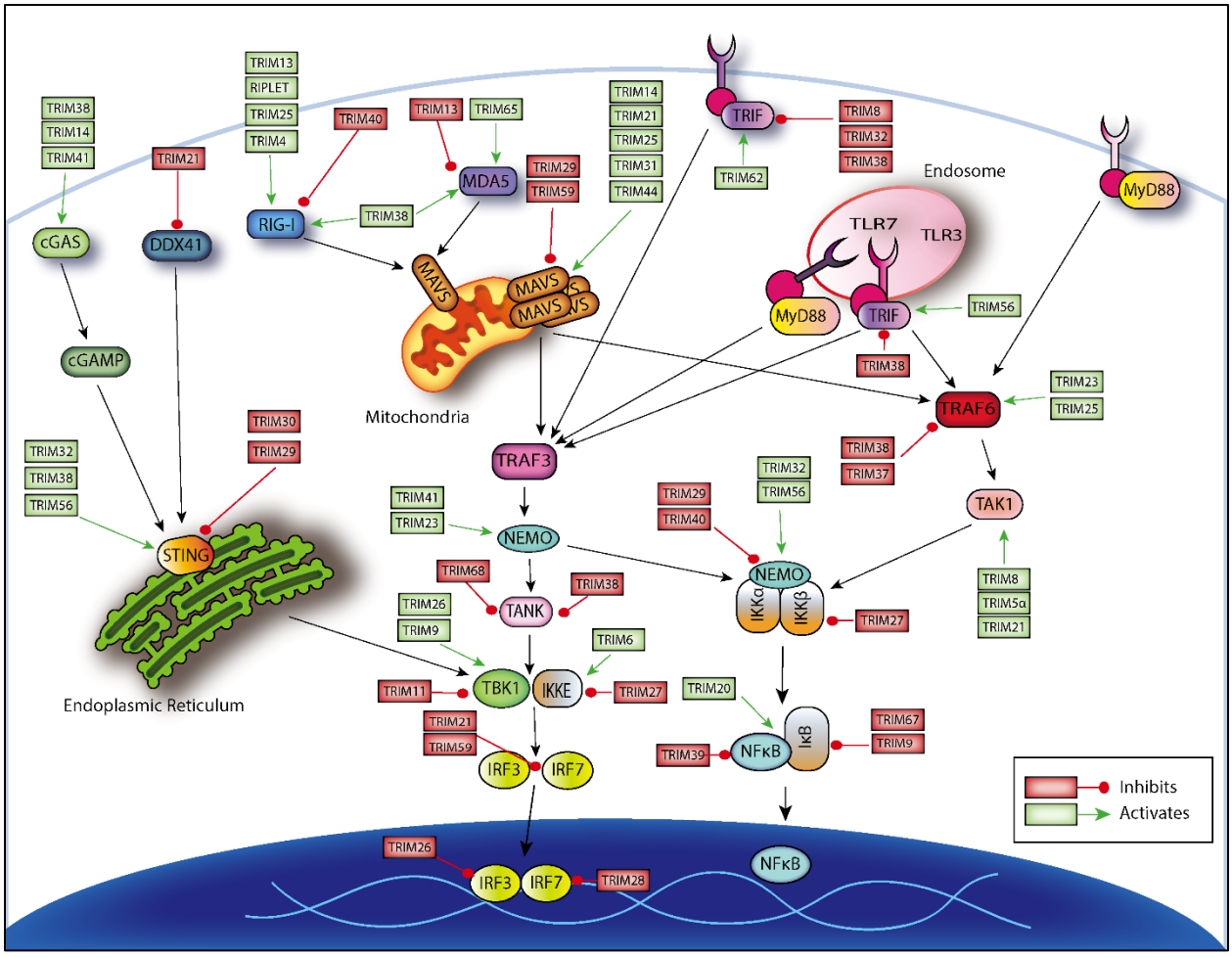


Figure 2.2. TRIM proteins involved in augmentation of anti-viral signaling pathways in mammals. Influenza A virus RNA can be recognized through RLRs or TLRs and signal downstream to activate IRF or NF-κB induced cytokine and chemokine expression. The cGAS/STING pathway can also be activated through influenza membrane fusion or cellular damage, leading to increases in antiviral signaling. Many TRIM proteins augment these pathways through E3 ubiquitin ligase activity, SUMOlyation, neddylation or other modulatory mechanisms to either increase or decrease antiviral signaling.

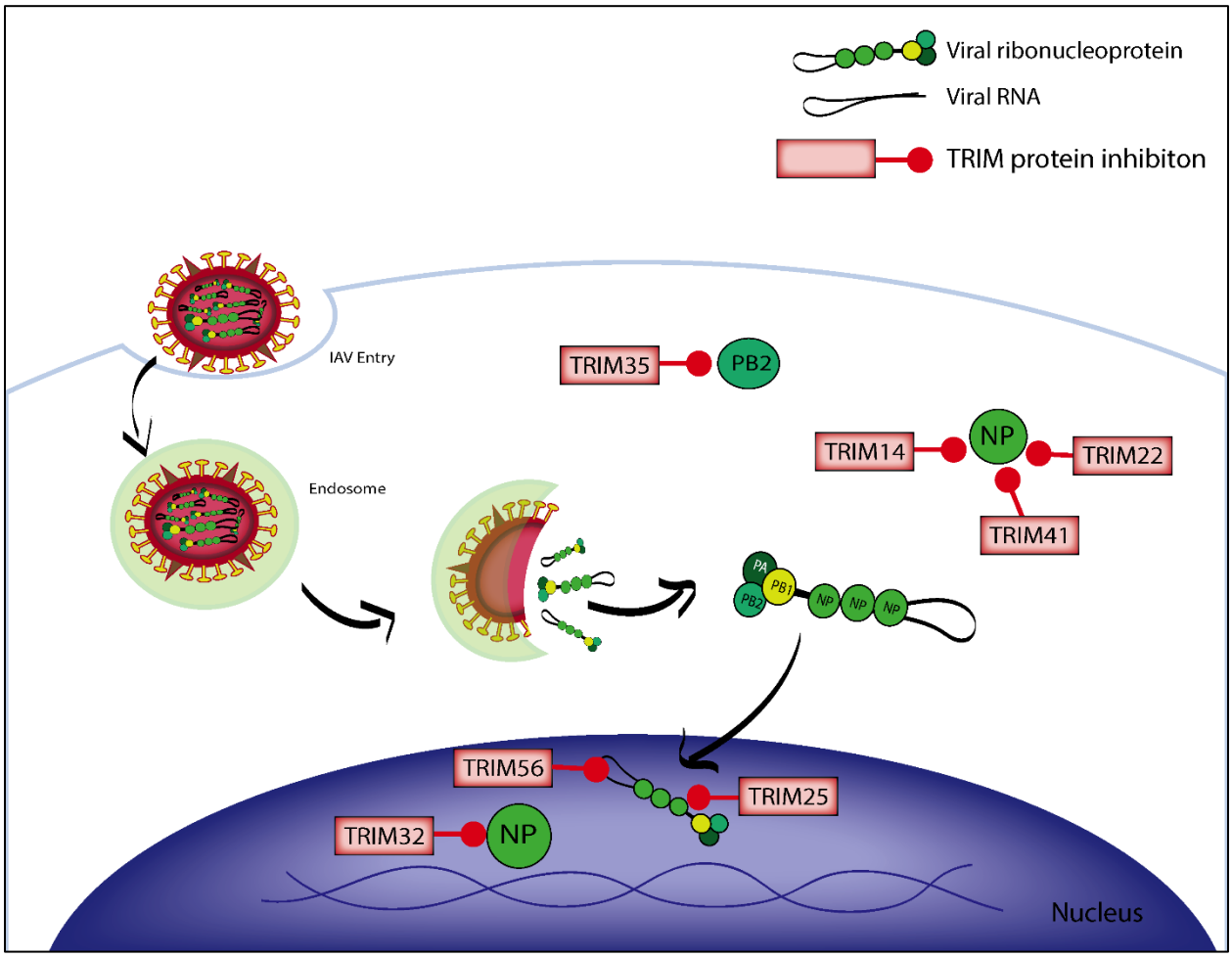


Figure 2.3. TRIM proteins that directly restrict influenza A virus replication in the cell. Influenza virus ribonucleoprotein components are targets of TRIM protein direct antiviral activity. Nucleoprotein (NP) is targeted by TRIM14, TRIM22 and TRIM41 for degradation in the cytoplasm. TRIM35 targets PB2 in the cytoplasm for degradation. TRIM32 translocates with PB1 to the nucleus where it aids in PB1 proteasomal degradation. TRIM25 targets both the viral ribonucleoprotein complex and viral RNA, restricting viral RNA elongation in the nucleus, while TRIM56 directly targets viral RNA in the nucleus and restricts influenza A virus replication.

CHAPTER 3

A version of Chapter 3 was published as Campbell LK, Fleming-Canepa X, Webster RG and Magor KE. 2021. Tissue Specific Transcriptome Changes Upon Influenza A Virus Replication in the Duck. Frontiers of Immunology 12(4653). doi:10.3389/fimmu.2021.786205

Tissue Specific Transcriptome Changes Upon Influenza A Virus Replication in the Duck

3.1 Introduction

Influenza A virus (IAV) causes disease in both humans and animals, resulting in periodic epidemics and potentially global pandemics. Mallard ducks (*Anas platyrhynchos*) are the natural host and reservoir IAV, and as such are highly resistant to viral pathology or mortality while still being permissive to viral replication (Webster et al., 1978; Webster et al., 1992; Taubenberger et al., 2010). In birds, the virus is categorized as either highly or low-pathogenic avian influenza (HPAI or LPAI, respectively) depending on the outcome of infection in chicken Alexander et al., 1986; Burggraaf et al., 2014). Ducks are resistant to both HPAI and LPAI viral strains of IAV, although it is of note that some H5 strains can cause severe pathology or even mass die offs in ducks (Sturm-Ramirez et al., 2004; Bingham et al., 2009; Haider et al., 2017). HPAI strains replicate in the lungs of infected ducks and chickens causing more pathology to infected animals, and these strains can also cause systemic dissemination of viral particles (Bingham et al., 2009; Vidana et al., 2018). LPAI strains replicate in the intestines of ducks to high titres without causing serious lesions (Webster et al., 1978; Daoust et al., 2011). This adaption allows the virus to be spread in excrement, and transferred through shared waterways, or when ducks fly over poultry farms, giving the ducks the moniker of the "Trojan horses" of infection (Kim et al., 2009). In particular, H5Ny strains of influenza continue to be enzootic in ducks and remain of concern for their pandemic potential ((Shi et al., 2021).

The duck mounts a robust immune response to IAV, involving key viral detectors and effectors, as recently reviewed (Magor et al., 2013; Evseev et al., 2019; Campbell et al., 2020). Key to the duck's innate defense is the cytoplasmic sensor DExD/H-Box Helicase 58/retinoic acid-inducible gene I (DDX58/RIG-I) which detects IAV, and the mitochondrial antiviral-signaling protein (MAVS) signaling pathway. Notably, components of this pathway differ between ducks and chickens (Barber et al., 2010; Karpala et al., 2011; Liniger et al., 2012; Barber et al., 2013; Burggraaf et al., 2014; Miranzo-Navarro et al., 2014; Blaine et al., 2015; Blyth et al., 2016). We have postulated that RIG-I being absent in chickens is one of the main reasons why chickens are so susceptible to IAV when compared to ducks (Barber et al., 2010).

While there are significant differences in basal expression of duck innate receptors and downstream effectors between tissues (Campbell et al., 2020), it is still unknown how different

tissues contribute to resistance to IAV and yet control damage from IAV infection despite high viral load. As ducks share a long evolutionary history with IAV (Webster et al., 1992) it is likely that global changes, not just the immune response, contribute to protection. Additionally, due to constant selective pressures from the virus, the duck may have unique antiviral effectors or splice isoforms. By comparing immune responses in tissues during replication we may discern differences in global response to avian influenza in ducks that are key to surviving highly pathogenic viruses that replicate systemically, while also permitting replication of harmless strains in intestine.

Several groups have examined gene expression in duck tissues following challenge with H5N1 strains of influenza. Transcriptome sequencing was performed on Shaoxin ducks infected with high and low pathogenic H5N1 strains (Huang et al., 2013), but this study was limited to lung tissues. Kumar and colleagues examined genome wide gene expression patterns to high and low pathogenic H5N1 viruses in ducks and compared lung transcriptomes at 5 days post-infection (Kumar et al., 2017). Smith and colleagues sequenced RNA from domestic Gray mallards and chickens infected with high and low pathogenic H5 strains (Smith et al., 2015) and focus on gene expression contributing to species differences in IAV susceptibility. However, these studies did not compare differences in gene expression between duck tissues involved in viral infection.

In our previous study comparing duck responses to highly and low pathogenic viruses, ducks infected with rgA/Vietnam 1203/2004 (H5N1) and A/British Columbia 500/2005 (H5N2) upregulated key innate immune genes, and although we characterized only a limited number of genes using qPCR, the ducks rapidly cleared both viruses with robust early responses (Fleming-Canepa et al., 2019). We also documented the viral kinetics of the rgA/Vietnam H5N1 virus and demonstrated that in spleen and lung from infected ducks, the influenza A M1 gene peaked at 1 day post infection, and on days 2 and 3 M1 expression only slightly decreased (Fleming-Canepa et al., 2019). Our aim is to extend this study by aligning pair-ended RNA-seq data to the current Pekin duck genome assembly (ZJU1.0) to analyze the global differential expression patterns in tissues involved in viral replication (lungs or intestine) and the lymphatic response (spleen) and identify novel candidate genes for future exploration.

Here we obtain transcriptome information from polyadenylated RNA of ducks infected with a reverse genetics version of the highly pathogenic H5N1 strain rgA/Vietnam/1203/04

(VN1203) and the low pathogenic H5N2 strain A/mallard/BC/500/05 (BC500). We look at the global differential expression (DE) in lung and spleen of Pekin ducks infected with VN1203; and lung, spleen and intestines of ducks infected with BC500. We have highlighted differences and similarities in differential expression of transcripts and identified sets of genes that have arisen by duplication and may contribute to host specific resistance. Our results suggest that tissue specific regulation mechanisms may play an integral role in both providing protection against IAV replication while limiting inflammatory responses.

3.2 Materials and methods

3.2.1 Viral infection and RNA collection

Viral infection and tissue collection were described previously (17, 27). Briefly, the VN1203 strain A/Vietnam/1203/04 (H5N1) was recreated using reverse genetics (28), while the BC500 strain A/mallard/BC/500/05 (H5N2) was collected during routine surveillance of wild ducks in British Columbia, Canada. Outbred 6-week-old Pekin ducks from Metzer Farm or Ideal Poultry were used for this study. Ducks were mock treated with PBS or infected with 10^6 of 50% egg infectious doses (EID_{50}) of VN1203 or BC500 using the natural route of infection by dripping virus in nares, eyes and trachea. Viral replication was tracked by taking tracheal and cloacal swabs from some ducks and reported previously (Vanderven et al., 2012). For BC500, cloacal swabs were positive at 2- and 3-days post-infection (dpi), and tracheal swabs were negative. For VN1203, tracheal swabs were positive at 3 dpi, while cloacal swabs were negative. Influenza RNA quantification showed M gene was highly expressed in lung and spleen for all three days in VN1203 infected ducks (Fleming-Canepa et al., 2019) (Supplementary Figure S3.1). At 1, 2 or 3 dpi, ducks were euthanized, tissues were harvested, and RNA was extracted using TRIzol reagent (Invitrogen). Samples were DNAse treated and stored at -80°C. Lung, spleen and intestine samples from mock treated animals were collected from 3 ducks at each time point (1, 2 and 3 dpi), however due to RNA quality, only 1 duck from mock infected animals on 2 dpi was used. RNA samples from lung and spleen from VN1203 infected ducks were used from 4 ducks at each time point, while RNA samples from lung, spleen and intestines from BC500 infected ducks were from 3 ducks at each time point. The sex of each duck was determined from raw read counts of the SWIM6 gene (LOC101797738) in each RNA sample. SWIM6 is located on the W chromosome found in WZ females, but not ZZ males, and was established as a valid determinant of avian embryo sex by He and colleagues (He et al., 2019). A

table of each sample name and corresponding sex of the duck can be found in Supplementary File S3.1.

3.2.2 Library construction and sequencing

Library preparation and poly-adenylated RNA sequencing were performed by LC Sciences (https://www.lcsciences.com/). Briefly, RNA-seq paired end libraries were created using Illumina's TruSeq-stranded-mRNA sample preparation protocol (Illumina, San Diego, CA). Integrity of RNA was checked using an Agilent Technologies 2100 Bioanalyzer. Two rounds of purification of poly(A) containing mRNAs were performed using oligo-dT magnetic beads. cDNA libraries were made and quality was assessed using Agilent Technologies 2100 Bioanalyzer High Sensitivity DNA Chip. Paired-ended sequencing of the cDNA libraries was performed using llumina's NovaSeq 6000 sequencing system. The sequencing resulted in paired 150 bp reads with approximately 6GB of data per run, resulting in a sequencing depth of approximately 40 million reads per sample.

Sequence data was submitted to NCBI sequence read archive (SRA) under the BioProject ID PRJNA767080.

3.2.3 Sequence analysis and differential expression

LC Sciences used CutAdapt (30) to remove adaptors and low-quality bases and then verified for quality using FastQC (Available online at: http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). We used Trimmomatic (Bolger et al., 2014) on these reads to further separate paired end reads and remove unpaired reads from the data. RNA sequence reads were aligned to the NCBI genome of the mallard duck (Anas platyrhynchos, assembly ZJU1.0) using HISAT2 version 2.20 (Kim et al., 2019). Reads were counted and sorted using FeatureCounts version 2.0.0 (Liao et al., 2014). Due to the overall rate of unassessed gene duplications in the genome, multi-mapped reads were counted and fractionally assigned to features. Reads were sorted by "feature" for differential expression between exons, and "Meta-feature" for differential expression between transcripts.

Differential expression (DE) analysis was performed in the R studio environment version 4.0.0 using EdgeR (Robinson et al., 2010). Library sizes were normalized using the trimmed mean of M-values (TMM). Fisher's exact test was used to determine the number of DE genes of

infected tissues compared to their internal control samples. Genes were considered DE if FDR<0.05 and fold change (FC) >2 and were used in downstream analysis. All DE genes for both VN1203 and BC500 experiments can be found in Supplementary File S3.2. Venn diagrams comparing expressed genes were made using DiVenn (35) or Venny 2.1 (Oliveros, 2007).

The EdgeR function diffspliceDGE was used to assess alternative splicing events during infection. All alternatively spliced genes for both VN1203 and BC500 experiments can be found in Supplementary File 3.

3.2.4 Enrichment and STRING analysis

Differentially expressed genes were combined for all three days of infection in each different tissue, infected with VN1203 or BC500. These genes were subjected to over-representation analysis (ORA) for gene ontology biological process (GO BP) terms, with the noRedundant filter added to reduce redundant enrichment terms on WebGestalt (Wang et al., 2017).

Search Tool for the Retrieval of Interacting Genes/Proteins (STRING) diagrams were made using Cytoscape version 3.8.0 (Shannon et al., 2003). Enrichment analysis of differentially expressed genes (DEGs) was performed in Cytoscape using the STRING app for STRING diagrams and enrichment (Doncheva et al., 2019). Due to the relative incompleteness of avian enrichment and interaction databases, all gene names were changed to the human equivalent and searched against the human database.

3.2.5 Gene annotation and identification

Many of the annotations in the NCBI file had only numerical descriptions, and not annotated gene names. To assign names to those with significant hits from the DEG lists, we searched on NCBI and if available from the description, the gene name was manually assigned. Gene names were changed using a find and replace macro in excel (Supplementary File S3.4) written by www.extendedoffice.com (https://www.extendoffice.com/documents/excel/1873-excel-find-and-replace-multiple-values-at-once.html). Any genes flagged in the datasets named with location numbers only were submitted to NCBI BLAST to search against both the bird and mammalian databases. For genes without significant BLAST orthologs, the location number identifier was not changed. Genes flagged as significant which had the same gene name as others

in the list were investigated to determine if this was a genome mis-assembly, paralogous gene or misidentification through BLAST, using protein alignments, and chromosomal locations.

To differentiate genes in our results we applied certain rules to naming related genes. Genes with the "pseudogene" designation in NCBI were assigned a "-pseu" suffix. Likewise, genes classified as noncoding RNA (ncRNA) were given a "-ncRNA" suffix. Genes which were identified as "like" another gene on NCBI were given the "-L" suffix. In cases where multiple genes in one dataset were classified with the same name with the "-L" suffix, the genes were numbered as "-L#", with the lowest number being closest in chromosomal location to the presumed ortholog or the annotated gene on NCBI. Genes which shared identical names, that could not be clarified by the above methods kept their gene names but were given a numerical suffix in the order of the genes on their respective chromosomes (i.e. "PARP14.1, PARP14.2).

3.3 Results

3.3.1 RNA sequencing reads from ducks infected with VN1203 or BC500 align to genome and cluster by tissue and virus infection

To determine the quality of our RNA reads and amount of coverage over the duck genome, we aligned our RNA-seq data to the NCBI Anas platyrhynchos genome assembly ZJU1.0. The average alignment rate of RNA sequencing reads to genome was 92%. Counted and sorted reads were successfully assigned to features at an average rate of 75%, indicating good coverage and sequencing depth of samples. The counted and assigned reads for each sample when graphed using multidimensional scaling (MDS) clustered with similar tissue (Figure 3.1A) and virus type (Figure 3.1B). Samples from lung and spleen tissues from VN1203 infected ducks were very distinct from tissues from BC500 infected and mock treated ducks, while tissues from infected ducks were distinct from mock treated samples. As intestine samples were taken only from ducks infected with BC500, we expanded our MDS analysis to include day post infection (dpi), where 1 and 3 dpi clustered more closely than 2 dpi. One mock treated intestine sample (MI1) was an outlier in this group, however as it clustered with other intestine samples, and did not significantly change the overall analysis when removed (data not shown), we kept it in in the data set for further analysis. Sex of ducks is indicated by color coding of samples (Figure 3.1B). Of note, control birds were a mix of male versus female ducks (4:3). Ratios of male to female ducks infected with LPAI were 1:2, 2:1, and 2:1 on 1, 2 and 3 dpi respectively. Ratios of male to

female ducks infected with HPAI were 3:1, 3:1 and 2:2 on 1, 2 and 3 dpi. The MDS plot suggests some segregation of samples according to sex.

3.3.2 VN1203 Infected Ducks Have More Differential Gene Expression Than Those Infected With BC500

To evaluate the number of genes differentially expressed in lungs, spleens and intestines of ducks infected with VN1203 or BC500, we used Fisher's exact test to compare infected tissues to control on 1, 2 and 3 dpi. Lung and spleen samples from VN1203 infected ducks had the most statistically significant (FDR<0.05) DEGs on all three days of infection, while BC500 infected ducks had much less DE in lungs and spleen (Table 3.1). We have previously reported much higher transcript levels following VN1203 than BC500 infection when analyzing expression of individual immune genes (26). In contrast to other tissues sampled in BC500 challenged ducks, the intestines had more DEGs, although most were below the threshold for fold change cut off (Table 3.1).

To determine how many genes were similarly upregulated or downregulated on all three days of infection, genes were filtered by FDR (>0.05) and log2(FC). Although each tissue had a number of unique altered genes there was considerable overlap of DE genes in lung and spleen on each day post infection (Figure 3.2A). At 1 dpi with VN1203, 852 DE genes are common between lung and spleen, with most genes upregulated. However, at 3 dpi the number of DEGs unique to lung are greatly increased.

To determine if the same subsets of genes were either up or down regulated on all days of VN1203 infection, we created VENN diagrams to compare gene regulation within each tissue. Genes that were up or downregulated during VN1203 infection in lung (Figure 3.2B) or spleen (Figure 3.2C) were analyzed to see how many were expressed during all 3 dpi, and how many changed expression depending on day. In the lung only 20% of upregulated genes were upregulated on all days of infection. This is similar in the spleen, where 18% of genes were upregulated on all days of infection. In both lung and spleen, the most DE specific to day of infection happens on 1 dpi (36% in lung, 31% in spleen), while 2 dpi has the lowest number of genes specific to that day differentially expressed (5% in lung, 6% in spleen). A similar pattern emerges for the downregulated genes, as only 13% of the downregulated DEGs were

downregulated all days of infection in both lung and spleen. Once again, 2 dpi had the lowest number of downregulated DEG specific to that day (8% in lung, 6% in spleen).

3.3.3. GO Biological Process Enrichment Analyses Find Commonalities and Dissimilarities Between VN1203 and BC500 Infection

To determine which pathways were enriched in each tissue during infection with VN1203 or BC500, we submitted DE genes to the WebGestalt server. All DE genes were clustered together to allow for analysis of enriched GO terms on all three days post infection, and the top 10 statistically significant (FDR<0.05) hits were reported in Figure 3. Results were separated into terms in common (GO terms enriched in all tissues sampled) or unique to tissue (spleen and lung in VN1203 infection, spleen, lung, and intestine for BC500 infection). During VN1203 infection, most of the commonly enriched pathways represented by upregulated DEGs were involved in immune responses (Figure 3.3A). Both innate and adaptive immune terms were enriched (GO:0045088 and GO:0002250). Likewise, response to virus was also highly enriched (GO:0009615). Pathways that were enriched by unique upregulated DEGs in spleen included many terms involved in protein folding, and misfolded protein responses (GO:0034976, GO:0035966, GO:0032527 and GO:0018196). Likely, these pathways and those involved in cell motility (GO:2000147) are enriched due to immune cell accumulation and activation. The lung of VN1203 infected ducks was enriched with terms involving actin and cytoskeleton rearrangement (GO:0031532 and GO:0043062), and cell surface signaling pathways (GO:0007186).

GO term enrichment resulting from downregulated DEGs found common terms in cell adhesion (GO:0031589) and cell growth (GO:0016049) (Figure 3B). Interestingly, bone mineralization or ossification terms were also enriched in the common downregulated DEG population (GO:0060348, GO:0001503 and GO:0060348). GO terms enriched in spleens of VN1203 infected ducks due to downregulation were mostly involved in neuronal signaling and cell development, while terms enriched by downregulated DEGs in lungs were comprised of pathways involved in muscle development (GO:0060537, GO:0042692, GO:0007517 and GO:0003012) and cell cycle progression (GO:0007059, GO:0061641 and GO:0051383).

DEGs upregulated during BC500 infection enriched similar pathways as those found in VN1203, however BC500 enriched the type I interferon pathway the most (Figure 3.3C).

Surprisingly, upregulated DEG in the intestine enriched many pathways involving not only cell cycle progression, but RNA processing. Due to the overall low number of DEGs in lungs infected with BC500, there were not enough uniquely expressed DEGs to properly analyze enrichment. Upregulated genes in BC500 infected spleens only enriched five pathways and all of those were involved in immunity. There were not enough total down regulated DEGs to analyze common enrichment or unique enrichment for spleens and lungs (Figure 3.3D). The intestines did have unique enrichment of many pathways involved in metabolic processing of glycerolipids and fatty acids (GO:0046486 and GO:0006631) among the downregulated DEGs.

3.3.4 VN1203 Infection Greatly Increases Alternative Splicing Events in Lung

To determine if IAV infection alters alternative splicing (AS) events in lung, spleen and intestine of infected ducks, we analyzed alternative splicing events by counting differences in reads mapped to individual exons between infected and control samples. As with total DE expression, VN1203 infected lungs and spleens had many more AS events than BC500 infected tissues (Table 3.2), with infected lungs having the most AS events on all 3 days post infection. Lungs from ducks infected with VN1203 had 456, 50, and 267 AS events at 1, 2 and 3 dpi. Spleens from VN1203 infected ducks had 70, 57, and 106 AS events on 1, 2 and 3 dpi respectively. Interestingly, while intestine tissues from BC500 infected ducks had a much greater amount of total DE genes than other tissues infected with the same virus, intestines only had 7, 37 and 9 AS events detected on 1, 2 and 3 dpi (respectively). As IAV proteins, such as NS1, can influence AS events in the cell (40), it is possible that VN1203 infection itself is responsible for this dramatic increase in AS events.

To determine the GO terminology associated with these AS events, all significant (FDR < 0.05) AS genes were submitted to the WebGestalt server. Only VN1203 infected ducks had enough genes to produce statistically significant (FDR < 0.05) GO results (Supplementary Figure S3.2). Lungs of ducks infected with VN1203 at 1 dpi have AS events in genes associated with muscle cell proliferation and migration (GO:0033002 and GO:0014812) (Supplementary Figure S3.2A). Curiously, respiratory system terms are also enriched by these AS genes (GO:0060541 and GO:0030323). Both spleens and lungs of VN1203 ducks had statistically significant enrichment of GO terms on 3 dpi (Supplementary Figure S3.2B). There were distinct subsets of GO terms enriched in each tissue, with lung AS genes enriching more general terms associated

with actin organization (GO:0007015), protein signal transduction (GO:0051056 and GO:0007265) and general cellular and tissue growth processes. AS events in the spleen however, enriched terms involving inflammatory responses (GO:0002526 and GO:0050727), platelets (GO:0002576), humoral immune responses (GO:0006959) and migration of leukocytes (GO:0050900).

The lack of statistically significant GO terms on other days in lung and spleen is likely due to the relatively low number of genes associated with AS events as well as the various functions of these genes. Repeating the RNA-sequencing with a greater sequencing depth would likely result in a more accurate sampling of AS events in these tissues.

3.3.5 VN1203 and BC500 Infections Upregulate Shared Sets Of Genes In Spleens, Lungs, and Intestines

To determine which genes were shared in response to virus in all three tissues sampled, we inspected the lists of DEGs on 1, 2 and 3 dpi for each tissue infected with either VN1203 or BC500 for genes in common. Between the VN1203 and BC500 infected ducks, there were 65 upregulated shared genes (Figure 3.4A). We subjected this set of genes to Reactome enrichment analysis using Cytoscape STRING app (Figure 3.4A). The largest group of genes enriched the "Reactome: Immune system" pathway. The expression patterns of genes in this group are different between VN1203 and BC500 infection (Figure 3.4A). VN1203 caused highest gene expression at 1 dpi while BC500 infected ducks had highest expression of most of these immune genes at 2 dpi. Indeed, when looking at statistical significance of these genes, the FDR is < 0.05 for most of these genes at 2 dpi in spleen, lung and intestine (Supplementary File S3.1), however this is not the case for most genes 1 and 3 dpi. The large increase of DEGs in ducks infected with BC500 at 2 dpi corresponds to viral titres, as we previously reported that these ducks had cloacal swabs negative for virus on 1 dpi, with significant viral titres on 2 and 3 dpi (Vanderven et al., 2012).

To determine which highly upregulated genes were in common between different tissues in VN1203 infected ducks, we filtered significant DEGs by $\log2(FC)>1.5$. Genes that reached these parameters on 1, 2 or 3 dpi were kept, combined into a list for each tissue, then compared. We removed the 65 genes found upregulated in all tissues during both VN1203 and BC500 infection to reduce redundancy in the datasets. Repeat non-coding RNA (ncRNA) and pseudogenes were

also removed. A heatmap of most upregulated DEGs in VN1203 infection was plotted showing genes corresponding to different Reactome pathways placed together (Figure 3.4B). The resulting STRING diagram demonstrates that most of the protein products of these genes have predicted interactions (Figure 3.4B). Out of the 67 total genes shared between tissues in VN1203 infection, 44 were associated with the "Reactome: Immune system" pathway. The genes enriched in the classical complement pathway have slightly different expression patterns between lung and spleen. In lungs many of these genes were upregulated 1 dpi, whereas in spleen the expression of these complement genes was sustained through all 3 days. Genes which enriched the "Reactome: Signaling by interleukins" pathway were predominantly upregulated 1 dpi. Proinflammatory interleukins IL-6, IL-1β and IL-18 are all upregulated 1 dpi, consistent with our previous qPCR analyses (Saito et al., 2018). Genes that enriched both the IFNα/β and IFNγ signaling pathways were mostly upregulated in both tissues 1 dpi. Some, such as IFNB and IFITM1 show sustained expression on all 3 days pi.

Ducks infected with both VN1203 and BC500 upregulate 65 genes in common, most of which produce proteins that respond to interferons or have immune function. VN1203 strongly upregulates a different subset of genes, including genes involved in the complement cascade, proinflammatory cytokines and interleukins and various other genes involved in immune responses. While VN1203 upregulates most immune genes on 1 dpi, BC500 upregulates these genes on 2 dpi, the delay likely due to the time needed for virus to reach the intestine for replication.

Unfortunately, there were no available enrichment databases that accurately placed all genes in enrichment categories. Many genes that are involved in immunity, antiviral defense or are interferon inducible are not yet added to the Reactome databases or without enough additional terms to be considered enriched in this dataset. Genes such as IFIT5 (Zhang et al., 2013; Zhou et al., 2013; Rohaim et al., 2018; Santhakumar et al., 2018), DDX60 (Miyashita et al., 2011; Oshiumi et al., 2015), SAMD9 (Liu et al., 2014) and SAMD9L (Boon et al., 2014) are potentially involved in innate immunity or antiviral defense, yet are unclassified in this dataset.

3.3.6 VN1203 Infection Preferentially Upregulates PRR and Signal Transduction Genes in the Lung and Interleukins in the Spleen of Infected Ducks

To determine which highly upregulated genes were uniquely expressed in lung or spleen of ducks infected with VN1203, we filtered significant DEGs by log2(FC)>1.5 for all three days post infection. Genes which were found in both tissues were removed from the dataset. We removed the 65 genes found upregulated in all tissues during both VN1203 and BC500 infection, as well as repeat ncRNA and pseudogenes, as described previously.

Many of the genes upregulated in lung have immune functions, however, only 20 out the total 54 "unique to lung" genes were found enriched in the "Reactome: Immune system" dataset (Figure 3.5A). Within this cluster there is significant overlap between accessory genes in both the "Reactome: Cytokine signaling in the immune system" and "Reactome: Signaling by interleukins" category. These genes are all upregulated on 1 dpi, with the notable exception of IFNG. IFNG is primarily upregulated in lungs and has increased expression on both 1 and 3 dpi, but curiously not on 2 dpi. Lungs of ducks infected with VN1203 also see unique expression of many PPRs, including TLR1A, 1B, 2A, 2B and 4. All of these TLRs have the highest expression on 1 dpi. The other primary enrichment pathway in lungs is the "Reactome: Signal transduction" which includes "Reactome: Class A/1 (Rhodopsin-like receptors)". The expression of these pathways is more varied, with some having highest expression at 1 dpi while others at 3 dpi.

Among the genes uniquely expressed in the spleen of VN1203 infected ducks, almost half enrich the "Reactome: Immune system" pathway (Figure 3.5B). As the spleen is secondary lymphatic tissue, it is perhaps not surprising that genes that enriched the "Reactome: MHC II antigen presentation" pathway were preferentially upregulated here. A subset of these genes also enriched the "Reactome: Cytokine signaling in the immune system" pathway, however unlike in the lung, most of these genes code for interleukins and not accessory proteins. A good proportion of the interleukin genes flagged here enriched the "Reactome Interleukin-20 family signaling" pathway. These genes have peak upregulation on 1 dpi, and interestingly, many are also greatly downregulated in lungs.

VN1203 infection causes upregulation of different and specific subsets of immune genes in the lung and spleen. In lung, more genes involved in pathogen recognition and signal

transduction were upregulated, while in spleen gene upregulation is centred around pro-inflammatory interleukins and peptide processing.

3.3.7 BC500 Infection Causes Up or Downregulation of Distinct Subsets of Genes in the Intestines

To determine which highly upregulated genes were uniquely expressed in spleen, lung or intestine of BC500 infected ducks, we filtered significant DEGs by log2(FC)>1.5 for all three days post infection (Figure 3.6). Genes which were found in 2 out of 3 tissues were included in this dataset (Figure 3.6A). For this analysis, we removed the 65 genes expressed in all tissues identified in Figure 4A. Out of the 64 genes highlighted in this dataset, 22 enriched the "Reactome: Immune system" pathway. While most genes found in this dataset in lung or spleens are also upregulated in VN1203 infection, CRISP3 is upregulated by BC500 in the lung on all 3 dpi, but not at all in VN1203 infection. Many of these genes enrich the term "Reactome: Cytokine signaling in the immune system". These are mostly specific to spleen and intestines of BC500 infected ducks and peak at 2 dpi. This is also true for a smaller number of genes which enriched the "Reactome: Signaling by interleukins" pathway. A subset of genes enriches the "Reactome: Regulation of genes in early pancreatic cells" pathway. These genes are all transcription factors and are primarily upregulated in intestines at 2 dpi. A group of solute carrier (SLC) family member genes enriched the "Reactome: SLC-mediated membrane transport" term uniquely in intestines from BC500 ducks. These SLC genes are upregulated starting at 2 dpi. Components of the complement pathway enriched the "Reactome: Regulation of complement cascade", similar to what is seen in VN1203 infection (Figure 3.5B). All three of these genes are upregulated 2 dpi in intestines, while their expression is variable in spleen and lung. It is of note that many of the highly upregulated genes specific to intestine during BC500 infection are not characterized in infection. As with the previous datasets, some genes in this dataset such as *CCL28* (Mar et al., 2018) are involved in immune cell responses and others such as *LY6E* (Mar et al., 2018), are interferon stimulated genes (ISGs).

As IAV can replicate in the intestines to high titres without causing significant damage or pathology, we investigated the differences in downregulated genes in the intestines compared to all other tissues to see if downregulation of specific genes might minimize pathology. DEGs in the intestines were filtered by significance (FDR<0.05) and log2(FC) (<-1). From this list, we

manually compared genes of interest to infection in lung and spleen of VN1203 and BC500 infected ducks. Genes which were highly downregulated in intestines that may play a role in either immune responses or viral restriction were examined in each tissue and visualized using a heatmap (Figure 3.6B). Many of the genes in this dataset of downregulated genes enrich the Reactome pathway "Reactome: Immune System". These genes also enrich the "Reactome: Cytokine signaling in the immune system" and "Reactome: Signaling by interleukins". The downregulation of these genes predominantly happens on 2 dpi, the time point when many other immune genes are upregulated in the intestines of BC500 infected ducks. The Reactome pathway "Reactome: Regulation of complement cascade" is also enriched by a subset of these downregulated genes, however unlike in Figure 3.6A, the genes that enrich this term also enrich the "Reactome: Activation of C3 and C5" pathway. While BC500 infection in the duck causes less DE than VN1203, there are many genes unique to the intestinal response to this virus. Many of these genes are uncharacterized in viral infection and warrant further study.

3.3.8 Interferon and Cytokine Responses Peak at 1 dpi With VN1203, and ISGs Peak at 2 dpi With BC500

To identify the genes contributing to the peak immune response in the sites of replication, we examined gene expression patterns in infected ducks. Because many important immune genes were upregulated in the lungs of ducks infected with VN1203 on 1 dpi, and most gene regulation in intestines of ducks infected with BC500 was at 2 dpi, we filtered all statistically significant (FDR < 0.05) genes on these days by expression levels and compiled lists of the top 100 most up or downregulated genes. We removed genes designated on NCBI as ncRNA or pseudogenes to limit the lists to genes that presumably code for protein. VN1203 induces a much more robust response than BC500 (Figure 3.7), with half of the top upregulated genes being upregulated by as much as log2(FC) of 4.5 or more, while only the top 13 genes in the BC500 dataset are above a log2(FC) of 4. Many of the top upregulated genes of the VN1203 infected lungs are cytokines (For example: *IFNA*, *IFNB* and *IL12A*) or interferon inducible genes (for example *IFIT5*, *OASL* and *Mx*). The top upregulated genes in BC500 however, are lacking high cytokine gene expression, but still have high expression of some interferon inducible genes (notably *Mx*).

We also expanded this analysis to the top 100 genes most downregulated on 1 dpi in VN1203 infected ducks, and on 2 dpi in BC500 infected ducks (Supplementary Figure S3.3). While initially it appears that downregulation is less robust in the lungs of VN1203 infected

ducks, this is only on 1 dpi. Inspection of the data reveals that more significant downregulation of genes in the lungs of ducks infected with VN1203 happens at 2 and 3 dpi.

3.4 Discussion

Here we compare global changes in gene expression of ducks infected with the IAV strains VN1203 or BC500 using RNA-seq analysis. We compared RNA profiles of tissues sampled to identify genes which were similarly upregulated in all infected tissues as well as genes which were uniquely upregulated in specific sites. This enabled us to delineate specific responses in sites of virus replication (lungs for VN1203, intestines for BC500) compared to lymphoid tissues (spleen). Ducks respond to VN1203 infection with a high interferon signature at 1 dpi in lung, yet soon down regulate key proinflammatory cytokines. BC500 infection stimulates the highest gene upregulation at 2 dpi in intestine together with downregulation of leukocyte recruitment cytokines. A global picture emerges of a robust and rapid interferon response to VN1203 in lung, and a significant response to BC500 in intestine, yet both responses were tempered to limit damage. As part of the sequencing effort for the *Anas platyrhynchos* genome, Huang and consortium performed transcriptomic analysis of Shaoxin lung tissues following infection with H5N1 strains (Huang et al., 2013). They saw a similarly robust interferon response early in infection and elaborated on cytokine and defensin expression. Our work builds on this by investigating the global regulation of genes in lung, spleen and intestine using the most current version of the Pekin duck genome (November 15, 2020). Smith and colleagues sequenced lung and intestine RNA from domestic Gray mallards infected with similar viruses, but used single-stranded reads at a lower sequencing depth on 1 and 3 dpi only (Smith et al., 2015). The Gray mallard ducks also had a much more robust response to VN1203 infection than they did to BC500 infection on 1 and 3 dpi. Many of the highly expressed genes are in common with our results, however the additional depth of sequencing reveals less abundant genes and allows us to analyze alternate splicing in the sampled tissues.

Overall, we found that there were 65 upregulated genes common to all tissues following infection with both viruses, while lungs, spleens and intestines had many genes uniquely differentially expressed. Of these 65 similarly upregulated genes, pattern recognition receptors (PRRs) which can detect RNA viruses were highly upregulated in all tissues. This includes RIG-I and the related interferon induced with helicase C domain 1/melanoma differentiation-

associated protein 5 (*IFIH1/MDA5*) and toll-like receptor 3 (TLR3). VN1203 infection induces a much more rapid and robust response of IFN inducible genes (such as *RSAD2/*Viperin and *IFIT5*) in the duck than BC500 infection does. The interferon response peaks at 1 dpi for VN1203, while the response to BC500 peaks at 2 dpi. The expression of genes in the RIG-I pathway from the RNA-seq data mostly match their expression profiles we previously determined using qPCR (Barber et al., 2013; Saito et al., 2018; Fleming-Canepa et al., 2019), with highest expression *of DDX58/RIG-I*, *IFIH1/MDA5*, *RNF135/RIPLET*, *OASL*, *IFITM3 and IRF7* on 1 dpi in ducks infected with VN1203. Exceptions include *IFNB* expression and tripartite motif protein 25 (TRIM25) expression in VN1203 infected ducks. In our previous studies, the qPCR results suggested these two genes were robustly expressed on 1 dpi, and rapidly dropped to basal levels by 3 dpi, while our RNA-seq analysis suggests these genes exhibit sustained expression across all 3 dpi. Likely these discrepancies are due to the different techniques of normalization used between the two studies, with the qPCR experiments normalized to a single gene (GAPDH) and the RNA-seq data normalized using TMM and library size. Additionally, RNA-sequencing data will still count splice variants in its read counts, while qPCR may miss some of these variants due to primer design and placement. Some of the changes in gene expression seen in tissues are likely due to infiltrating immune cells responding to infection. We previously reported aggregates of leukocytes in lung tissues and leukocyte depletion of spleen tissues in VN1203 infected ducks (Fleming-Canepa et al., 2019). Others have also noted infiltration of immune cells to lung, spleen and intestine following influenza infection (Cooley et al., 1989; Cornelissen et al., 2013). We previously showed upregulation of CCL19 and CCL21 chemokines, responsible for homing of dendritic cells and naïve lymphocytes (Förster et al., 2008), in VN1203 infected lung (Fleming-Canepa et al., 2011). In this study, we see upregulation of these transcripts on all days following VN1203 infection in both lung and spleen, but only in spleens of BC500 infected ducks. Indeed, CCL19 is one of the most upregulated genes. Higher expression of CCL19 and other ISGs was seen in genotyped Ri chicken lines that were more resistant to H5N1 infection (Vu et al., 2021).

Infection with BC500 induces the most DEGs on 2 dpi. This is especially evident in the site of BC500 replication, the intestine. This is not surprising, given that cloacal swabs from these ducks were negative for virus on 1 dpi, but they were shedding high titres of virus on days 2 and 3 (Vanderven et al., 2012). It seems that the virus replicates in intestine at 2 dpi, and the

host tissue responds accordingly. Tracheal swabs from BC500 ducks were also negative and this is consistent with our result of relatively low changes in DEGs in the lung. While this virus produces no observable symptoms in the duck, it replicates to high titres and as such can disseminate into the environment. Since we do not see abundant viral transcripts in the intestinal tissues, either the tissue collection missed the major sites of replication, or the cells actively producing virus are present only transiently or rapidly destroyed. The 2 dpi timepoint was not examined by Smith and colleagues in their RNA sequencing data, however comparable to our data, they found similar genes upregulated at 1 dpi in the ileum of BC500 infected mallards, including *IFIT5, ESPTI1*, *Mx*, *OASL*, *DHX58* and *SAMD9L* (Smith et al., 2015).

We see many PRR and IFN-inducible genes upregulated in tissues infected with either VN1203 or BC500. We had previously looked at expression of both ring finger protein 135 (*RNF135*/Riplet) and TRIM25 (Fleming-Canepa et al., 2019), which both augment RIG-I signaling during viral infection. Here we see that *RNF135* is upregulated by infection with either BC500 or VN1203 in all tissues, while *TRIM25* is upregulated in both lung and spleen during VN1203 infection. We also see DExD/H-Box Helicase 60 (*DDX60*) upregulated by both VN1203 and BC500 in all tissues sampled. In mammals, DDX60 binds RIG-I and promotes RNA binding and downstream type I IFN production during viral infection (Miyashita et al., 2011 but has not been studied in birds. We also see strong upregulation of IFN inducible genes such as radical SAM domain-containing 2 (*RSAD2*/Viperin), 2′-5′-oligoadenylate synthetase-like (*OASL*), interferon induced protein with tetratricopeptide repeats 5 (*IFIT5* and *IFIT5-L*, a likely mistake in genome assembly) and *Mx*. *RSAD2*, *OASL* and *Mx* are all highly upregulated by VN1203 and BC500 in all tissues sampled. Recent experiments establish the antiviral function of these duck homologues. Overexpression of duck IFIT5 reduces viral titre at early timepoints but appears to inhibit innate immunity later (Wu et al., 2020). Duck OASL activates the OAS/RNaseL pathway (Rong et al., 2018). Similarly, overexpression of duck Viperin reduces viral replication (Xiang et al., 2020).

Although upregulated, some genes may not be functional. For example, *Mx* is highly upregulated at 2 dpi in intestines of BC500 infected ducks, while many other PRR and IFN-inducible genes were only slightly upregulated in that tissue. The function of duck Mx has long been in question, as two alleles showed no antiviral activity in vitro (Bazzigher et al., 1993). Similarly, two members of the interferon induced transmembrane protein family IFITM1 and

IFITM2, small proteins capable of preventing viral hemi-fusion of membranes preventing entry, have highly upregulated transcripts in intestine, but we previously showed that neither restricts influenza viruses *in vitro* (Blyth et al., 2016). IFITM1 is mis-targeted to plasma membrane, rather than the endosomal compartment due to a unique insertion in ducks, not seen in chickens. Only IFITM3 restricts influenza viruses, and its expression is high in lung, spleen and intestine. Influenza viruses may exploit these adaptations to preferentially replicate in duck intestines.

Alternative splicing events are prevalent in lungs of ducks infected with VN1203, and rare in tissues of ducks infected with BC500. IAV modifies AS events through many mechanisms (Thompson et al., 2019; Thompson et al., 2020). Some alternative transcripts may have specific antiviral activity. For example, a short isoform of human nuclear receptor co-activator 7 (NCOA7) was induced by interferon and able to inhibit IAV entry through endosomal fusion (Doyle et al., 2018). The short isoform of NCOA7 was also identified in VN1203 infected lungs and spleens, and BC500 infected lungs. The presence of this NCOA7 isoform in ducks suggests it may have a conserved function of viral restriction in vertebrates. The high number of AS events in VN1203 infected tissues may be due to viral subversion of host response, as well as IFN induction of AS events. Interestingly, when subjected to GO analysis, many of the AS events in the lungs of VN1203 infected ducks enriched terms involved with physiology, rather than immunological responses. AS events in spleens of ducks infected with VN1203 found more enrichment in terms associated with humoral and inflammatory responses. These differences may be due to the abundance of each transcript type in each tissue, as there may be more inflammatory cells activated in the spleen during an infection. Human lung epithelial cells infected with the A/WSN/1933 strain of H1N1 also demonstrated an increase in AS events (Thompson et al., 2020). Thompson and colleagues demonstrated through siRNA screening that some of the alternatively spliced genes also were actively enhancing viral replication, and thus knocking these genes out reduced viral titre in infected cells. Of note, some of these genes are also present in our AS analysis in duck tissues, including: *RAB11F1P3*, *PAXBP1*, *IP6K2* and *TNRC6A*. These genes only show as AS in lungs of ducks infected with VN1203. As little is known about AS responses to infection in birds, future research should involve both sequencing

infected duck tissues at a greater depth to capture more AS events and investigating these alternate transcripts in duck cells to determine which aid or restrict IAV replication.

We found gene duplications unique to ducks particularly interesting, especially if the mammalian homologue has known antiviral activity. The poly-ADP-ribose polymerase (PARP) family of genes is largely understudied but is often associated with DNA repair and transcription. We found members of this family upregulated by both VN1203 and BC500 infection in ducks (*PARP9*, *PARP10*, *PARP12* and *PARP14*). Of interest, PARP12 can inhibit replication of RNA viruses (Atasheva et al., 2012). We found two presumed orthologs of human PARP12 in ducks (LOC101802866 and LOC101796889, with the former being named *PARP12-L* for this paper). The shorter gene, *PARP12-L* is only significantly upregulated in lungs of VN1203 infected ducks, suggesting it may play a tissue specific role in viral inhibition. *PARP12* was also upregulated in a previous study in both lungs and ileums of VN1203 and BC500 infected Gray mallard ducks (Smith et al., 2015). Neither of these genes (*PARP12* or *PARP12-L*) has yet been characterized in birds, and it is unknown if either can restrict RNA viral replication. *PARP14* appears to be duplicated in the duck, with two forms of *PARP14* sharing equal percent identities to human *PARP14* (~43%), but only 52% identity to each other (data not shown). *PARP14* deletion reduces proinflammatory responses in murine macrophages (Iwata et al., 2016), and in another study *PARP14* deletion was found to reduce IFN-β and ISG response (Caprara et al., 2018). Both duck *PARP14* genes also have a predicted RNA binding domain, which is not present in the human *PARP14*. *PARP14.1* (LOC101789908) is upregulated by both BC500 and VN1203 infection in ducks, while *PARP14.2* (LOC101798744) is only upregulated by VN1203 in lungs and spleens of infected ducks. Members of the PARP gene family appear to be expanded in the duck, making them interesting candidates for further study of proteins which may play lineage specific roles in immune responses to IAV in the duck.

We postulate that specific responses to the virus that limit damage from infection may have been selected in ducks. Notably, lung tissues in VN1203 infected ducks show downregulation of some proinflammatory cytokines, including IL-17 and IL-8. In humans, IL-17 is elevated in patients who were infected with the 2009 S-OIV H1N1 IAV (Li et al., 2012). Mice infected with the 2009 S-OIV H1N1 had a significant increase in survival when treated with anti-IL-17A monoclonal antibodies. In this study, we see a large increase of *IL17A* expression in spleens but not lungs of VN1203 infected ducks. Additionally, there was a significant decrease in expression

of this gene, especially on 2 and 3 dpi, in the lungs of these infected ducks. This response may help lessen damage in the lungs from infection. We see a similar pattern in the proinflammatory cytokine IL-8, of which ducks have two presumed orthologous genes, *IL8* (LOC101804010) and *IL8-L* (LOC101803817). Both genes show strong upregulation in the spleen, particularly on 1 dpi. However, in lungs there is significant downregulation on 2 and 3 dpi of both *IL8* and *IL8-L*. In humans, IL8 is secreted by alveolar epithelial cells infected by IAV (Ito et al., 2015). As IL-8 is a potent neutrophil chemoattractant (Proost et al., 1993; Henkels et al., 2011; Ito et al., 2015), decreasing the expression of the *IL8* gene in the site of VN1203 replication may reduce bystander damage to the tissues from excessive neutrophil accumulation. Interestingly, the downregulation of *IL17A, IL8* and *IL8-L* in lungs seems to be unique to our experiment. Huang et al. also investigated cytokine expression in lungs of Shaoxin ducks infected with DK/49, a HPAI H5N1 and GS/65 a LPAI H5N1 (Huang et al., 2013). In these experiments, IL17A was upregulated in the lungs of ducks infected with DK/49 on all three days, while it was downregulated in ducks infected with GS/65 on 1 dpi and increased in expression on 2 and 3 dpi (Huang et al., 2013). A similar pattern is seen in this data when comparing *IL8* and *IL8-L* expression. This is likely due to the viral strains used as well as the differences in breeds of duck. In our experiments all ducks infected with VN1203 survived. While Huang et al. do not specifically mention survival rates of the ducks used, Song et al. demonstrated that DK/49 killed all infected Shaoxin ducks with viral titres as low as 10^3 EID_{50} (Song et al., 2011). They also noted that GS/65 did not cause any mortality in infected ducks, yet it spread systemically in infected birds. Additionally, while Shaoxin ducks and Pekin ducks did originate from the same lineage, they have been selectively bred for eggs or meat (respectively) and inhabit separate clades in phylogenetic analyses (Feng et al., 2021; Guo et al., 2021). It is also likely that this selective breeding has resulted in differences in immune responses.

As the intestine tissue both permits BC500 to reproduce to high titres, yet ultimately clears infection, we looked at both uniquely upregulated and downregulated gene expression, in comparison to other tissues. In intestines of BC500 ducks, we see upregulation of complement components *C1S* and *C4A* and strong downregulation of *C3* and *C5* as well as the complement receptor gene *CR1* and the *C5* receptor *CD88*. The C3 protein acts as a point of convergence to activate the classical, alternative and lectin pathways of complement activation (as reviewed by Zipfel et al., 2009). Mice with *C3* and *CR1* genes knocked out were deficient in forming long

term memory to IAV (Gonzalez et al., 2008) while C5 activation is associated with lung damage during IAV infection in mice (Garcia et al., 2013). Activated C5 is split into C5a, which is a potent chemoattractant of neutrophils (Price et al., 2015) and monocytes (Niyonzima et al., 2017). Limiting not only the key component of the complement cascade (C3) but also a potent activator of inflammatory cell subsets (C5) and their receptors likely decreases the inflammation in the intestine. Similarly, damage may be ameliorated by downregulation of *C3* and *C5* seen in lungs, but not spleens, of VN1203 infected ducks.

Because ducks are permissive to IAV replication while being resistant to pathology from replicating virus, we searched our data for genes that might assist in increased viral replication. In a recent review, Shaw and Stertz listed many genes that assisted in IAV replication in mammalian hosts (Shaw et al., 2018), however many of these genes were not differentially expressed in ducks above physiologically relevant thresholds. Indeed, some of the differentially expressed genes that would allow for increased entry/endosome trafficking were downregulated in both intestines of BC500 ducks and lungs of VN1203 infected ducks. For example, *DYNLT2*, *ACTG2, ACTA1*, *ACTN2* and *ACTC1* were all downregulated in lungs of VN1203 ducks. Both actin and dynein proteins can aid in endosomal trafficking of IAV during early stages of infection (Lakadamyali et al., 2003). Several genes which encode chemoattractant proteins were specifically downregulated in intestines including Leukocyte cell-derived chemotaxin-2 (*LECT2*), a chemoattractant for neutrophils and macrophages (Yamagoe et al., 1996; Slowik et al., 2017), *CCL26* a chemoattractant for eosinophils and basophils (Kitaura et al., 1999) and *IL15*, which has various functions in inflammatory responses and promoting immune cell maturation and proliferation (Perera et al., 2012). We did not take intestine samples from VN1203 infected ducks because the cloacal swabs of these ducks were negative. However, future research and analysis should include samples from tissues without virus present, to further elucidate which DEGs are from interferons and non-specific inflammatory responses, and which are caused by the presence of replicating virus.

Sex differences in immunity have evolved in all species from sea urchins to mammals, and where examined, innate and adaptive immunity is typically greater in females than males (Klein et al., 2016). Our study sampled a mix of male and female Pekin ducks in both our controls and infected birds. We observe some separation of samples according to sex in our MDS plot, thus it is possible that sex contributes to the differences in expression patterns seen, however this is

mostly obscured by variation in response between genetically diverse individuals. Due to our relatively small sample size on each day of infection, we do not have enough male and female animals to compare immune responses by sex. Previously, we did not find differences in viral load between male and female ducks infected with rgVN1203 (Fleming-Canepa et al., 2019). In wild ducks, most studies show males carry more IAV (Ip et al., 2008; Parmley et al., 2008; Farnsworth et al., 2012), while one showed more female ducks infected (Runstadler et al., 2007), and one found differences in viral load between male and female ducks depending on geographic location (Papp et al., 2017). In the wild, host ecology contributes to prevalence of IAV infection in mallards including dabbling in infected water, flock density and migration (Olsen et al., 2006).

Our results highlight the incredible complexity of tissue responses to both highly pathogenic and low pathogenic strains of IAV. Ducks are well equipped to control IAV replication, demonstrated by the shared expression of key IAV detectors and innate effectors in all tissues, notably the RIG-I pathway and interferon stimulated genes. The early timing of this robust early interferon response to VN1203 at 1 dpi may also be protective, while peak ISG responses are seen at 2 dpi for low pathogenic avian influenza. Many genes uniquely upregulated have as yet unknown roles in the physiological changes or immune response during infection, as thorough literature searches fail to link these genes to inflammatory modulators or viral restriction. It is suspected that recruitment of leukocytes contributes to DE of genes, but the responding cell types are not known. Ducks also have tissue-specific mechanisms in place to prevent damage and out-of-control inflammation, including downregulation of complement components *C3* and *C5*. The downregulation of certain proinflammatory genes, with the upregulation of other proinflammatory genes in the same tissues suggests the protection is from a dampening, rather than an all-out inhibition of the inflammatory response.

3.5 References

Alexander, D.J., Parsons, G., and Manvell, R.J. (1986). Experimental assessment of the pathogenicity of eight avian influenza A viruses of H5 subtype for chickens, turkeys, ducks and quail. *Avian Pathol* 15(4)**,** 647-662. doi: 10.1080/03079458608436328.

Atasheva, S., Akhrymuk, M., Frolova, E.I., and Frolov, I. (2012). New PARP gene with an anti-alphavirus function. *Journal of Virology* 86(15)**,** 8147. doi: 10.1128/JVI.00733-12.

Barber, M.R., Aldridge, J.R., Jr., Webster, R.G., and Magor, K.E. (2010). Association of RIG-I with innate immunity of ducks to influenza. *Proc Natl Acad Sci U S A* 107(13)**,** 5913-5918. doi: 10.1073/pnas.1001755107.

Barber, M.R.W., Aldridge, J.R., Jr., Fleming-Canepa, X., Wang, Y.-D., Webster, R.G., and Magor, K.E. (2013). Identification of avian RIG-I responsive genes during influenza infection. *Molecular immunology* 54(1)**,** 89-97. doi: 10.1016/j.molimm.2012.10.038.

Bazzigher, L., Schwarz, A., and Staeheli, P. (1993). No enhanced influenza virus resistance of murine and avian cells expressing cloned duck Mx protein. *Virology* 195(1)**,** 100-112. doi: 10.1006/viro.1993.1350.

Bingham, J., Green, D.J., Lowther, S., Klippel, J., Burggraaf, S., Anderson, D.E., et al. (2009). Infection studies with two highly pathogenic avian influenza strains (Vietnamese and Indonesian) in Pekin ducks (*Anas platyrhynchos*), with particular reference to clinical disease, tissue tropism and viral shedding. *Avian Pathol* 38(4)**,** 267-278. doi: 10.1080/03079450903055371.

Blaine, A.H., Miranzo-Navarro, D., Campbell, L.K., Aldridge, J.R., Jr., Webster, R.G., and Magor, K.E. (2015). Duck TRIM27-L enhances MAVS signaling and is absent in chickens and turkeys. *Mol Immunol* 67(2 Pt B)**,** 607-615. doi: 10.1016/j.molimm.2015.07.011.

Blyth, G.A., Chan, W.F., Webster, R.G., and Magor, K.E. (2016). Duck interferon-inducible transmembrane protein 3 mediates restriction of influenza viruses. *J Virol* 90(1)**,** 103-116. doi: 10.1128/JVI.01593-15.

Bolger, A.M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30(15)**,** 2114-2120. doi: 10.1093/bioinformatics/btu170.

Boon, A.C.M., Williams, R.W., Sinasac, D.S., and Webby, R.J. (2014). A novel genetic locus linked to pro-inflammatory cytokines after virulent H5N1 virus infection in mice. *BMC Genomics* 15(1)**,** 1017. doi: 10.1186/1471-2164-15-1017.

Burggraaf, S., Karpala, A.J., Bingham, J., Lowther, S., Selleck, P., Kimpton, W., et al. (2014). H5N1 infection causes rapid mortality and high cytokine levels in chickens compared to ducks. *Virus Res* 185**,** 23-31. doi: 10.1016/j.virusres.2014.03.012.

Campbell, L.K., and Magor, K.E. (2020). Pattern recognition receptor signaling and innate responses to influenza A viruses in the mallard duck, compared to humans and chickens.

Frontiers in Cellular and Infection Microbiology 10(209). doi: 10.3389/fcimb.2020.00209.

Caprara, G., Prosperini, E., Piccolo, V., Sigismondo, G., Melacarne, A., Cuomo, A., et al. (2018). PARP14 Controls the Nuclear Accumulation of a Subset of Type I IFN–Inducible Proteins. *The Journal of Immunology* 200(7)**,** 2439. doi: 10.4049/jimmunol.1701117.

Cooley, A.J., Van Campen, H., Philpott, M.S., Easterday, B.C., and Hinshaw, V.S. (1989). Pathological lesions in the lungs of ducks infected with influenza A viruses. *Vet Pathol* 26(1)**,** 1-5. doi: 10.1177/030098588902600101.

Cornelissen, J.B., Vervelde, L., Post, J., and Rebel, J.M. (2013). Differences in highly pathogenic avian influenza viral pathogenesis and associated early inflammatory response in chickens and ducks. *Avian Pathol* 42(4)**,** 347-364. doi: 10.1080/03079457.2013.807325.

Daoust, P.Y., Kibenge, F.S., Fouchier, R.A., van de Bildt, M.W., van Riel, D., and Kuiken, T. (2011). Replication of low pathogenic avian influenza virus in naturally infected Mallard ducks (Anas platyrhynchos) causes no morphologic lesions. *J Wildl Dis* 47(2)**,** 401-409. doi: 10.7589/0090-3558-47.2.401.

Doncheva, N.T., Morris, J.H., Gorodkin, J., and Jensen, L.J. (2019). Cytoscape StringApp: Network analysis and visualization of proteomics data. *J Proteome Res* 18(2)**,** 623-632. doi: 10.1021/acs.jproteome.8b00702.

Doyle, T., Moncorgé, O., Bonaventure, B., Pollpeter, D., Lussignol, M., Tauziet, M., et al. (2018). The interferon-inducible isoform of NCOA7 inhibits endosome-mediated viral entry. *Nat Microbiol* 3(12)**,** 1369-1376. doi: 10.1038/s41564-018-0273-9.

Evseev, D., and Magor, K.E. (2019). Innate immune responses to avian influenza viruses in ducks and chickens. *Vet Sci* 6(1)**,** 5-5. doi: 10.3390/vetsci6010005.

Farnsworth, M.L., Miller, R.S., Pedersen, K., Lutman, M.W., Swafford, S.R., Riggs, P.D., et al. (2012). Environmental and demographic determinants of avian influenza viruses in waterfowl across the contiguous United States. *PLoS One* 7(3)**,** e32729. doi: 10.1371/journal.pone.0032729.

Feng, P., Zeng, T., Yang, H., Chen, G., Du, J., Chen, L., et al. (2021). Whole-genome resequencing provides insights into the population structure and domestication signatures of ducks in eastern China. *BMC Genomics* 22(1)**,** 401. doi: 10.1186/s12864-021-07710-2.

Fleming-Canepa, X., Aldridge, J.R., Jr., Canniff, L., Kobewka, M., Jax, E., Webster, R.G., et al. (2019). Duck innate immune responses to high and low pathogenicity H5 avian influenza viruses. *Vet Microbiol* 228**,** 101-111. doi: 10.1016/j.vetmic.2018.11.018.

Fleming-Canepa, X., Brusnyk, C., Aldridge, J.R., Ross, K.L., Moon, D., Wang, D., et al. (2011). Expression of duck CCL19 and CCL21 and CCR7 receptor in lymphoid and influenza-infected tissues. *Mol Immunol* 48(15-16)**,** 1950-1957. doi: 10.1016/j.molimm.2011.05.025.

Förster, R., Davalos-Misslitz, A.C., and Rot, A. (2008). CCR7 and its ligands: balancing immunity and tolerance. *Nat Rev Immunol* 8(5)**,** 362-371. doi: 10.1038/nri2297.

Garcia, C.C., Weston-Davies, W., Russo, R.C., Tavares, L.P., Rachid, M.A., Alves-Filho, J.C., et al. (2013). Complement C5 activation during influenza A infection in mice contributes to neutrophil recruitment and lung injury. *PLOS ONE* 8(5)**,** e64443. doi: 10.1371/journal.pone.0064443.

Gonzalez, S.F., Jayasekera, J.P., and Carroll, M.C. (2008). Complement and natural antibody are required in the long-term memory response to influenza virus. *Vaccine* 26**,** I86-I93. doi: https://doi.org/10.1016/j.vaccine.2008.11.057.

Guo, X., He, X.X., Chen, H., Wang, Z.C., Li, H.F., Wang, J.X., et al. (2021). Revisiting the evolutionary history of domestic and wild ducks based on genomic analyses. *Zool Res* 42(1)**,** 43-50. doi: 10.24272/j.issn.2095-8137.2020.133.

Haider, N., Sturm-Ramirez, K., Khan, S.U., Rahman, M.Z., Sarkar, S., Poh, M.K., et al. (2017). Unusually high mortality in waterfowl caused by highly pathogenic avian influenza A(H5N1) in Bangladesh h. *Transboundary and Emerging Diseases* 64(1)**,** 144-156. doi: 10.1111/tbed.12354.

He, L., Martins, P., Huguenin, J., Van, T.N., Manso, T., Galindo, T., et al. (2019). Simple, sensitive and robust chicken specific sexing assays, compliant with large scale analysis. *PLoS One* 14(3)**,** e0213033. doi: 10.1371/journal.pone.0213033.

Henkels, K.M., Frondorf, K., Gonzalez-Mejia, M.E., Doseff, A.L., and Gomez-Cambronero, J. (2011). IL-8-induced neutrophil chemotaxis is mediated by Janus kinase 3 (JAK3). *FEBS letters* 585(1)**,** 159-166. doi: 10.1016/j.febslet.2010.11.031.

Huang, Y., Li, Y., Burt, D.W., Chen, H., Zhang, Y., Qian, W., et al. (2013). The duck genome and transcriptome provide insight into an avian influenza virus reservoir species. *Nat Genet* 45(7)**,** 776-783. doi: 10.1038/ng.2657.

Ip, H.S., Flint, P.L., Franson, J.C., Dusek, R.J., Derksen, D.V., Gill, R.E., Jr., et al. (2008). Prevalence of Influenza A viruses in wild migratory birds in Alaska: patterns of variation in detection at a crossroads of intercontinental flyways. *Virol J* 5**,** 71. doi: 10.1186/1743-422x-5-71.

Ito, Y., Correll, K., Zemans, R.L., Leslie, C.C., Murphy, R.C., and Mason, R.J. (2015). Influenza induces IL-8 and GM-CSF secretion by human alveolar epithelial cells through HGF/c-Met and TGF-α/EGFR signaling. *American journal of physiology. Lung cellular and molecular physiology* 308(11)**,** L1178-L1188. doi: 10.1152/ajplung.00290.2014.

Iwata, H., Goettsch, C., Sharma, A., Ricchiuto, P., Goh, W.W.B., Halu, A., et al. (2016). PARP9 and PARP14 cross-regulate macrophage activation via STAT1 ADP-ribosylation. *Nature Communications* 7(1)**,** 12849. doi: 10.1038/ncomms12849.

Karpala, A.J., Stewart, C., McKay, J., Lowenthal, J.W., and Bean, A.G. (2011). Characterization of chicken Mda5 activity: regulation of IFN-beta in the absence of RIG-I functionality. *J Immunol* 186(9)**,** 5397-5405. doi: 10.4049/jimmunol.1003712.

Kim, D., Paggi, J.M., Park, C., Bennett, C., and Salzberg, S.L. (2019). Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. *Nature Biotechnology* 37(8)**,** 907-915. doi: 10.1038/s41587-019-0201-4.

Kim, J.-K., Negovetich, N.J., Forrest, H.L., and Webster, R.G. (2009). Ducks: the "Trojan horses" of H5N1 influenza. *Influenza and other respiratory viruses* 3(4)**,** 121-128. doi: 10.1111/j.1750-2659.2009.00084.x.

Kitaura, M., Suzuki, N., Imai, T., Takagi, S., Suzuki, R., Nakajima, T., et al. (1999). Molecular cloning of a novel human CC chemokine (Eotaxin-3) that is a functional ligand of CC chemokine receptor 3. *J Biol Chem* 274(39)**,** 27975-27980. doi: 10.1074/jbc.274.39.27975.

Klein, S.L., and Flanagan, K.L. (2016). Sex differences in immune responses. *Nature Reviews Immunology* 16(10)**,** 626-638. doi: 10.1038/nri.2016.90.

Kumar, A., Vijayakumar, P., Gandhale, P.N., Ranaware, P.B., Kumar, H., Kulkarni, D.D., et al. (2017). Genome-wide gene expression pattern underlying differential host response to

high or low pathogenic H5N1 avian influenza virus in ducks. *Acta Virol* 61(1)**,** 66-76. doi: 10.4149/av_2017_01_66.

Lakadamyali, M., Rust, M.J., Babcock, H.P., and Zhuang, X. (2003). Visualizing infection of individual influenza viruses. *Proc Natl Acad Sci U S A* 100(16)**,** 9280-9285. doi: 10.1073/pnas.0832269100.

Li, C., Yang, P., Sun, Y., Li, T., Wang, C., Wang, Z., et al. (2012). IL-17 response mediates acute lung injury induced by the 2009 pandemic influenza A (H1N1) virus. *Cell research* 22(3)**,** 528-538. doi: 10.1038/cr.2011.165.

Liao, Y., Smyth, G.K., and Shi, W. (2014). FeatureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* 30(7)**,** 923-930. doi: 10.1093/bioinformatics/btt656.

Liniger, M., Summerfield, A., Zimmer, G., McCullough, K.C., and Ruggli, N. (2012). Chicken cells sense influenza A virus infection through MDA5 and CARDIF signaling involving LGP2. *J Virol* 86(2)**,** 705-717. doi: 10.1128/JVI.00742-11.

Liu, J., and McFadden, G. (2014). SAMD9 is an innate antiviral host factor with stress response properties that can be antagonized by poxviruses. *Journal of Virology***,** JVI.02262-02214. doi: 10.1128/JVI.02262-14.

Magor, K.E., Miranzo Navarro, D., Barber, M.R., Petkau, K., Fleming-Canepa, X., Blyth, G.A., et al. (2013). Defense genes missing from the flight division. *Dev Comp Immunol* 41(3)**,** 377-388. doi: 10.1016/j.dci.2013.04.010.

Mar, K.B., Rinkenberger, N.R., Boys, I.N., Eitson, J.L., McDougal, M.B., Richardson, R.B., et al. (2018). LY6E mediates an evolutionarily conserved enhancement of virus infection by targeting a late entry step. *Nature Communications* 9(1)**,** 3603. doi: 10.1038/s41467-018-06000-y.

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal; Vol 17, No 1: Next Generation Sequencing Data Analysis*. doi: 10.14806/ej.17.1.200.

Miranzo-Navarro, D., and Magor, K.E. (2014). Activation of duck RIG-I by TRIM25 is independent of anchored ubiquitin. *PLoS One* 9(1)**,** e86968. doi: 10.1371/journal.pone.0086968.

Miyashita, M., Oshiumi, H., Matsumoto, M., and Seya, T. (2011). DDX60, a DEXD/H box helicase, is a novel antiviral factor promoting RIG-I-like receptor-mediated signaling. *Molecular and cellular biology* 31(18)**,** 3802-3819. doi: 10.1128/MCB.01368-10.

Mohan, T., Deng, L., and Wang, B.-Z. (2017). CCL28 chemokine: An anchoring point bridging innate and adaptive immunity. *International Immunopharmacology* 51**,** 165-170. doi: https://doi.org/10.1016/j.intimp.2017.08.012.

Niyonzima, N., Freeley, S., Arbore, G., Le Friec, G., Lappegård, K.T., Mollnes, T.E., et al. (2017). The intracellular C5 system is critical to DAMP sensing and cellular responses in human monocytes. *The Journal of Immunology* 198(1 Supplement)**,** 75.78.

Oliveros, J.C. (2007). *An interactive tool for comparing lists with Venn's diagrams.* [Online]. Available: https://bioinfogp.cnb.csic.es/tools/venny/index.html [Accessed].

Olsen, B., Munster, V.J., Wallensten, A., Waldenstrom, J., Osterhaus, A.D., and Fouchier, R.A. (2006). Global patterns of influenza a virus in wild birds. *Science* 312(5772)**,** 384-388. doi: 10.1126/science.1122438.

Oshiumi, H., Miyashita, M., Okamoto, M., Morioka, Y., Okabe, M., Matsumoto, M., et al. (2015). DDX60 is involved in RIG-I-dependent and independent antiviral responses, and its function is attenuated by virus-induced EGFR activation. *Cell Rep* 11(8)**,** 1193-1207. doi: 10.1016/j.celrep.2015.04.047.

Papp, Z., Clark, R.G., Parmley, E.J., Leighton, F.A., Waldner, C., and Soos, C. (2017). The ecology of avian influenza viruses in wild dabbling ducks (Anas spp.) in Canada. *PLoS One* 12(5)**,** e0176297. doi: 10.1371/journal.pone.0176297.

Parmley, E.J., Bastien, N., Booth, T.F., Bowes, V., Buck, P.A., Breault, A., et al. (2008). Wild bird influenza survey, Canada, 2005. *Emerg Infect Dis* 14(1)**,** 84-87. doi: 10.3201/eid1401.061562.

Perera, P.-Y., Lichy, J.H., Waldmann, T.A., and Perera, L.P. (2012). The role of interleukin-15 in inflammation and immune responses to infection: implications for its therapeutic use. *Microbes and infection* 14(3)**,** 247-261. doi: 10.1016/j.micinf.2011.10.006.

Price, P.J.R., Bánki, Z., Scheideler, A., Stoiber, H., Verschoor, A., Sutter, G., et al. (2015). Complement component C5 recruits neutrophils in the absence of C3 during respiratory infection with modified vaccinia virus Ankara. *The Journal of Immunology* 194(3)**,** 1164. doi: 10.4049/jimmunol.1301410.

Proost, P., De Wolf-Peeters, C., Conings, R., Opdenakker, G., Billiau, A., and Van Damme, J. (1993). Identification of a novel granulocyte chemotactic protein (GCP-2) from human tumor cells. In vitro and in vivo comparison with natural forms of GRO, IP-10, and IL-8. *The Journal of Immunology* 150(3)**,** 1000.

Robinson, M.D., McCarthy, D.J., and Smyth, G.K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26(1)**,** 139-140. doi: 10.1093/bioinformatics/btp616.

Rohaim, M.A., Santhakumar, D., Naggar, R.F.E., Iqbal, M., Hussein, H.A., and Munir, M. (2018). Chickens expressing IFIT5 ameliorate clinical outcome and pathology of highly pathogenic avian influenza and velogenic Newcastle disease viruses. *Front Immunol* 9(SEP)**,** 2025. doi: 10.3389/fimmu.2018.02025.

Rong, E., Wang, X., Chen, H., Yang, C., Hu, J., Liu, W., et al. (2018). Molecular mechanisms for the adaptive switching between the OAS/RNase L and OASL/RIG-I pathways in birds and mammals. *Front Immunol* 9**,** 1398. doi: 10.3389/fimmu.2018.01398.

Runstadler, J.A., Happ, G.M., Slemons, R.D., Sheng, Z.M., Gundlach, N., Petrula, M., et al. (2007). Using RRT-PCR analysis and virus isolation to determine the prevalence of avian influenza virus infections in ducks at Minto Flats State Game Refuge, Alaska, during August 2005. *Arch Virol* 152(10)**,** 1901-1910. doi: 10.1007/s00705-007-0994-1.

Saito, L.B., Diaz-Satizabal, L., Evseev, D., Fleming-Canepa, X., Mao, S., Webster, R.G., et al. (2018). IFN and cytokine responses in ducks to genetically similar H5N1 influenza A viruses of varying pathogenicity. *J Gen Virol* 99(4)**,** 464-474. doi: 10.1099/jgv.0.001015.

Salomon, R., Franks, J., Govorkova, E.A., Ilyushina, N.A., Yen, H.-L., Hulse-Post, D.J., et al. (2006). The polymerase complex genes contribute to the high virulence of the human H5N1 influenza virus isolate A/Vietnam/1203/04. *The Journal of experimental medicine* 203(3)**,** 689-697. doi: 10.1084/jem.20051938.

Santhakumar, D., Rohaim, M., Hussein, H.A., Hawes, P., Ferreira, H.L., Behboudi, S., et al. (2018). Chicken interferon-induced protein with tetratricopeptide repeats 5 antagonizes replication of RNA viruses. *Sci Rep* 8(1)**,** 6794. doi: 10.1038/s41598-018-24905-y.

Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., et al. (2003). Cytoscape: A software environment for integrated models of biomolecular interaction networks. *Genome Res* 13(11)**,** 2498-2504. doi: 10.1101/gr.1239303.

Shaw, M.L., and Stertz, S. (2018). Role of host genes in influenza virus replication. *Curr Top Microbiol Immunol* 419**,** 151-189. doi: 10.1007/82_2017_30.

Shi, W., and Gao, G.F. (2021). Emerging H5N8 avian influenza viruses. *Science* 372(6544)**,** 784. doi: 10.1126/science.abg6302.

Slowik, V., and Apte, U. (2017). Leukocyte cell-derived chemotaxin-2: It's role in pathophysiology and future in clinical medicine. *Clinical and translational science* 10(4)**,** 249-259. doi: 10.1111/cts.12469.

Smith, J., Smith, N., Yu, L., Paton, I.R., Gutowska, M.W., Forrest, H.L., et al. (2015). A comparative analysis of host responses to avian influenza infection in ducks and chickens highlights a role for the interferon-induced transmembrane proteins in viral resistance. *BMC Genomics* 16(1)**,** 574. doi: 10.1186/s12864-015-1778-8.

Song, J., Feng, H., Xu, J., Zhao, D., Shi, J., Li, Y., et al. (2011). The PA protein directly contributes to the virulence of H5N1 avian influenza viruses in domestic ducks. *J Virol* 85(5)**,** 2180-2188. doi: 10.1128/jvi.01975-10.

Sturm-Ramirez, K.M., Ellis, T., Bousfield, B., Bissett, L., Dyrting, K., Rehg, J.E., et al. (2004). Reemerging H5N1 influenza viruses in Hong Kong in 2002 are highly pathogenic to ducks. *Journal of virology* 78(9)**,** 4892-4901. doi: 10.1128/jvi.78.9.4892-4901.2004.

Sun, L., Dong, S., Ge, Y., Fonseca, J.P., Robinson, Z.T., Mysore, K.S., et al. (2019). DiVenn: An interactive and integrated web-based visualization tool for comparing gene lists. *Frontiers in Genetics* 10(421). doi: 10.3389/fgene.2019.00421.

Taubenberger, J.K., and Kash, J.C. (2010). Influenza virus evolution, host adaptation, and pandemic formation. *Cell host & microbe* 7(6)**,** 440-451. doi: 10.1016/j.chom.2010.05.009.

Thompson, M.G., Dittmar, M., Mallory, M.J., Bhat, P., Ferretti, M.B., Fontoura, B.M., et al. (2020). Viral-induced alternative splicing of host genes promotes influenza replication. *eLife* 9**,** e55500. doi: 10.7554/eLife.55500.

Thompson, M.G., and Lynch, K.W. (2019). Functional and mechanistic interplay of host and viral alternative splicing regulation during influenza infection. *Cold Spring Harb Symp Quant Biol* 84**,** 123-131. doi: 10.1101/sqb.2019.84.039040.

Thompson, M.G., Muñoz-Moreno, R., Bhat, P., Roytenberg, R., Lindberg, J., Gazzara, M.R., et al. (2018). Co-regulatory activity of hnRNP K and NS1-BP in influenza and human mRNA splicing. *Nature Communications* 9(1)**,** 2407. doi: 10.1038/s41467-018-04779-4.

Vanderven, H.A., Petkau, K., Ryan-Jean, K.E., Aldridge, J.R., Jr., Webster, R.G., and Magor, K.E. (2012). Avian influenza rapidly induces antiviral genes in duck lung and intestine. *Mol Immunol* 51(3-4)**,** 316-324. doi: 10.1016/j.molimm.2012.03.034.

Vidana, B., Dolz, R., Busquets, N., Ramis, A., Sanchez, R., Rivas, R., et al. (2018). Transmission and immunopathology of the avian influenza virus A/Anhui/1/2013 (H7N9) human isolate in three commonly commercialized avian species. *Zoonoses Public Health* 65(3)**,** 312-321. doi: 10.1111/zph.12393.

Vu, H.T., Hong, Y., Truong, A.D., Lee, J., Lee, S., Song, K.-D., et al. (2021). Cytokine-cytokine receptor interactions in the highly pathogenic avian influenza H5N1 virus-infected lungs of genetically disparate Ri chicken lines. *Asian-Australas J Anim Sci* 0(0)**,** 0-0. doi: 10.5713/ab.21.0163.

Wang, J., Vasaikar, S., Shi, Z., Greer, M., and Zhang, B. (2017). WebGestalt 2017: a more comprehensive, powerful, flexible and interactive gene set enrichment analysis toolkit. *Nucleic Acids Research* 45(W1)**,** W130-W137. doi: 10.1093/nar/gkx356.

Webster, R.G., Bean, W.J., Gorman, O.T., Chambers, T.M., and Kawaoka, Y. (1992). Evolution and ecology of influenza A viruses. *Microbiol Rev* 56(1)**,** 152-179.

Webster, R.G., Yakhno, M., Hinshaw, V.S., Bean, W.J., and Murti, K.G. (1978). Intestinal influenza: replication and characterization of influenza viruses in ducks. *Virology* 84(2)**,** 268-278. doi: 10.1016/0042-6822(78)90247-7.

Wu, X., Liu, K., Jia, R., Pan, Y., Wang, M., Chen, S., et al. (2020). Duck IFIT5 differentially regulates Tembusu virus replication and inhibits virus-triggered innate immune response. *Cytokine* 133**,** 155161. doi: 10.1016/j.cyto.2020.155161.

Xiang, C., Huang, M., Xiong, T., Rong, F., Li, L., Liu, D.X., et al. (2020). Transcriptomic analysis and functional characterization reveal the duck interferon regulatory factor 1 as an important restriction factor in the replication of Tembusu virus. *Front Microbiol* 11**,** 2069. doi: 10.3389/fmicb.2020.02069.

Yamagoe, S., Yamakawa, Y., Matsuo, Y., Minowada, J., Mizuno, S., and Suzuki, K. (1996). Purification and primary amino acid sequence of a novel neutrophil chemotactic factor

LECT2. *Immunology Letters* 52(1)**,** 9-13. doi: https://doi.org/10.1016/0165-2478(96)02572-2.

Zhang, B., Liu, X., Chen, W., and Chen, L. (2013). IFIT5 potentiates anti-viral response through enhancing innate immune signaling pathways. *Acta Biochim Biophys Sin (Shanghai)* 45(10)**,** 867-874. doi: 10.1093/abbs/gmt088.

Zhou, X., Michal, J.J., Zhang, L., Ding, B., Lunney, J.K., Liu, B., et al. (2013). Interferon induced IFIT family genes in host antiviral defense. *Int J Biol Sci* 9(2)**,** 200-208. doi: 10.7150/ijbs.5613.

Zipfel, P.F., and Skerka, C. (2009). Complement regulators and inhibitory proteins. *Nature Reviews Immunology* 9(10)**,** 729-740. doi: 10.1038/nri2620.

Table 3.1. Differentially expressed genes in tissues from ducks infected with VN1203 or BC500. Total DEGs were sorted by false discovery rate (FDR) >0.05. Genes were considered upregulated if the log2FC>1 and downregulated if the log2FC< -1.

	Total DEGs (FDR < 0.05)	Total DEGs (FC >2 and FC <-2)	Upregulated (FC >2)	Downregulated (FC <-2)
VN1203 - Lung 1 dpi	4204	1804	1063	741
VN1203 - Lung 2 dpi	2808	1235	542	693
VN1203 - Lung 3 dpi	4719	1939	772	1167
VN1203 - Spleen 1 dpi	4894	2738	1054	1684
VN1203 - Spleen 2 dpi	3572	1575	698	877
VN1203 - Spleen 3 dpi	4465	2121	943	1178
BC500 - Lung 1 dpi	53	51	50	1
BC500 - Lung 2 dpi	110	93	82	11
BC500 - Lung 3 dpi	10	10	10	0
BC500 - Spleen 1 dpi	84	75	72	3
BC500 - Spleen 2 dpi	320	217	202	15
BC500 - Spleen 3 dpi	75	65	55	10
BC500 - Intestine 1 dpi	73	64	55	9
BC500 - Intestine 2 dpi	3732	1583	551	1032
BC500 - Intestine 3 dpi	401	187	106	81

Total DEGs were sorted by false discovery rate (FDR) <0.05. Genes were considered upregulated if the log2(FC)>1 and downregulated if the log2(FC)<-1.

Table 3.2. Counts of alternatively spliced (AS) transcripts in tissues of ducks infected with VN1203 or BC500. Individual exon counts in VN1203 or BC500 infection were compared to controls. Alternative splicing events were considered significant (FDR<0.05) when analyzed using EdgeR diffSpliceDGE program, using the “Simmes” method.

	Total AS Genes (Simmes FDR < 0.05)
VN1203 - Lung 1 dpi	456
VN1203 - Lung 2 dpi	50
VN1203 - Lung 3 dpi	267
VN1203 - Spleen 1 dpi	70
VN1203 - Spleen 2 dpi	57
VN1203 - Spleen 3 dpi	106
BC500 - Lung 1 dpi	7
BC500 - Lung 2 dpi	14
BC500 - Lung 3 dpi	7
BC500 - Spleen 1 dpi	14
BC500 - Spleen 2 dpi	15
BC500 - Spleen 3 dpi	21
BC500 - Intestine 1 dpi	7
BC500 - Intestine 2 dpi	37
BC500 - Intestine 3 dpi	9

Individual exon counts in VN1203 or BC500 infection were compared to controls. Alternative splicing events were considered significant (FDR<0.05) when analyzed using EdgeR diffSpliceDGE program, using the "Simmes" method.

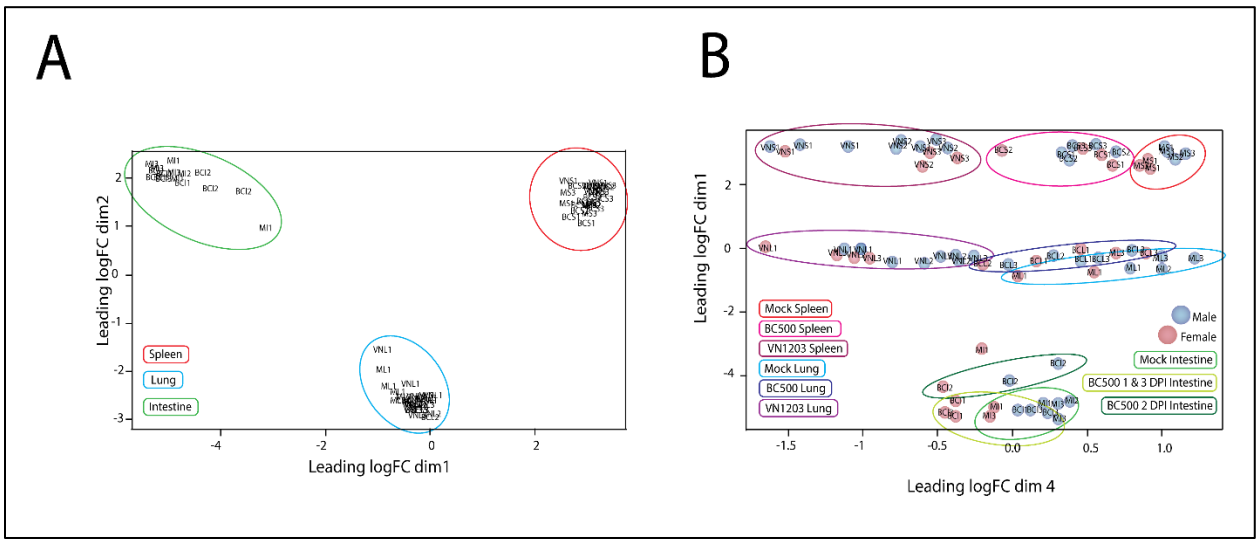


Figure 3.1. Multidimensional scaling (MDS) plot of normalized individual RNA-sequencing experiments. MDS plots were made in the EdgeR program comparing the top 200 logFC results between each sample. Individual samples and their relative similarity and differences were compared using dimensions 1 and 2 (A) and dimensions 1 and 4 (B). Individual plot points were named by treatment (VN, VN1203; BC, BC500 and M, Mock), tissue (L, Lung; S, Spleen and I, intestine) and dpi (1, 2 and 3). Ex: Spleen from mock treated duck 1 dpi = MS1. Individual male ducks are identified by a blue dot and females by a red dot.

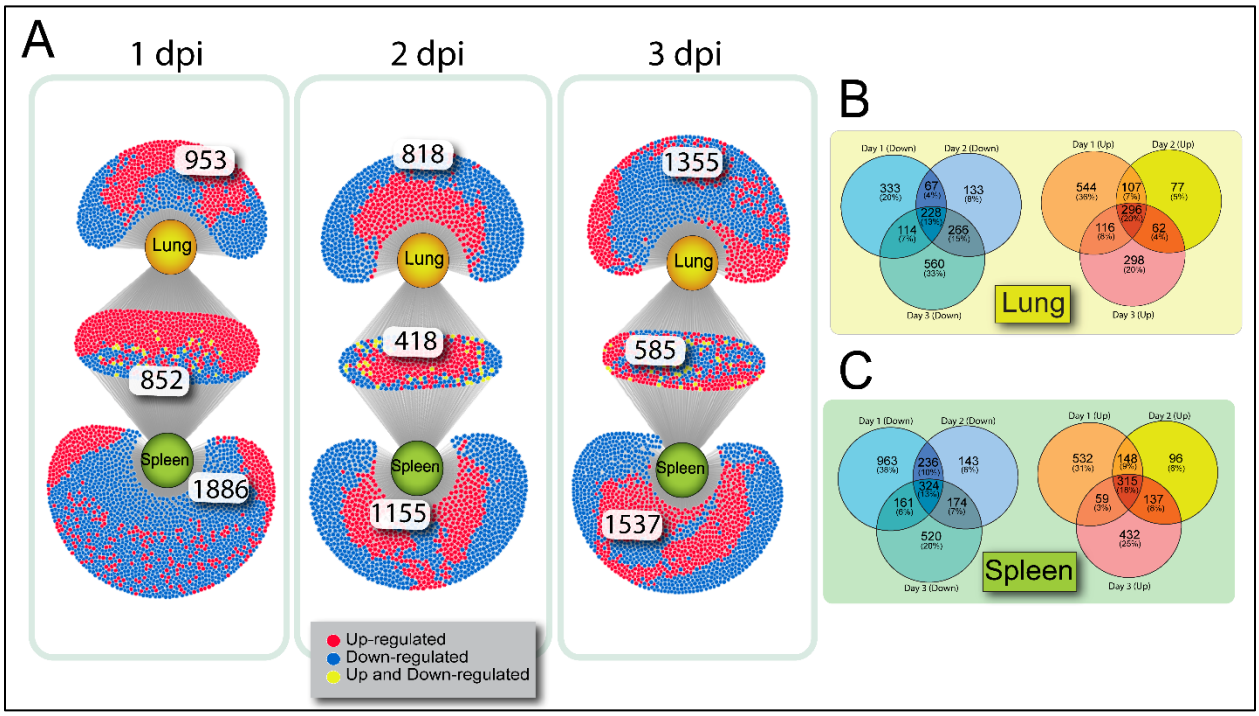


Figure 3.2. Venn diagrams showing overlap of gene expression on 1, 2 and 3 dpi in VN1203 infected ducks. Differentially expressed genes were assigned up or down regulated based on log2(FC) (upregulated>1, downregulated<-1) and overlap of DEGs was compared between tissues using DiVenn (A). Overlap of up or downregulated gene populations specific to each tissue in ducks infected with VN1203 in lung (B) or spleen (C) created using Venny 2.1.

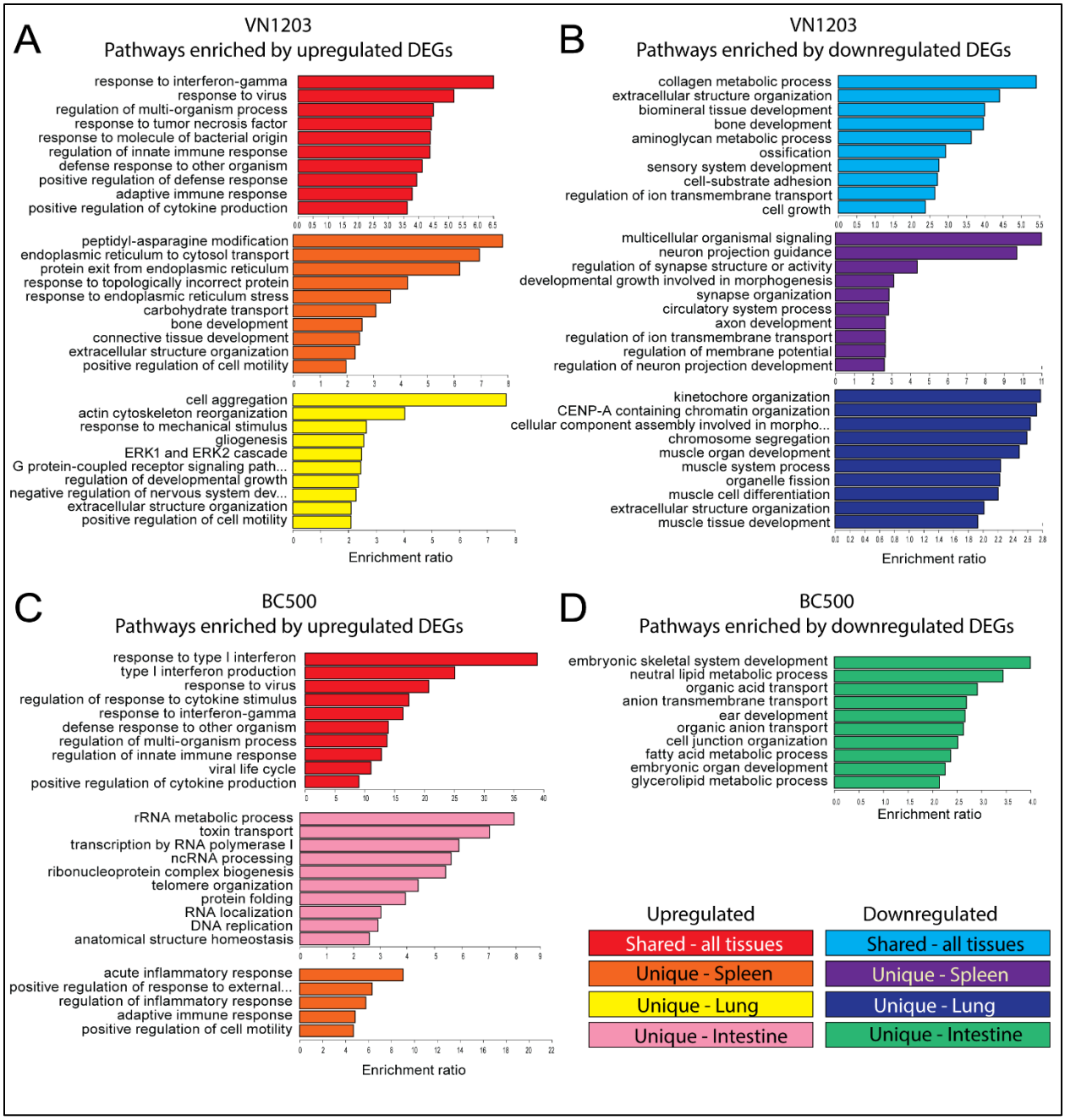


Figure 3.3. Gene ontology (GO) analysis of common and uniquely differentially expressed transcripts between VN1203 and BC500 infected ducks. Differentially expressed genes were assigned up or down regulated based on log2(FC) (upregulated>1, downregulated<-1) lists and were arranged as "similar between tissues", "unique to spleen", "unique to lung" or "unique to intestine". For ducks infected with VN1203, the top 10 most highly enriched terms in the GO biological function category were graphed for upregulated genes (A) and downregulated genes (B). For ducks infected with BC500, the top 10 most highly enriched terms derived from

upregulated genes (C) or downregulated genes (D) were identified and enrichment ratio for each term is graphed.

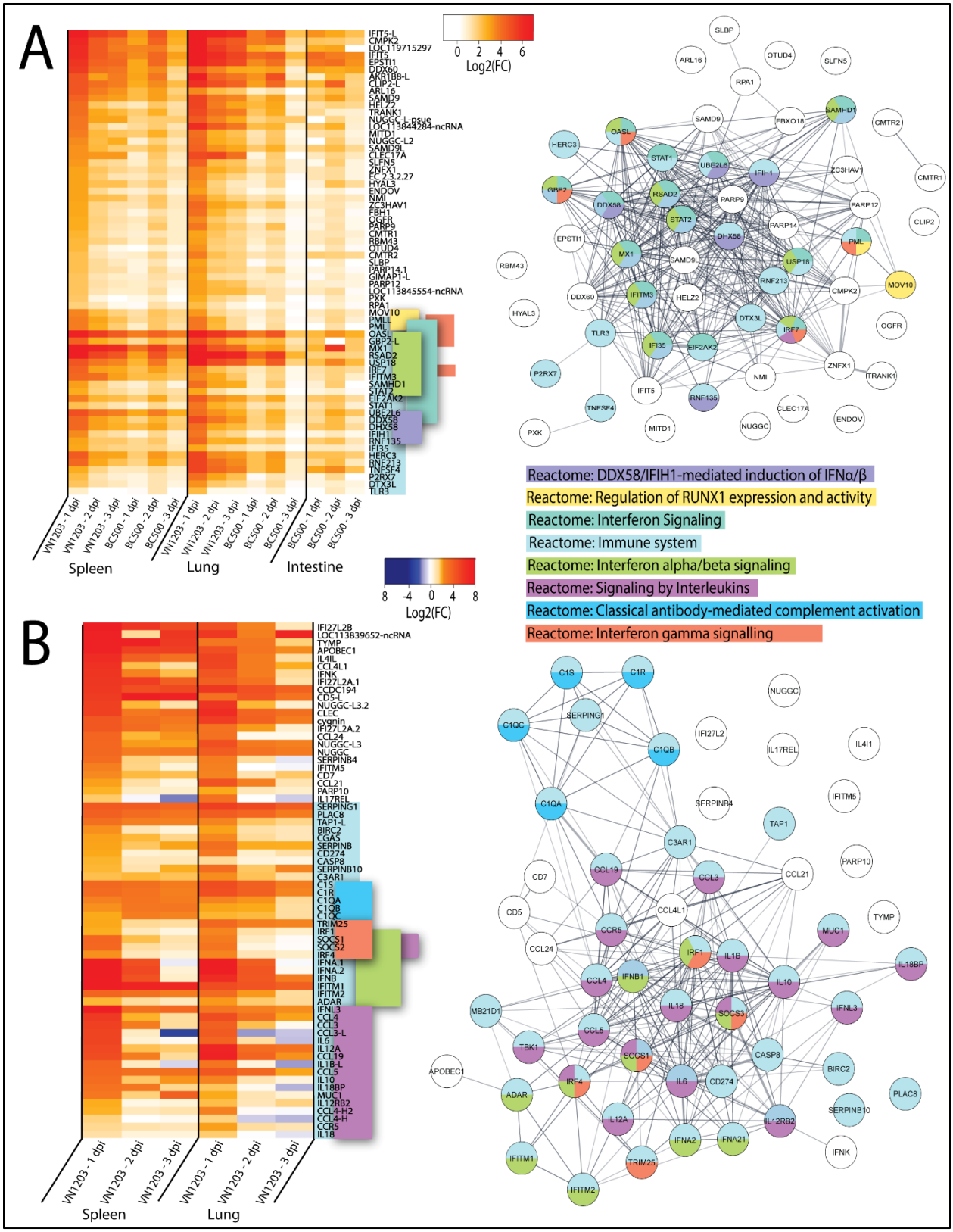

A
Log2(FC)
0 2 4 6
IFIT5-L
CMPK2
LOC119715297
IFIT5
EPSTI1
DDX60
AKR1B8-L
CLIP2-L
ARL16
SAMD9
HELZ2
TRANK1
NUGGC-L-psue
LOC113844284-ncRNA
MITD1
NUGGC-L2
SAMD9L
CLEC17A
SLFN5
ZNFX1
EC 2.3.2.27
HYAL3
ENDOV
NMI
ZC3HAV1
FBH1
OGFR
PARP9
CMTR1
RBM43
OTUD4
CMTR2
SLBP
PARP14-L
GIMAP1-L
PARP12
LOC113845554-ncRNA
PXK
RPA1
MOV10
PML
OASL
GBP2-L
MX1
RSAD2
USP18
IRF7
IFITM3
SAMHD1
STAT2
EIF2AK2
STAT1
UBE2L6
DDX58
DHX58
IFIH1
RNF135
IFI35
HERC3
RNF213
TNFSF4
P2RX7
DTX3L
TLR3
VN1203 - 1 dpi
VN1203 - 2 dpi
VN1203 - 3 dpi
BC500 - 1 dpi
BC500 - 2 dpi
BC500 - 3 dpi
Spleen
Lung
Intestine
8 -4 0 4 8
Log2(FC)
SLBP
ARL16
OTUD4
SLFN5
RPA1
SAMHD1
SAMD9
FBXO18
CMTR2
OASL
HERC3
STAT1
UBE2L6
IFIH1
ZC3HAV1
CMTR1
GBP2
RSAD2
PARP9
DDX58
PARP12
STAT2
PARP14
CLIP2
EPSTI1
DHX58
PML
MX1
SAMD9L
USP18
RBM43
RNF213
MOV10
IFITM3
HELZ2
CMPK2
DDX60
HYAL3
DTX3L
IRF7
OGFR
TLR3
IFI35
EIF2AK2
ZNFX1
P2RX7
NMI
TRANK1
IFIT5
RNF135
TNFSF4
CLEC17A
ENDOV
NUGGC
MITD1
PXK
Reactome: DDX58/IFIH1-mediated induction of IFNα/β
Reactome: Regulation of RUNX1 expression and activity
Reactome: Interferon Signaling
Reactome: Immune system
Reactome: Interferon alpha/beta signaling
Reactome: Signaling by Interleukins
Reactome: Classical antibody-mediated complement activation
Reactome: Interferon gamma signalling
B
IFI27L2B
LOC113839652-ncRNA
TYMP
APOBEC1
IL4I1
CCL4L1
IFNK
IFI27L2A.1
CCDC194
CD5-L
NUGGC-L3.2
CLEC
cygnin
IFI27L2A.2
CCL24
NUGGC-L3
NUGGC
SERPINB4
IFITM5
CD7
CCL21
PARP10
IL17REL
SERPING1
PLAC8
TAP1-L
BIRC2
CGAS
SERPINB
CD274
CASP8
SERPINB10
C3AR1
C1S
C1R
C1QA
C1QB
C1QC
TRIM25
IRF1
SOCS1
SOCS2
IRF4
IFNA.1
IFNA.2
IFNB
IFITM1
IFITM2
ADAR
IFNL3
CCL4
CCL3
CCL3-L
IL6
IL12A
CCL19
IL18-L
CCL5
IL10
IL18BP
MUC1
IL12RB2
CCL4-H2
CCL4-H
CCR5
IL18
VN1203 - 1 dpi
VN1203 - 2 dpi
VN1203 - 3 dpi
Spleen
Lung
C1S
C1R
NUGGC
C1QC
SERPING1
IFI27L2
IL17REL
IL4I1
C1QB
C1QA
SERPINB4
IFITM5
TAP1
C3AR1
PARP10
CD7
CCL19
CCL3
CCL21
CD5
CCL4L1
TYMP
CCR5
MUC1
CCL24
IRF1
IL1B
IL18BP
CCL4
IFNB1
IL10
MB21D1
IL18
IFNL3
SOCS3
CCL5
TBK1
SOCS1
CASP8
BIRC2
APOBEC1
IRF4
IL6
CD274
PLAC8
ADAR
SERPINB10
IL12A
IL12RB2
IFNA2
IFNA21
IFNK
IFITM1
TRIM25
IFITM2

Figure 3.4. VN1203 and BC500 upregulate genes shared between all tissues sampled. Lists of statistically significant (FDR<0.05) genes were compared for similarity in expression (Log2(FC)>1) and clustered together if they were expressed in (A) all tissues in VN1203 and BC500 infected ducks or (B) spleens and lungs of VN1203 infected ducks. STRING diagrams depict predicted protein-protein interactions between the products of each gene, while colour coding is based on term enrichment of Reactome pathways. No colour means that the genes did not enrich any of the Reactome terms. Reactome terms were considered significant if FDR<0.05. Enrichment and STRING diagram was created using the STRINGTIE app in Cytoscape.

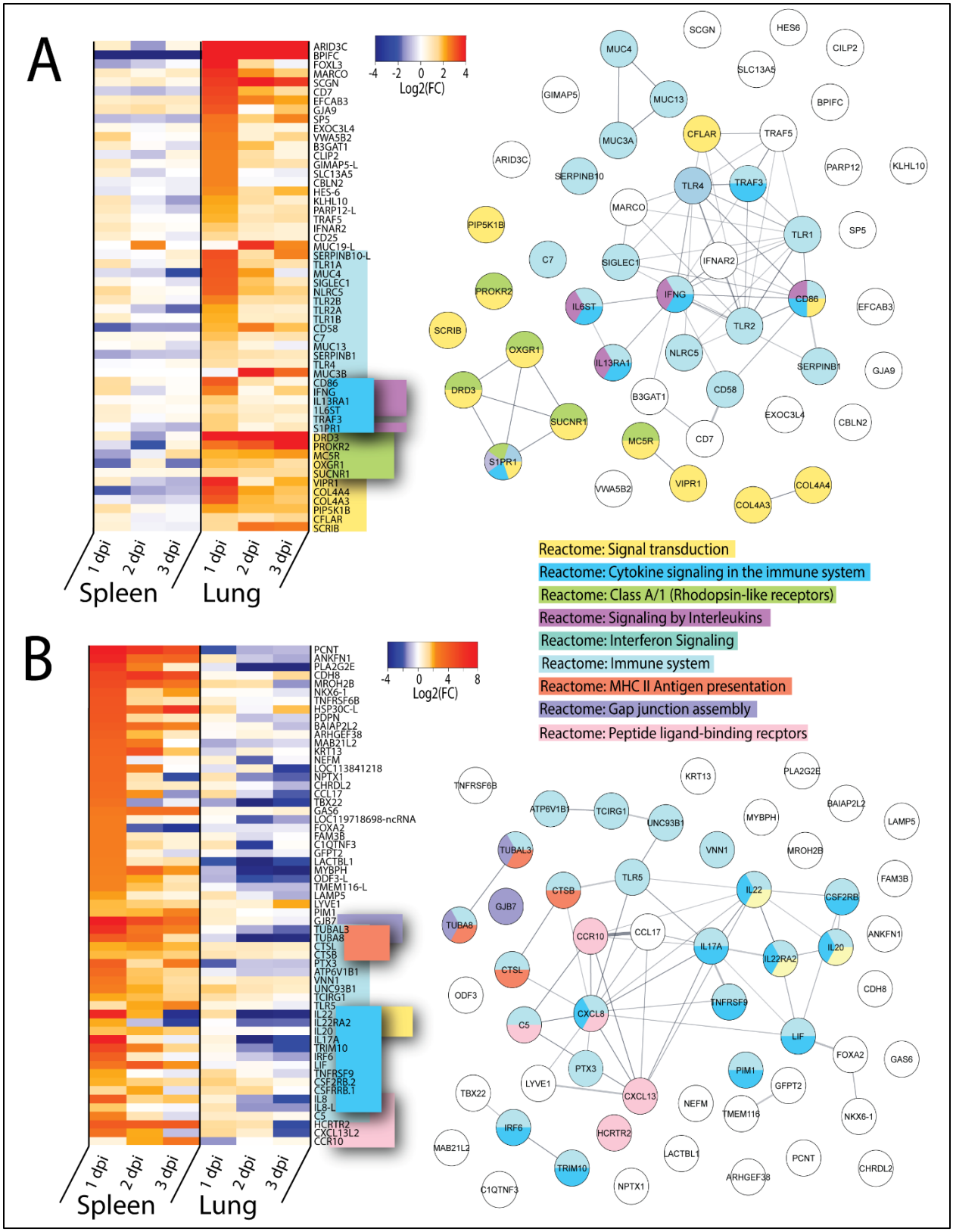

A
ARID3C
BPIFC
FOXL3
MARCO
SCGN
CD7
EFCAB3
GJA9
SP5
EXOC3L4
VWA5B2
B3GAT1
CLIP2
GIMAP5-L
SLC13A5
CBLN2
HES-6
KLHL10
PARP12-L
TRAF5
IFNAR2
CD25
MUC19-L
SERPINB10-L
TLR1A
MUC4
SIGLEC1
NLRC5
TLR2B
TLR2A
TLR1B
CD58
C7
MUC13
SERPINB1
TLR4
MUC3B
CD86
IFNG
IL13RA1
1L6ST
TRAF3
S1PR1
DRD3
PROKR2
MC5R
OXGR1
SUCNR1
VIPR1
COL4A4
COL4A3
PIP5K1B
CFLAR
SCRIB
-4 -2 0 2 4
Log2(FC)
1 dpi
2 dpi
3 dpi
1 dpi
2 dpi
3 dpi
Spleen
Lung
SCGN
HES6
CILP2
MUC4
SLC13A5
GIMAP5
MUC13
BPIFC
MUC3A
CFLAR
TRAF5
ARID3C
SERPINB10
PARP12
KLHL10
TLR4
TRAF3
MARCO
PIP5K1B
TLR1
SP5
C7
SIGLEC1
IFNAR2
PROKR2
IL6ST
IFNG
CD86
EFCAB3
SCRIB
TLR2
OXGR1
NLRC5
IL13RA1
SERPINB1
GJA9
DRD3
B3GAT1
CD58
SUCNR1
EXOC3L4
CBLN2
MC5R
CD7
S1PR1
VWA5B2
VIPR1
COL4A4
COL4A3
Reactome: Signal transduction
Reactome: Cytokine signaling in the immune system
Reactome: Class A/1 (Rhodopsin-like receptors)
Reactome: Signaling by Interleukins
Reactome: Interferon Signaling
Reactome: Immune system
Reactome: MHC II Antigen presentation
Reactome: Gap junction assembly
Reactome: Peptide ligand-binding recptors
B
PCNT
ANKFN1
PLA2G2E
CDH8
MROH2B
NKX6-1
TNFRSF6B
HSP30C-L
PDPN
BAIAP2L2
ARHGEF38
MAB21L2
KRT13
NEFM
LOC113841218
NPTX1
CHRDL2
CCL17
TBX22
GAS6
LOC119718698-ncRNA
FOXA2
FAM3B
C1QTNF3
GFPT2
LACTBL1
MYBPH
ODF3-L
TMEM116-L
LAMP5
LYVE1
PIM1
GJB7
TUBAL3
TUBA8
CTSL
CTSB
PTX3
ATP6V1B1
VNN1
UNC93B1
TCIRG1
TLR5
IL22
IL22RA2
IL20
IL17A
TRIM10
IRF6
LIF
TNFRSF9
CSF2RB.2
CSFRRB.1
IL8
IL8-L
C5
HCRTR2
CXCL13L2
CCR10
-4 0 4 8
Log2(FC)
1 dpi
2 dpi
3 dpi
1 dpi
2 dpi
3 dpi
Spleen
Lung
KRT13
PLA2G2E
TNFRSF6B
BAIAP2L2
ATP6V1B1
TCIRG1
MYBPH
LAMP5
UNC93B1
VNN1
MROH2B
TUBAL3
TLR5
FAM3B
CTSB
IL22
CSF2RB
GJB7
TUBA8
CCR10
CCL17
ANKFN1
IL17A
IL22RA2
IL20
CTSL
ODF3
CDH8
TNFRSF9
CXCL8
C5
LIF
FOXA2
GAS6
PTX3
PIM1
LYVE1
GFPT2
TBX22
CXCL13
NEFM
TMEM116
NKX6-1
IRF6
HCRTR2
MAB21L2
LACTBL1
PCNT
CHRDL2
TRIM10
ARHGEF38
C1QTNF3
NPTX1

Figure 3.5. Genes uniquely upregulated in lungs or spleens of VN1203 infected ducks. Lists of statistically significant (FDR<0.05) genes were compared for similarity in expression (Log2(FC)>1.5) and clustered together if they were uniquely upregulated in (A) lungs of VN1203 infected or (B) spleens of VN1203 infected ducks. STRING diagrams depict predicted protein-protein interactions between the products of each gene, while colour coding is based on term enrichment of Reactome pathways. No colour means that the genes did not enrich any of the Reactome terms. Reactome terms were considered significant if FDR<0.05. Enrichment and STRING diagram was created using the STRINGTIE app in Cytoscape.

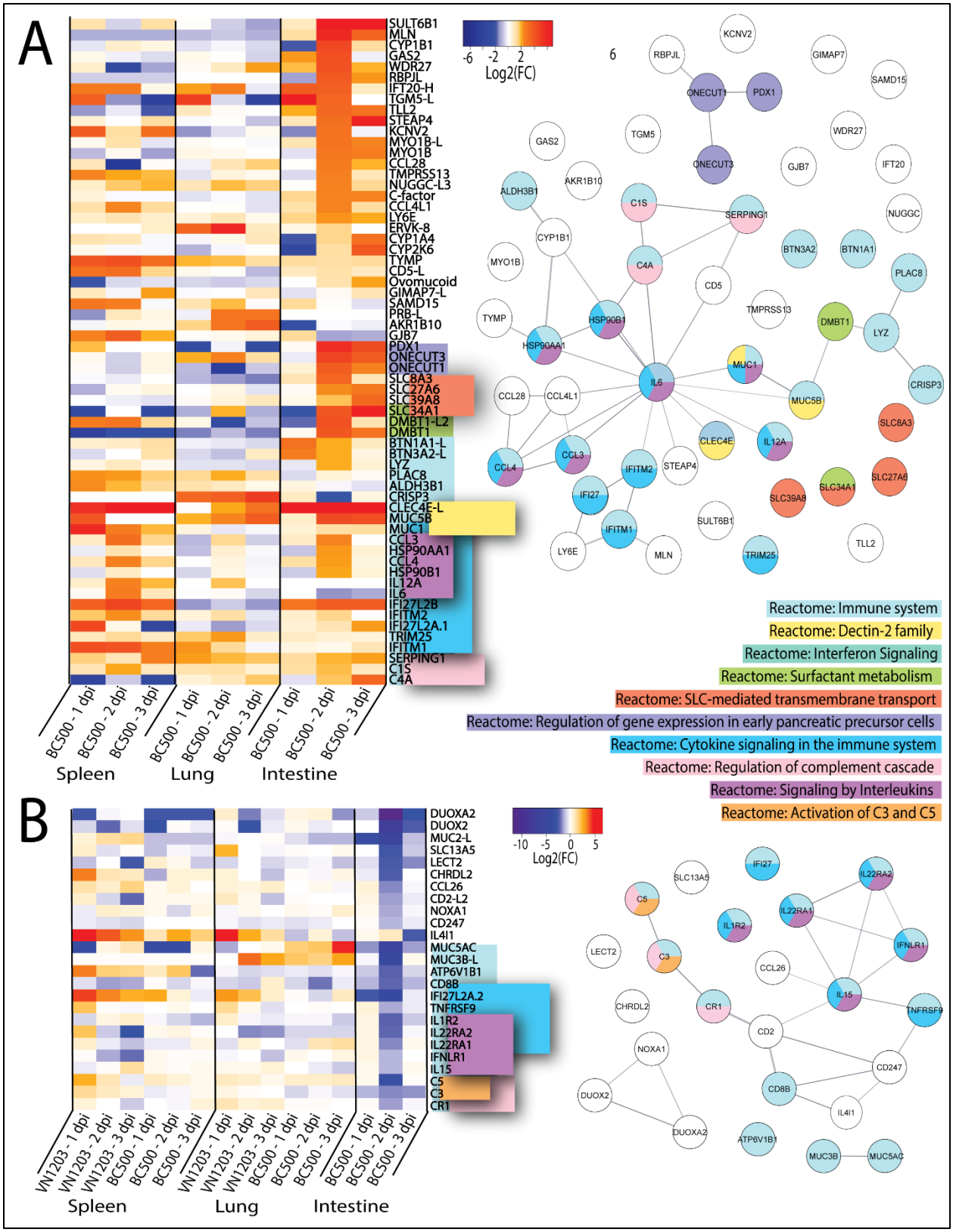

A
SULT6B1
MLN
CYP1B1
GAS2
WDR27
RBPJL
IFT20-H
TGM5-L
TLL2
STEAP4
KCNV2
MYO1B-L
MYO1B
CCL28
TMPRSS13
NUGGC-L3
C-factor
CCL4L1
LY6E
ERVK-8
CYP1A4
CYP2K6
TYMP
CD5-L
Ovomucoid
GIMAP7-L
SAMD15
PRB-L
AKR1B10
GJB7
PDX1
ONECUT3
ONECUT1
SLC8A3
SLC27A6
SLC39A8
SLC34A1
DMBT1-L2
DMBT1
BTN1A1-L
BTN3A2-L
LYZ
PLAC8
ALDH3B1
CRISP3
CLEC4E-L
MUC5B
MUC1
CCL3
HSP90AA1
CCL4
HSP90B1
IL12A
IL6
IFI27L2B
IFITM2
IFI27L2A.1
TRIM25
IFITM1
SERPING1
C1S
C4A
BC500 - 1 dpi
BC500 - 2 dpi
BC500 - 3 dpi
Spleen
Lung
Intestine
-6 -2 2
Log2(FC)
KCNV2
RBPJL
GIMAP7
SAMD15
ONECUT1
PDX1
WDR27
GAS2
TGM5
ONECUT3
GJB7
IFT20
ALDH3B1
AKR1B10
C1S
SERPING1
NUGGC
CYP1B1
BTN3A2
BTN1A1
MYO1B
C4A
CD5
PLAC8
TYMP
TMPRSS13
DMBT1
LYZ
HSP90B1
HSP90AA1
MUC1
IL6
CRISP3
CCL28
CCL4L1
MUC5B
SLC8A3
CLEC4E
IL12A
CCL4
CCL3
IFITM2
STEAP4
SLC34A1
SLC27A6
IFI27
SLC39A8
SULT6B1
IFITM1
LY6E
MLN
TRIM25
TLL2
Reactome: Immune system
Reactome: Dectin-2 family
Reactome: Interferon Signaling
Reactome: Surfactant metabolism
Reactome: SLC-mediated transmembrane transport
Reactome: Regulation of gene expression in early pancreatic precursor cells
Reactome: Cytokine signaling in the immune system
Reactome: Regulation of complement cascade
Reactome: Signaling by Interleukins
Reactome: Activation of C3 and C5
B
DUOXA2
DUOX2
MUC2-L
SLC13A5
LECT2
CHRDL2
CCL26
CD2-L2
NOXA1
CD247
IL4I1
MUC5AC
MUC3B-L
ATP6V1B1
CD8B
IFI27L2A.2
TNFRSF9
IL1R2
IL22RA2
IL22RA1
IFNLR1
IL15
C5
C3
CR1
VN1203 - 1 dpi
VN1203 - 2 dpi
VN1203 - 3 dpi
BC500 - 1 dpi
BC500 - 2 dpi
BC500 - 3 dpi
Spleen
Lung
Intestine
-10 -5 0 5
Log2(FC)
IFI27
SLC13A5
IL22RA2
C5
IL22RA1
IL1R2
IFNLR1
LECT2
C3
CCL26
IL15
CHRDL2
CR1
TNFRSF9
CD2
NOXA1
CD247
CD8B
DUOX2
IL4I1
DUOXA2
ATP6V1B1
MUC3B
MUC5AC

Figure 3.6. Genes up or downregulated following BC500 infection of ducks. Lists of statistically significant (FDR<0.05) genes were compared for similarity in expression (Log2(FC)>1.5) and clustered together if they were uniquely upregulated in at least 2 out of 3 tissues in BC500 infected ducks (A). Lists of statistically significant (FDR<0.05) downregulated (Log2(FC)<-1) in intestine of BC500 ducks were compared to other tissues of both VN1203 and BC500 infected ducks to determine uniquely downregulated genes in intestine (B). STRING diagrams depict predicted protein-protein interactions between the products of each gene, while colour coding is based on term enrichment of Reactome pathways. No colour means that the genes did not enrich any of the Reactome terms. Reactome terms were considered significant if FDR<0.05. Enrichment and STRING diagram was created using the STRINGTIE app in Cytoscape.

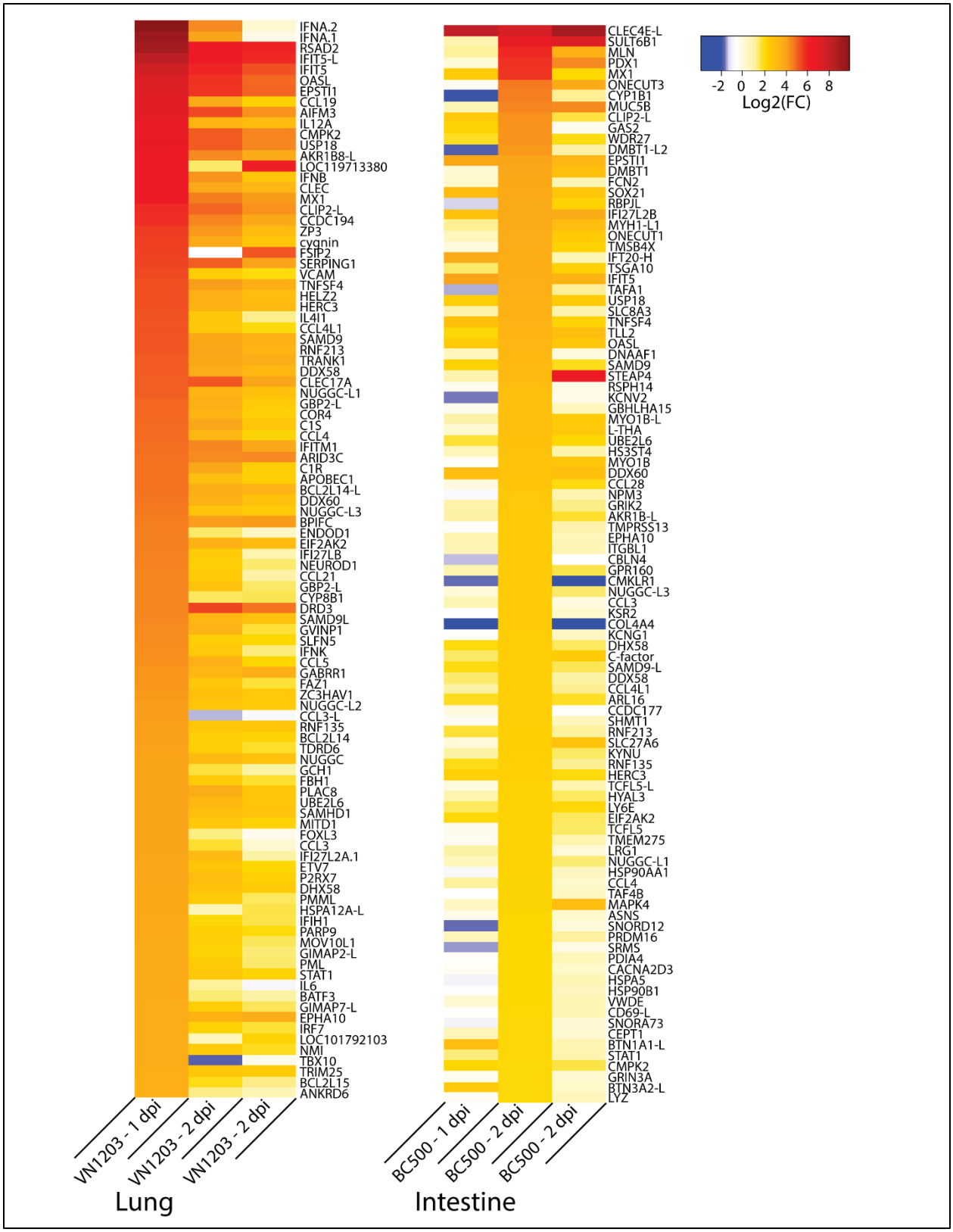
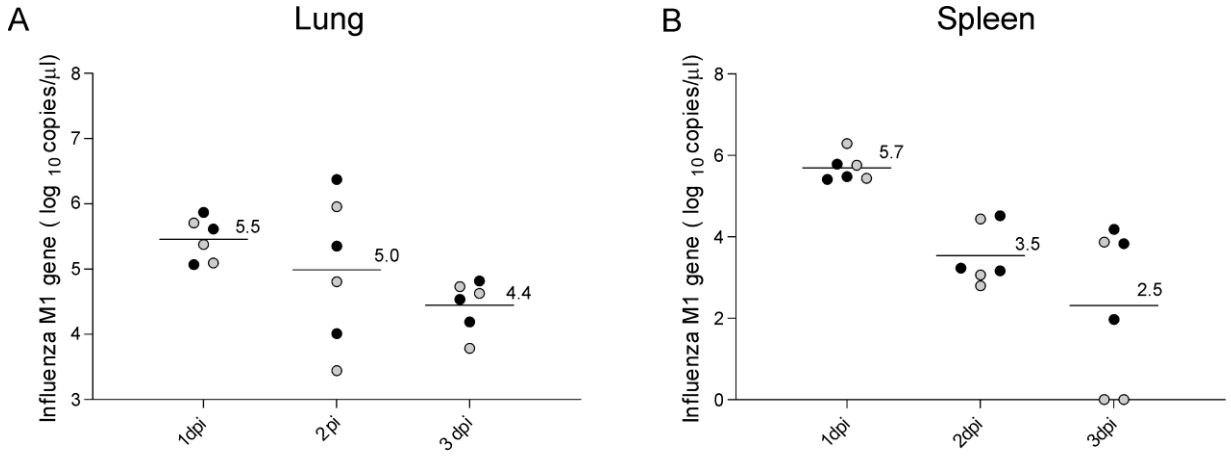
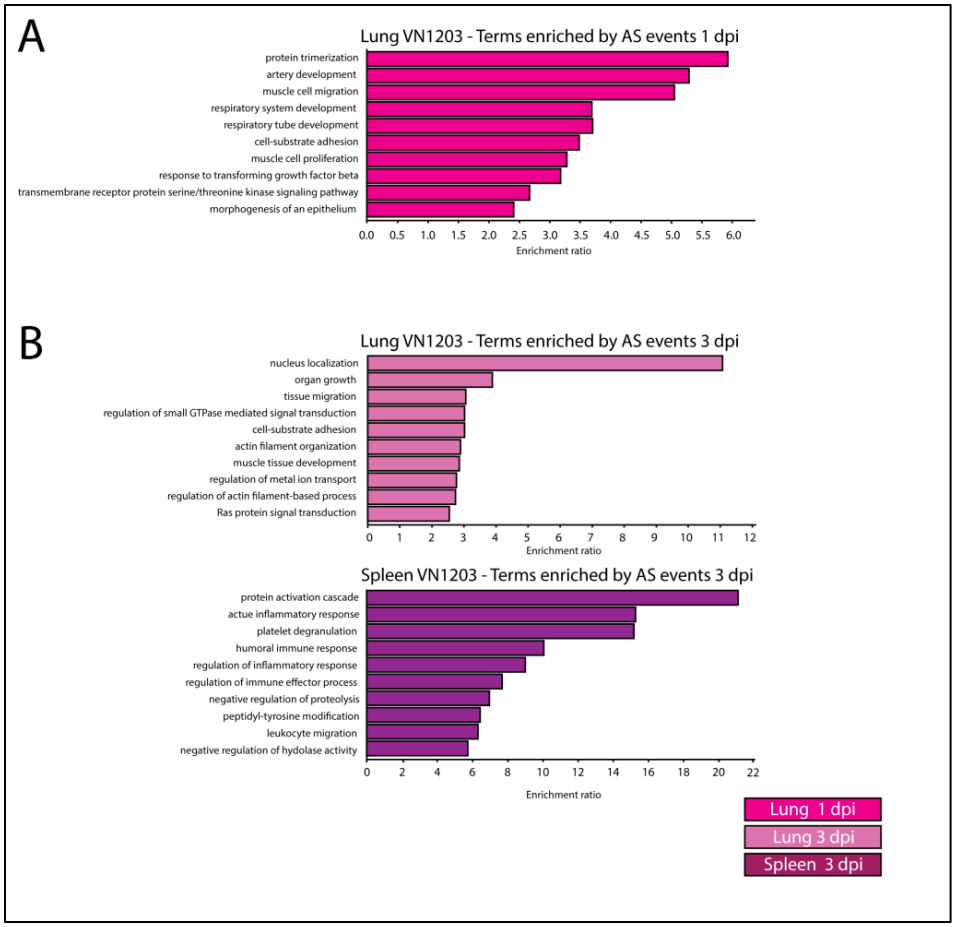

IFNA.2
IFNA.1
RSAD2
IFIT5-L
IFIT5
OASL
EPSTI1
CCL19
AIFM3
IL12A
CMPK2
USP18
AKR1B8-L
LOC119713380
IFNB
CLEC
MX1
CLIP2-L
CCDC194
ZP3
cygnin
FSIP2
SERPING1
VCAM
TNFSF4
HELZ2
HERC3
IL4I1
CCL4L1
SAMD9
RNF213
TRANK1
DDX58
CLEC17A
NUGGC-L1
GBP2-L
COR4
C1S
CCL4
IFITM1
ARID3C
C1R
APOBEC1
BCL2L14-L
DDX60
NUGGC-L3
BPIFC
ENDOD1
EIF2AK2
IFI27LB
NEUROD1
CCL21
GBP2-L
CYP8B1
DRD3
SAMD9L
GVINP1
SLFN5
IFNK
CCL5
GABRR1
FAZ1
ZC3HAV1
NUGGC-L2
CCL3-L
RNF135
BCL2L14
TDRD6
NUGGC
GCH1
FBH1
PLAC8
UBE2L6
SAMHD1
MITD1
FOXL3
CCL3
IFI27L2A.1
ETV7
P2RX7
DHX58
PMML
HSPA12A-L
IFIH1
PARP9
MOV10L1
GIMAP2-L
PML
STAT1
IL6
BATF3
GIMAP7-L
EPHA10
IRF7
LOC101792103
NMI
TBX10
TRIM25
BCL2L15
ANKRD6
VN1203 - 1 dpi
VN1203 - 2 dpi
VN1203 - 2 dpi
Lung
CLEC4E-L
SULT6B1
MLN
PDX1
MX1
ONECUT3
CYP1B1
MUC5B
CLIP2-L
GAS2
WDR27
DMBT1-L2
EPSTI1
DMBT1
FCN2
SOX21
RBPJL
IFI27L2B
MYH1-L1
ONECUT1
TMSB4X
IFT20-H
TSGA10
IFIT5
TAFA1
USP18
SLC8A3
TNFSF4
TLL2
OASL
DNAAF1
SAMD9
STEAP4
RSPH14
KCNV2
GBHLHA15
MYO1B-L
L-THA
UBE2L6
HS3ST4
MYO1B
DDX60
CCL28
NPM3
GRIK2
AKR1B-L
TMPRSS13
EPHA10
ITGBL1
CBLN4
GPR160
CMKLR1
NUGGC-L3
CCL3
KSR2
COL4A4
KCNG1
DHX58
C-factor
SAMD9-L
DDX58
CCL4L1
ARL16
CCDC177
SHMT1
RNF213
SLC27A6
KYNU
RNF135
HERC3
TCFL5-L
HYAL3
LY6E
EIF2AK2
TCFL5
TMEM275
LRG1
NUGGC-L1
HSP90AA1
CCL4
TAF4B
MAPK4
ASNS
SNORD12
PRDM16
SRMS
PDIA4
CACNA2D3
HSPA5
HSP90B1
VWDE
CD69-L
SNORA73
CEPT1
BTN1A1-L
STAT1
CMPK2
GRIN3A
BTN3A2-L
LYZ
BC500 - 1 dpi
BC500 - 2 dpi
BC500 - 2 dpi
Intestine
-2 0 2 4 6 8
Log2(FC)

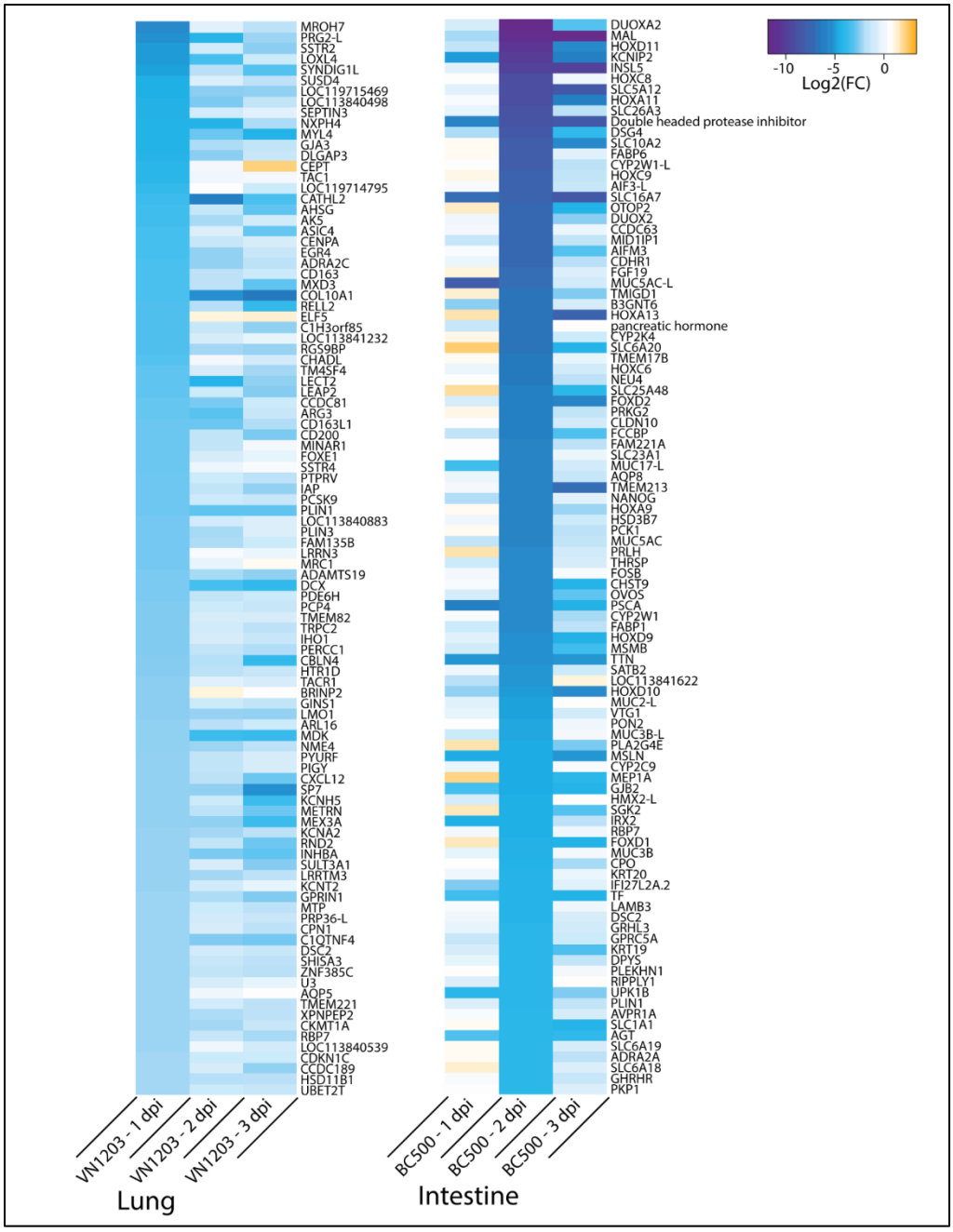
Figure 3.7. Top 100 upregulated genes in lungs of VN1203 and intestines of BC500 infected ducks. Lists of statistically significant (FDR<0.05) genes were filtered for the top 100 genes expressed in lungs of VN1203 infected ducks on 1 dpi, and intestines of BC500 infected ducks on 2 dpi. Redundant genes, pseudo genes and genes denoted as ncRNA in NCBI were manually removed from lists.



Supplementary figure S3.2. Influenza M1 gene has sustained expression in VN1203 infected ducks. Figure adapted from Figure 1 Fleming-Canepa et al., 2019. Influenza A matrix gene (M1) was amplified from RNA collected from lung (A) and spleen (B) from ducks infected with VN1203. Influenza matrix gene copy number is determined against a known copy number of an influenza matrix M1 clone. Each dot represents one duck and mean fold-induction is indicated for the ducks (n = 6). Dark circles (males) and light circles (females). Significant differences in mean viral titre on each day were determined by two-way ANOVA ($P < 0.05$) *$P < 0.05$, **$P < 0.01$, ****$P < 0.0001$. Non-significant differences are not indicated.



Supplementary figure S3.2. Gene ontology (GO) analysis of alternatively spliced genes in VN1203 infected ducks. Statistically significant (FDR < 0.05) alternatively spliced genes were submitted to enrichment analysis and the top 10 most enriched terms in the category of GO biological process are graphed for lungs of ducks infected with VN1203 on 1 dpi (A) and lungs and spleens of ducks infected with VN1203 on 3 dpi (B).



Supplementary figure S3.3. Top 100 downregulated genes in lungs of VN1203 and intestines of BC500 infected ducks. Lists of statistically significant (FDR<0.05) genes were filtered for the 100 genes most downregulated in lungs of VN1203 infected ducks on 1 dpi, and intestines of BC500 ducks on 2 dpi. Redundant genes, pseudo genes and genes denoted as ncRNA in NCBI were manually removed from lists.

Supplementary files S3.1, S3.2, S3.3 and S3.4 all stored in an online repository located here: https://www.frontiersin.org/articles/10.3389/fimmu.2021.786205/full#supplementary-material

CHAPTER 4

Evolution and expression of TRIM protein genes in the mallard duck

4.1 Introduction

Tripartite motif (TRIM) proteins comprise a large family with important roles in development (Reymond et al., 2001; Berti et al., 2002a), cell cycle regulation (Venuto et al., 2019), immunity (Nisole et al., 2005; Ozato et al., 2008; Rajsbaum et al., 2014; Hatakeyama, 2017), autophagy (Hatakeyama, 2017; Venuto et al., 2019) and various other intracellular signaling functions (Marín, 2012; Hatakeyama, 2017). Comparisons of TRIM proteins across species demonstrate a rapidly expanding repertoire during vertebrate evolution. Humans have more than 80 TRIM genes, while mice have approximately 60, and 208 have been reported in zebrafish (Ozato et al., 2008; Sardiello et al., 2008; Carthagena et al., 2009; Hatakeyama, 2011; Meroni, 2012). The avian TRIM repertoire has not been systematically analyzed. An early estimate identified 37 in chicken (Sardiello et al., 2008), but this has not been updated with improved genome resources. The ambiguity of TRIM gene number in each species arises from their location on many different chromosomes, each evolving independently of each other, as well as having species-specific expansions (Sawyer et al., 2005; Song et al., 2005; Sawyer et al., 2007).

TRIM proteins are characterized by the presence of three domains: the really interesting new gene (RING) domain, one or two B-box domains and a coiled-coil (CC) domain. These domains together are termed the RBCC motif. TRIM proteins possess E3 ubiquitin ligase activity due to their RING domains which aids in conjugation of polyubiquitin to target proteins (Joazeiro et al., 2000; Metzger et al., 2014). The RING domain is not confined to catalyzing ubiquitination as some TRIM proteins in mammals also can conjugate ubiquitin-like proteins, such as SUMO (Ma et al., 2019). B-box domains have a more complex and perhaps less well understood role in TRIM protein function. B-box domains contain a zinc binding domain like the RING domain, and thus can also perform E3 ligase activity (Han et al., 2011; Bell et al., 2012; Zheng et al., 2015). The B-box domain is also implicated in higher order multimerization and

binding substrate proteins (Massiah et al., 2006; Wagner et al., 2016; Li et al., 2019b; Massiah, 2019). The CC domain is involved in homo or heteromeric assemblies and can be necessary for function (Sanchez et al., 2014; Weinert et al., 2015). The C-terminal domains are often responsible for substrate recognition. Substrates can range from intracellular proteins targeted for degradation or activation, to pathogen proteins or nucleotides (James et al., 2007; D'Cruz et al., 2013; Choudhury et al., 2017; Wu et al., 2019).

TRIM proteins are divided into 11 subfamilies by variable C-terminal domains, which are termed C-I to C-XI (Short et al., 2006; Ozato et al., 2008; Meroni, 2012; Watanabe et al., 2017). Sardiello suggested the TRIM family separates into two groups (Sardiello et al., 2008). The first, group I, is more conserved through evolution and is composed of all 11 subfamilies. Group II contains only TRIM proteins belonging to the C-IV subfamily, which contain a C-terminal B30.2/PRY-SPRY domain. Members of group II appear to evolve faster than members in group I (Sardiello et al., 2008). Marín and colleagues demonstrated that the evolutionary relationships between TRIM proteins are more complex, and different subfamilies arose at distinct points in eukaryote evolution, with the C-IV subfamily evolving and drastically expanding in vertebrates only (Marín, 2012). Many members of the C-IV subfamily are regulated by immune responses in the host (Versteeg et al., 2013; Rajsbaum et al., 2014). Furthermore, several members of the C-IV family have direct roles in immunity and viral restriction (Nisole et al, 2005; Ozato et al, 2008). Also, several of the C-IV type TRIM genes are present in the MHC locus of humans (Meyer et al., 2003), chickens and ducks (Ruby et al., 2005; Blaine et al., 2015), and fish (Boudinot et al., 2011). This suggests that TRIM genes were part of the ancestral MHC, and intriguingly they have undergone independent duplication events in each species.

Many TRIM proteins are modulators of innate immunity and mediators of direct viral restriction. Hence the repertoire of the mallard duck (*Anas platyrhynchos*) is of interest, as they are the reservoir host of influenza A viruses (Webster et al., 1978; Webster et al., 1992). Comparison to the repertoire of chickens is of value since they are an important agricultural species and an established model for vertebrate development. A few functional studies of individual TRIM proteins of birds demonstrate their importance in immunity. TRIM25 is perhaps the best studied TRIM protein in birds with both characterization and functional studies published in chicken (Feng et al., 2015; Zhou et al., 2020; Wang et al., 2021), duck (Miranzo-Navarro et al., 2014; Kaikai et al., 2021) and goose (Wei et al., 2016). In mammals and ducks,

TRIM25 catalyzes addition of polyubiquitin for activation of RIG-I in the innate immune signaling pathway (Gack et al., 2007; Miranzo-Navarro et al., 2014). Chicken TRIM39 is predominately expressed in the spleen (Pan et al., 2011). TRIM62 has been investigated for its antiviral properties in chicken and has demonstrated antiviral activity against reticuloendotheliosis virus (Li et al., 2020) and avian leukosis virus subgroup J (Li et al., 2019a). TRIM32, known for its antiviral activity in mammals (Zhang et al., 2012; Fu et al., 2015; Cui et al., 2017), can restrict influenza (Wu et al., 2020) and Tembusu virus (Li et al., 2021) in ducks.

In this study, we characterized the duck TRIM gene repertoire by utilizing the NCBI databases, as well as de novo transcriptome assembly to identify candidates. We investigated their coding regions, domain architecture and phylogenetic relationships to each other. Once we had characterized the duck TRIM repertoire, we compared these sequences to those published in the chicken to look at species specific differences between these two birds. We also looked at both abundance and relative expression of these TRIM genes in duck tissues. Our results demonstrated that ducks have 57 TRIM or TRIM-like genes, compared to 52 in chicken. Both ducks and chickens have TRIM genes specific to their respective lineages. Additionally, while some TRIM genes were ubiquitously expressed in all duck tissues available on NCBI, other TRIM genes were highly tissue specific.

4.2 Materials and methods

4.2.1 Data mining

To generate a master transcriptome of duck sequence reads, the NCBI short read archive (SRA) database (https://www.ncbi.nlm.nih.gov/sra) was mined for projects involving domestic mallard ducks (*Anas platyrhynchos*). Wild mallard and Muscovy ducks (*Cairina moschata*) were excluded. Project numbers and individual samples included in this study are listed in supplementary table S4.1. All SRA libraries were uploaded to Compute Canada's WestGrid computing environment (www.computecanada.ca) for subsequent processing. Data files with ambiguous descriptions, questionable content or failed quality checks were excluded from our analysis. To generate a reference dataset of all known avian TRIM sequences, we manually searched the NCBI protein databases for TRIM sequences in avian species. Redundant and misannotated sequences were removed. From the avian TRIM list we made two additional

databases, one composed of mallard duck TRIM proteins only, and one composed of chicken TRIM proteins only.

4.2.2 Transcriptome assembly

All SRA libraries were initially checked for quality and adaptor content using fastQC Version 0.11.9 (Andrews, 2010). Samples used in this study passed fastQC analysis and were between 100-150 bp in length. Reads were trimmed using Trimmomatic version 0.36 (Bolger et al., 2014)**.** Trim settings were set to retain reads with a minimum length of 33 and using a sliding window of 15 bp.

A total of 180 libraries were successfully assembled using 7 Kmer values ranging from 25 to 85 using TRANS-ABYSS 2.0.1 (Birol et al., 2009). After each individual library was assembled, they were binned by tissue type and duplicate contigs found within a tissue type were collapsed into a consensus contig using CAP3 (4) with a cut-off value of 95% identity and CD-HIT-EST version 4.8.1 (5) with a cutoff value of 97% identity. Finally, all tissue type assemblies were collapsed into one assembly using CAP3. Singlet files were merged separately to reduce loss of genes due to excessive reduction in putative duplicate contigs. Merged singlet contigs were then compared back to the master transcriptome for a final master assembly. Sequences less than 200 bp were pruned from the master assembly using in-house scripts published at https://github.com/rmpeery/dataProcessing. Two quality control measures were employed to assess the final assembly. We checked the number and average size of contigs using the abyss-fac command in ABYSS v 2.0.1 (Birol et al., 2009). To ensure that transcriptome collapsing was not impacting gene content, BUSCO version 3.0.2 (Simao et al., 2015) was used to determine common orthologous gene content. To remove duplicate copies of genes (putative orthologs remaining due to assembly strategy), we used reciprocal blast hits (RBH) and applied a leave one out method to remove contigs with ≥ 97% similarity (perl script published at https://github.com/rmpeery/dataProcessing).

4.2.3 Duck TRIM gene identification

The master assembly was translated into all 6 reading frames using EMBOSS 6.6.0 (Rice et al., 2000). All avian TRIM reference proteins were compiled into a BLAST+ database using the makeblastdb command in BLAST+ version 2.7.1 (Camacho et al., 2009). We ran BLASTp (BLAST+) against the assembled transcriptome, and all hits were parsed from the master transcriptome using in-house scripts (published at https://github.com/rmpeery/dataProcessing).

Our newly identified, putative avian TRIM proteins were aligned to the reference TRIM proteins and neighbor-joining (NJ) phylogenetic trees were inferred using CLUSTAL OMEGA (Sievers et al., 2011)**.** We compared full length transcriptome contigs to the assembled list of annotated duck TRIM proteins to both confirm identity and to validate the master assembly. Any TRIM proteins which were detected by the BLAST search but were not present in ducks were aligned against the avian TRIM protein database to confirm identity. Any other ambiguous hits were submitted to SMART (Letunic et al., 2015) to verify protein domain composition. Many butyrophilin (BTN) proteins were pulled during our searches, as BTN genes are their own separate defined family of genes, they were excluded from this analysis. To identify any TRIM genes assumed to be missing in birds or ducks, orthologous TRIM sequences from various species were aligned using COBALT (https://www.ncbi.nlm.nih.gov/tools/cobalt/) and submitted to HMMer (v3.3.2) (hmmer.org) to search for domain profiles of these missing TRIMs in our master assembly. Any hits were further analyzed using HMMer (Finn et al., 2011), UniProt (UniProt Consortium, 2018) and SMART (Letunic et al., 2015). Domain composition of amino acid sequences were verified using SMART. Duck TRIM genes found in this analysis are summarized in Table 4.1.

4.2.4 Comparison between duck and chicken TRIM protein repertoires

The newly identified duck TRIM nucleotide and protein sequences were compared to the chicken TRIM protein sequences that we had previously downloaded in FASTA format from NCBI. Any sequences present in the duck but presumed to be missing in the chicken were submitted to BLASTn and queried against the current chicken genome (bGalGal1.mat.broilerGRCg7b) using the BLAST+ online portal (https://blast.ncbi.nlm.nih.gov/Blast). Annotations and identification numbers of these genes were compiled into Table 2.

4.2.6 Mapping to chromosomes

To find chromosomal location of all duck TRIM genes, we queried nucleotide sequences against the current NCBI duck genome (assembly ZJU1.0) using the online version of BLASTn. Chromosome lengths were taken from the reference duck genome, and locations were assigned from the start of the TRIM gene. TRIM genes were assigned chromosomal location using karyoplotR (Gel et al., 2017) in the R studio environment (RStudio Team, 2020). The resulting map was edited graphically using Adobe Illustrator for clarity and readability.

Sizes and locations of TRIM genes located in the MHC-B locus of chickens, mallard and tufted ducks (*Aythya fuligula*) were approximated from genomic data on NCBI. Data for mallard TRIM genes were extracted from the reference genome ZJU1.0, while data for the tufted duck and chicken MHC-B locus were from the reference genomes bAytFul2.pri and bGalGal1.mat.broiler.GRCg7b, respectively. Likewise, the sizes and distances of TRIM25, 27 and 65 in chicken and mallard duck were approximated from data in the chicken and mallard duck genomes previously mentioned. The resulting distances and approximations of gene sizes were edited graphically using Adobe illustrator.

4.2.7 Phylogenetic trees and minimum spanning networks

We made two initial alignments of TRIM proteins, the first aligning all duck TRIM proteins and the second aligning both duck and chicken TRIM protein sequences. A third phylogenetic tree was made using C-IV subfamily members from representative species to help in properly assigning annotations. The species used were human, mouse or alternative non-primate placental mammal, a representative sequence from marsupials, a representative sequence from turtles and a representative sequence from lizards. Occasionally a gene not annotated in chicken or duck would be found in another bird, and those would also be added to our alignments. All accession numbers for protein sequences used can be found in Supplementary table S4.2. All TRIM proteins were initially aligned using the online MAFFT alignment program (Katoh et al., 2019). All alignments used the E-INS-I refinement method. Alignments were further refined by eye in unipro UGENE (Rose et al., 2019). The best protein model was determined for each tree using ModelFinder (Kalyaanamoorthy et al., 2017) in the IQtree environment. Maximum-likelihood (ML) phylogenetic trees of these alignments were inferred using IQTree (Nguyen et al., 2014) resulting in one phylogeny for the duck TRIM protein repertoire and one phylogeny for the duck and chicken TRIM proteins. The Ultrafast bootstrap algorithm (Minh et al., 2013) was run with 10,000 bootstrap replications on the duck and chicken TRIM ML phylogenies. The Ultrafast bootstrap algorithm was run with 1000 bootstrap replications on the C-IV TRIM protein subfamily tree. The consensus of these replicates were visualized in FigTree v1.4.3 (http://tree.bio.ed.ac.uk/software/figtree/). Phylogenetic trees were then further edited using Adobe Illustrator for clarity and to add additional information.

To make minimum spanning networks with duck TRIM proteins, the duck TRIM protein alignment (as described above) was converted to a distance matrix using msa (Bodenhofer et al.,

2015) and seqinr (Charif et al., 2007) in the Rstudio (v4.0) environment. A minimum spanning network (MSN) was created using the prim algorithm provided by the RGBL r package (Vince Caery, 2021) and visualized using ggplot2 (Wickham, 2016). The resulting MSN was edited in Adobe illustrator for clarity.

4.2.8 Assigning names to duck TRIM genes

We interrogated our master *de novo* transcriptome assembly for TRIM proteins not annotated on NCBI. To resolve ambiguous names and incorrect NCBI annotations, we used names previously assigned in the literature, or genes annotated in related species. Due to the redundant nature of naming putative TRIM genes on NCBI (avian TRIM genes are named by their closest BLAST hit to human TRIM proteins, and then named as TRIM-like), many names needed to be resolved through literature or phylogenetic analysis. Three genes described as "zinc protein RFP" by NCBI (gene ID: 101791103, 101791534 and 101790919), were previously assigned the names TRIM27.1, TRIM27.2 and TRIM27L, respectively (Blaine et al., 2015). TRIM59L (Gene ID: 101789404) is annotated as LOC101789404 and incorrectly described as serine-rich adhesin for platelets. Our domain prediction for TRIM59L using SMART finds a RING, B-box and CC domain, indicative of TRIM gene lineage. However, when we compare the sequences, there is a single nucleotide difference, which causes a frameshift in the NCBI sequence that abrogates the tripartite motif of the predicted protein structure. In this case, we will refer to our sequence as the correct sequence because the transcriptome represents many individuals but acknowledge that either could be correct.

We resolved unannotated (TRIM genes assigned "LOC" numbers and a description rather than a definite annotation) using previously published annotations, structural analysis, and phylogenetic analysis.

4.2.9 Read mapping and differential expression of TRIM genes

Our newly identified TRIM contigs were trimmed to CDS regions and were concatenated into a multi-FASTA file and used as the reference for read mapping. We aligned RNA-seq reads to the reference TRIM genes using Bowtie2 v2.3.4.3 (Ben et al., 2011). We used FeatureCounts (Liao et al., 2014) to summarize, count reads, and assign features from the BowTie2 mapped outputs. Reads were normalized to individual library sizes using the EdgeR (Robinson et al., 2010) trimmed mean of M values (TMM) method. Reads were compared using plotMDS in the Rstudio environment. Normalized log2 counts per million Log2(CPM) were visualized using the

cim function in R (Eisen et al., 1998). We analyzed relative patterns of tissue distribution by setting the matrix intercept as the overall mean and using the generalized linear model Quasi-likelihood test (glmQL) to determine log fold change (FC) values of individual tissues when compared to the mean in EdgeR. All logFC values were visualized using a heatmap using the gplots heatmap.2 function (Warnes, 2022) in the Rstudio environment. Dendrograms were added using heatmap.2 function in gplots in the Rstudio environment. To determine the highest and lowest relatively expressed TRIM genes in different duck tissues, we sorted the relative expression results for each tissue by false discovery rate FDR (<0.05) and filtered out any TRIM genes above this threshold. We then sorted this data by log2(FC) and summarized the top 5 highest and top 5 lowest relatively expressed genes.

4.3 Results

4.3.1 Ducks have 57 genes in their TRIM repertoire

To determine how many TRIM or TRIM-like genes are present in the domestic mallard duck (*Anas platyrhynchos*) we first mined the NCBI genome and protein databases for all annotated avian TRIM proteins. From this list, we identified TRIM proteins annotated in the mallard duck and chicken. We used both BLAST and HMM based searches to identify 57 TRIM or TRIM-like genes in the duck. We used a neighbor-joining tree to cluster putative TRIM contigs with known avian TRIM proteins to verify identity and identify outliers. A flow chart of the workflow can be found in Supplementary figure S4.1**.** Any sequences discovered to be BTN proteins were omitted, although we acknowledge that many BTN proteins appear to be highly similar in C-terminal domain composition to C-IV subfamily TRIM proteins and as such TRIM proteins and BTN proteins may share similar evolutionary ancestry. All duck TRIM protein annotations were assembled into a table (Table 4.1). All but four of the mallard duck TRIM sequences we identified were previously annotated on NCBI. TRIM28 and FSD1 are not annotated in the mallard duck, however they are annotated in chicken and in tufted duck (*Aythya fuligula*).

For completeness, we have included 11 genes in this analysis that are classified as TRIM-like, in that they do not have the traditional “tripartite motif” domain structure the TRIM family is named for. Some of these, such as ring finger protein 207 (RNF207), fibronectin III and SPRY domain containing proteins 1 and 2 (FSD1 and FSD2), B-Box and SPRY domain containing (BSPRY) and NHL repeat containing E3 ubiquitin protein ligase 1 (NHLRC1) are ancestral

TRIM genes which have lost important domains during speciation events (Marín, 2012). RNF135, also referred to as RIPLET, is an E3 ubiquitin ligase that functions in innate immune pathway modulation (Oshiumi et al., 2010; Kato et al., 2021; Kouwaki et al., 2021). While it contains a RING, CC and PRY-SPRY domain, it is lacking B-box domains. However, due to its similarity to other TRIM genes, we included it in these analyses. The TRIM-like genes will be included in our analyses of the duck TRIM repertoire.

To determine proper names for both annotated and unannotated TRIM genes, we first resolved ambiguous annotations. For example, genes 101805457 and 101804875 are both described as "TRIM39-Like" by NCBI. We used the name TRIM39.2 (assigned by Blaine et al. 2015) for gene ID 101805457. Gene 101804875, described as TRIM39-like in ducks, does BLAST to mammalian TRIM39, however, it does so with less than 50% similarity. It appears this gene is specific to the group Diapsida (reptiles and birds) as a direct ortholog is not found in mammals. Phylogenetic analysis found this gene had closest ancestry to mammalian TRIM58, thus we named this gene diapsid-TRIM58 (diaTRIM58). This gene is annotated as TRIM39 in chicken, however our phylogenetic analyses suggest diaTRIM58 is not a direct ortholog of TRIM39. We did, however, find an unplaced TRIM gene in duck from our HMMER searched that was called "TRIM39-like" in the bird species. This protein clustered with TRIM39 from mammals and reptiles and thus we assigned the name TRIM39. A sequence identified in our HMMER searches which coded for only a RING domain clearly clustered with RNF39 of other species, so we designated this gene RNF39-RING (or RNF39R). The previously annotated RNF39L protein from ducks did not cluster with RNF39 of other species, instead it clustered with the Diapsid MHC-linked genes, and appears to be only in avian lineages. We named this avian-TRIM15L. It was named as a TRIM-like gene as it is missing B-box domains, and TRIM15L as it most closely BLASTS to marsupial TRIM15L, a PRY-SPRY only TRIM-like gene. TRIM15L does not have any orthologs in primates or placental mammals. For clarity, in this manuscript we will refer to the duck TRIM genes by the names we have assigned them in Table 1.

4.3.2 The duck TRIM gene repertoire spans 21 chromosomes

To determine chromosomal locations of the 57 TRIM or TRIM-like genes found in the duck, we interrogated the Pekin duck genome (assembly ZJU1.0) using our newly generated TRIM contigs. We were able to assign chromosomal location of 51 of 57 TRIM or TRIM-like

genes, which are located on 21 different duck chromosomes (Figure 4.1). Most duck TRIM genes are located in unique locations throughout the genome; however, two interesting clusters of genes are found on chromosome 17 and 19. Ducks also have a duplication of promyelocytic leukemia protein (TRIM19/PML), resulting in two very similar genes on chromosome 11 in opposite orientation.

A cluster of TRIM genes are found in the duck MHC region on chromosome 17 (Figure 4.1 inset). We placed 11 TRIM or TRIM-like genes on chromosome 17, with 10 of these found in the duck MHC locus. Of the 10 genes found in this region, 7 are full length TRIM proteins and the remaining are TRIM-like genes, most having lost their N-terminal motifs. All MHC-linked TRIM or TRIM-like proteins have a C-terminal PRY-SPRY domain. Similar TRIM genes had been previously identified in the chicken on their corresponding chromosome 16 (the location of the chicken MHC locus) (Ruby et al., 2005; Shiina et al., 2007). We have also previously compared this region in ducks to the same region in chickens and turkeys (Blaine et al., 2015). The newest assembly of the mallard genome allows TRIM10, another of these MHC-linked TRIMs, to be placed in this region. These data support the expansion of a group of related TRIM genes in the MHC region of birds.

An additional, smaller cluster of TRIM genes is located on chromosome 19. The TRIM-like gene RNF135 is located on chromosome 19 in between TRIM25 and TRIM65. This region also includes a related gene, TRIM47. A nucleotide sequence with close similarity to chicken TRIM25L is present in ducks and is located on chromosome 14. However, the duck TRIM25L gene located on chromosome 14 had premature stop codons in the sequence suggesting it may be a pseudogene. Indeed, its annotation on NCBI includes it in the 3′ untranslated region of a separate gene, described as *PPARGC1B (*GeneID: 101790707). Whether this is a result of mistakes in genome assembly/sequencing or gene fusion resulting in loss of function remains to be determined.

We were able to identify TRIM28, TRIM39, RNF39R or FSD1 within the duck transcriptome data, however these genes were not successfully mapped onto any chromosomes within the duck genome. TRIM28 is annotated in both chicken and tufted duck. In chicken, TRIM28 is located on chromosome 31 while in tufted duck it is located on chromosome 32, suggesting it is likely on chromosome 32 in mallard duck. Unfortunately, this chromosome has not yet been assembled for the mallard duck. A homolog for FSD1 is annotated in tufted duck on

chromosome 26. Two genes surrounding FSD1 in the tufted duck, MPND and YJU2, appear on mallard duck chromosome 29, however FSD1 does not appear between these genes in mallard. This suggests either FSD1 is located in an unassembled chromosome in the mallard duck, or the current chromosome 29 in the mallard duck is only partially complete.

4.3.4 Chicken and duck TRIM homologues share clades and common ancestors

To compare the chicken and duck TRIM gene families, we submitted our 57 duck TRIM protein sequences to NCBI blast search. This search yielded 52 chicken TRIM or TRIM-like proteins (Table 4.2). While many of these chicken TRIM proteins shared high percent identities to duck TRIM proteins, there were some TRIM proteins found only in chicken or only in duck**.** The duck TRIM25L nucleotide sequence contains a premature stop codon, which results in a non-protein coding sequence. The chicken TRIM25L sequence codes for a full-length TRIM protein, and is adjacent to an additional TRIM gene missing in ducks, described as MID2L (MID2 being an alternative gene name for TRIM1 in mammals). Closer inspection of the syntenic region of duck chromosome 14 revealed a deletion in that section of the chromosome in the duck. In ducks, TRIM25L is likely a pseudogene, with MID2-L completely missing. Alternatively, the identified deletion could also be due to misassembly, however HMMer searches of our duck transcriptome did not find any homologous MID2L genes. The phylogenetic analysis indicates that TRIM25L and MID2L are a sister group to TRIM25, 47 and 65. Chickens appear to be missing 8 other TRIM genes found in ducks: avTRIM15L, RNF39R, RNF135, TRIM11L, TRIM27L, TRIM39, and TRIM46. We previously noted TRIM27L to be absent in chickens and turkeys (Blaine et al., 2015). The TRIM-like gene RNF135 has previously been described as absent in chicken and quail (Smith et al., 2015; Morris et al., 2020). TRIM11L and TRIM46 both appear to be missing due to deletions in the chicken, as confirmed by synteny. As the gene RNF39R is unannotated and unplaced in the mallard duck genome, it is likely that it is also unannotated and unassembled in the chicken genome and potentially does exist in the chicken.

To inspect the relationships between the duck and chicken TRIM protein repertoires, we aligned all duck and chicken amino acid sequences using MAFFT, then used IQ-tree to build a ML tree using 10,000 Ultrafast bootstrap replications. Most of the duck and chicken TRIM proteins are very close to their homologs on this tree (Figure 4.2). However, the duck and chicken PML paralogs (TRIM19.1 and TRIM19.2) appear to be quite distant from their

homologous counterparts. This could be due to significant divergence in sequences, or misassembly of these sequences during assembly of the genome. AvTRIM15L clusters in closely with the chicken TRIM39.1, however there is low bootstrap support for this relationship. It appears that avTRIM15L, duck TRIM11L and chicken TRIM39.1 all share common ancestry.

4.3.5 Subfamily classification of human, duck and chicken TRIM proteins

To compare the duck TRIM repertoire to human and chicken, we grouped the subfamilies of TRIMs by the standard C-terminal domain nomenclature (Short et al., 2006; Ozato et al., 2008; Watanabe et al., 2017), while other nomenclature has been proposed (Sardiello et al., 2008; Marín, 2012), this remains the most standard for classifying TRIM subgroups. Many TRIM proteins are shared between human, chickens, and ducks there are some notable repertoire differences between species (Figure 4.3). All TRIM subfamilies are represented in the duck and chicken TRIM proteins.

The C-IV subfamily is highly expanded in humans when compared to chickens and ducks. While humans have a cluster of MHC-linked TRIMs including RNF39, TRIM39, 31, 40 10, 15, 26, 39 along with two singletons (TRIM 38 and 27) (Jia et al., 2021), ducks and chickens appear to have duplications of TRIM7, 10, 27, 39 and 41. An entire cluster of human C-IV family TRIM genes on chromosome 11, including TRIM5, 6, 22 and 34 appear to be absent from avian genomes. Humans and ducks have RNF135, which belongs in the C-IV subfamily and it is missing in chickens. Chickens, however, have other members of the TRIM25 lineage as they have TRIM25L and MID2L. Duck TRIM25L was not added to this table as it may be a pseudogene. Several members of the C-V family are not present in birds, however, both duck and chicken have two TRIM19 genes.

Ducks and chickens have an additional member of the C-XI subfamily, TRIM59L. The C-XI subfamily is classified by the presence of a C-terminal transmembrane domain, however both duck TRIM59L and TRIM13 appear to be missing this domain.

4.3.6 Birds, reptiles and mammals have independent expansions of MHC-linked TRIM genes

To examine the expansion within the C-IV family in higher-order vertebrates (reptiles, birds and mammals) we mined the NCBI protein database for representative TRIM genes from the C-IV subfamily. We assembled protein sequences from human, mouse, marsupials, birds and reptiles. We aligned these sequences and inferred a ML phylogenetic tree using IQTree with

1000 bootstrap replications (Figure 4.4). We combined external nodes when all members of the clade were clear orthologs and labelled these branches by the species or group of higher-order vertebrates. Many TRIM C-IV subfamily genes are conserved in all vertebrate species, while some are present in only mammals, and some unique to birds and/or reptiles. The C-IV subfamily broadly splits into three separate clades. The first clade contains many TRIM proteins present in all animals sampled, including TRIM14/25/47/65 and RNF135. There are some lineage specific TRIM proteins in this clade, with TRIM16 orthologs appearing only in mammals, TRIM25L and MID2L (proteins we found present in chicken but not ducks) appearing in other birds and some reptiles and one specific TRIM25-like protein present only in lizards. Notably, RNF135 is not present in the Galliformes but is found in birds other than duck, reptiles and mammals. The second clade contains TRIM35/62/69 and 72. TRIM35/50 and 62 are found in all higher-order vertebrates sampled, while TRIM72 orthologs appear to be present only in mammals and TRIM69 is limited to eutherian (placental) mammals.

The most divergent TRIM proteins cluster within the third major clade. Many of these TRIM proteins are known to have expanded in eutherian mammals such as the expansion of the TRIM5/6/22/34 locus (Sawyer et al., 2007; Hattlmann et al., 2012; Fernandes et al., 2022). The human MHC-linked TRIM proteins, TRIM10/15/26/21 cluster with orthologs from both mouse and marsupial. Interestingly, the avian and reptilian MHC-linked TRIM proteins (in duck: TRIM7.1/7.2/10/10L/27.1/27.2/27L) do not cluster as closely. Lizard MHC-linked proteins cluster closely together, as do most of the reptilian and avian TRIM proteins. It appears expansions of the diapsid MHC-linked TRIM genes happened more independently between diapsid species than between mammalian species. The exception to this is TRIM7.2, TRIM27L and diapsid TRIM11 (not to be confused with mammalian TRIM11, which does not share direct ancestry with diapsid TRIM11). Clear orthologs of TRIM7.2 are present in birds, lizards and turtles, while orthologs of TRIM27L are present in birds (excluding Galliformes) and lizards. Diapsid TRIM11 appears in both lizards and turtles, and a single avian ortholog was found in the Kiwi (*Apteryx rowi*). While our results suggest that the expansion of diapsid MHC-linked genes diverged from an ancestor of TRIM7, the bootstrap values are much too low to be conclusive. Our phylogenetic analysis of the C-IV TRIM proteins also suggests that the current methodology of naming non-mammalian TRIM orthologs is insufficient for adequately describing them. For example, neither TRIM39.1 and TRIM39.2 in birds clusters with TRIM39 orthologs from

vertebrates. Indeed, TRIM39.1 and TRIM39.2 appear to be derived from a shared common ancestor of the MHC-linked avian TRIM genes.

To compare the chromosomal regions containing the MHC-linked TRIM genes and the TRIM25 locus genes between species, we generated maps of chromosomal locations (Figure 4.5). We compared the MHC-linked TRIM genes of mallard duck, tufted duck and chicken (Figure 4.5A). Notably, tufted duck and chicken both contain the TRIM39.1/BR TRIM-like gene, while this gene appears to be missing in mallard duck. We can find the corresponding sequence of TRIM39.1/BR in the 3'-UTR of TRIM27.1 in the mallard duck, which suggests a misassembly. However, there are no detectable transcripts of this gene expressed in any mallard tissues, indicating it may no longer be functionally expressed in ducks. There is no evidence of TRIM27L in chickens while the gene is present in mallard and tufted duck. The TRIM25 locus has gone through a significant rearrangement in the chicken when compared to the mallard duck (Figure 4.5B). In the duck, genes are arranged TRIM25/ RNF135/TRIM65/TRIM47, while in the chicken they appear in the gene order of TRIM65/47/25 in the chicken. RNF135 is documented as missing in chicken (Magor et al., 2013; Smith et al., 2015) and quail genomes (Morris et al., 2020), but can be found in birds such as duck, corvids, raptors and songbirds (NCBI search). The deletion of chicken RNF135 appears to be independent of the rearrangement as only RNF135 is missing, however the surrounding genes ADAP2 and RHOT1 are still present in the chicken.

4.3.7 Duck TRIM proteins cluster within TRIM subfamilies

To inspect relatedness in the duck TRIM protein repertoire, we aligned all duck amino acid sequences using MAFFT and generated a ML tree using IQTree. We colour coded the tree based on the subfamily assigned by the C-terminal domain possessed by each TRIM protein (Figure 4.6A). TRIM genes segregate into two clades, with one clade largely composed of the C-IV genes. The C-IV subfamily, notably diversified in vertebrates and often associated with immune responses in mammals, is expanded in ducks, with the MHC-linked TRIM proteins located on chromosome 17 (TRIM7.1, 7.2, 10, 10L, 27.1, 27.2, 27L, 39.2 and 41) all segregating within the same clade. Interestingly diaTRIM58 (located on chromosome 20), avTRIM15L (currently unplaced) and TRIM39 (currently unplaced) also cluster with the MHC-linked TRIM proteins.

Within the other major clade, many of the subfamilies are represented, which supports the hypothesis that these subfamilies originated from a common ancestral TRIM gene. Typically,

members of a subfamily group together in a clade. Subfamilies C-I and C-II are related, since they segregate to the same clades. A notable exception are members of subfamily C-V, which do not share clades and are quite distant from each other. This is perhaps not unexpected as the C-V subfamily is classified as RING-B-BOX-CC domain containing TRIM proteins with unclassified C-terminal regions. Additionally, the TRIM-like gene NHLRC1 and TRIM32 do not share a clade with the other C-VII subfamily members, suggesting they could have arisen through exon shuffling events independently from the other C-VII family members TRIM2, 3 and 71 We only included TRIM or TRIM-like genes in this analysis that coded for full length proteins. TRIM25L had premature stop codons throughout the sequence and as such, was excluded.

To infer which duck TRIM proteins may have similar functions we generated a minimum spanning network (MSN) (Figure 4.6B), which connects protein sequences (nodes) based on the distance between proteins without inferring ancestry and can infer shared function between these proteins (Keiser et al., 2007). Members of the C-IV family group closely together, as do members of the C-VI subfamily. Many of the C-V members are quite distant in the MSN, while most of the other subfamily members cluster closely together or branch off from each other. TRIM proteins from the TRIM25/RNF135/TRIM47/TRIM65 expansion also show interesting branching patterns. TRIM25, RNF135 and TRIM47 all share a branch and close distance, while TRIM65 is more distant and branches with TRIM14. TRIM25, RNF135 both augment interferon signaling through ubiquitination of RIG-I, while TRIM65 augments interferon signaling through a similar modification of the RIG-I related helicase MDA5 (Kato et al., 2021). No function has yet been assigned to TRIM47. TRIM11L and avTRIM15L, both found in duck but appear to be missing in chicken, also share a more distant branch. TRIM27L and RNF135, which both appear to be missing in chickens, also branch off of each other.

4.3.6 While most duck TRIM genes are ubiquitously expressed, some show tissue specific patterning

To visualize expression of TRIM genes in duck tissues, we mined the NCBI SRA database for RNA-seq reads from various tissues and aligned these reads to our 57 TRIM or TRIM-like sequences. The MDS plots measured the leading log2(FC) of TRIM expression of each tissue in four dimensions and demonstrate that the types of tissues generally clustered together, however this was more dependent on what dimensions were visualized (Figure 4.7). When comparing the leading log2(FC) in dimensions 4 and 2 one lung sample is an outlier and

clusters with the spleen samples (Figure 4.7A). However, when we inspect dimensions 1 and 3 (Figure 4.7B) lung samples no longer overlap between the spleen, but there is more overlap between lung, fibroblast and adipose. These differences are likely due to many TRIM genes being more ubiquitously expressed, as well as experimental differences between samples.

To visualize abundance and relative expression of TRIM genes in each duck tissue we generated heatmaps **(**Figure 4.8**).** While many of the 57 duck TRIM genes are ubiquitously expressed in the tissues sampled, with varying levels of read counts per gene, there are some which are more specific **(**Figure 4.8A**).** TRIM10, 54, 55, 63, FSD2 and RNF207 have many more reads mapped to muscle than to other tissues. TRIM36 and TRIM42 were abundantly expressed in testis. TRIM9 appears to be predominantly expressed in brain tissues. TRIM7.2 and 41, two of the MHC-linked TRIM genes had high read counts in all tissues sampled. In contrast, TRIM29 had very few reads counted in any tissues sampled. Although TRIM25L encoded a sequence with a premature stop codon, it was still expressed in many tissues and exhibited more tissue specificity, as no reads were aligned from ovary or fibroblast tissue samples.

To determine relative expression of TRIM genes sampled in each tissue, we used EdgeR to compare expression of TRIM genes from each tissue to the average expression in all samples. The resulting heatmap visualizes the relative expression of TRIM genes in each tissue and demonstrates some of the more subtle differences in TRIM gene expression between the tissues. The expression patterns of TRIM genes in muscle remains the most unique while the remaining tissue expression patterns are more related. Hierarchical clustering demonstrates that the expression patterning of TRIM genes separates into three clades. The first clade contains adipose, lung, liver, spleen and intestine, with spleen and intestine sharing more similarities in expression patterns than adipose, lung and liver. The second major clade finds testes, brain, fibroblasts and ovaries being closer in expression patterning. The third clade contains muscle alone, as it has the most unique relative TRIM expression when compared to the other tissues.

We summarized the 5 highest and lowest relatively expressed TRIM genes in each duck tissue analyzed (Figure 4.9). As the C-IV subfamily of genes are often associated with inflammation and immune responses, perhaps unsurprisingly, immune relevant tissues such as lung, spleen and intestine had a predominance of C-IV family members expressed at a higher level than in other tissues. Interestingly, many of the MHC-linked genes also have higher relative expression in these immune relevant tissues. TRIM27.1, 27.2 and 39.1 have higher relative

expression in lung. TRIM27.2 and 39.2 have higher relative expression in the spleen. TRIM7.1, 27.2 and 39.2 have higher relative expression in the intestine. We also noted that in immune privileged tissues such as brain and gonads, the C-IV subfamily genes are among the least expressed. Fibroblasts unexpectantly had many C-IV family members which were much less relatively expressed than in other tissues, these expression patterns could be because TRIM genes are often differently regulated during development than during adulthood and most duck fibroblast cells used in experiments are from embryos.

4.4 Discussion

Here we identify TRIM and TRIM-like genes in the domestic mallard duck by mining data available on the SRA, gene and protein NCBI databases, generating and searching a *de novo* transcriptome assembly of ducks. From these searches we found 57 TRIM or TRIM-like genes in the duck, which we classified by their C-terminal domains and compared them to mammalian and chicken TRIM repertoires. Most duck TRIM proteins are similar to their chicken homologues. The duck TRIM sequences were aligned, and phylogenetic relationships between the TRIM proteins were inferred. Phylogenetic analyses show expansion of the C-IV TRIM family with PRY-SPRY domains. Finally, we aligned RNA-seq reads from different duck tissues to the TRIM or TRIM-like gene sequences to determine both abundance and relative expression levels of these TRIMs in spleen, lung, intestine, fibroblasts, adipose, brain, ovary, testis, muscle and liver tissues.

To our knowledge only one study has attempted to classify any TRIM family genes in birds. Sardiello and colleagues originally listed 37 chicken TRIM genes during their analysis of the evolution of group I and group II TRIM genes in vertebrates (Sardiello et al., 2008). Other reports on avian TRIM genes characterized the expanded B30.2/PRY-SPRY TRIM genes in chickens (Ruby et al., 2005; Shiina et al., 2007), turkey (Chaves et al., 2009) and ducks (Blaine et al., 2015). Outside of birds, there has also been lineage specific expansions of B30.2/PRY-SPRY TRIM genes noted in fish (van der Aa et al., 2009). Teleost fish have three separate expansions of B30.2/PRY-SPRY TRIM genes, with some specific to teleost fish as they are not found in amphibians, birds or mammals. A study from 2011 documented 208 TRIM genes in zebrafish (*Danio rerio*) and 67 in pufferfish (*Tetraodon nigroviridis*) (Boudinot et al., 2011), while a recent study found grass carp (*Ctenopharyngodon idella*) to have 42 TRIM genes (Qin et

al., 2021). The differences in TRIM gene repertoire numbers in fish are likely due to the whole genome duplication events (Glasauer et al., 2014).

From our data mining and transcriptome inspection we found 57 TRIM or TRIM-like genes in the duck. Fifty-three of these TRIM genes could be found on their respective chromosomal locations in the NCBI Pekin duck genome (assembly ZJU1.0). The duck TRIM gene repertoire spans 21 separate chromosomes, suggesting many of these genes have evolved independently from each other. Similar expansions can also be seen in human (Sardiello et al., 2008) and fish (van der Aa et al., 2009; Boudinot et al., 2011; Qin et al., 2021). As previously described, there is an expansion of PRY-SPRY containing TRIM genes within the MHC-B locus on chromosome 17 in the duck (Blaine et al., 2015). The human MHC-linked TRIM genes are tightly linked and include TRIM10, 15, 26, 31, 39, 40 and RNF39 while TRIM27 and 38 are telomeric to this region (Meyer et al., 2003). In mice TRIM27 and 38 are on chromosome 13 in the A3.1 region, while TRIM10, 15, 26, 31, 39, 40 and RNF39 are located on chromosome 17 in the B1 region (Jia et al., 2021). In chickens, the MHC-B locus is located on chromosome 16 (Ruby et al., 2005; Shiina et al., 2007; Kaufman, 2022) and contains many of the same TRIM genes found in ducks in the syntenic organization on chromosome 17 including TRIM 7.1, 7.2, 10, 10L, 27.1, 27.2, 39.2 and 41 (Ruby et al., 2005; Shiina et al., 2007; Blaine et al., 2015; Kaufman, 2022). One notable difference is TRIM27L, which appears missing in chickens and turkeys (Blaine et al., 2015), but is present in many other birds (Blaine et al., 2015). TRIM39.1/BR is present in chickens, but we could not find evidence of a coding gene in mallard ducks. Interestingly this gene is annotated in tufted ducks and shares synteny with the chicken TRIM39.1/BR gene. Thus, it appears that the TRIM genes associated with MHC can change over time (Kaufman, 2022). Indeed, we demonstrate that some of the MHC-linked genes have changed significantly between reptiles, birds and mammals. This is especially interesting as the MHC-linked TRIM genes in humans are associated with regulating inflammatory responses (Jia et al., 2021). As TRIM27.1 and TRIM27L in duck have both been characterized as augmenting the RIG-I signaling pathway (Blaine et al., 2015), it is possible that the other avian MHC-linked TRIM genes may have similar function.

Our phylogenetic trees showed ducks and chickens have direct orthologs for 50 TRIM genes, which segregate into clades of related sequences. Ducks have 7 proteins which are not identified in chickens: RNF39R, RNF135, avTRIM15L, TRIM11L, TRIM27L, TRIM39 and

TRIM46. Chickens have two TRIMs, TRIM25L and MID2L, which are either incomplete or missing in ducks. Duck RNF135, chicken TRIM25L and MID2L all fall within the clade containing TRIM25, TRIM47 and TRIM65. No functions have yet been published for TRIM25L and MID2L, however due to their location in this phylogeny they may be able to bind to helicase domains like other members of this clade do in mammals (Kato et al., 2021). TRIM46 also appears to be missing in chickens. TRIM46 is a TRIM protein important in polarization and axon formation in neuronal cells in mammals (van Beuningen et al., 2015), making its absence in chickens especially perplexing. Some avian genes have been notoriously difficult to identify due to high GC content and lack of representation of these regions in genomic libraries (Hron et al., 2015). Indeed, this may be the reason RNF39R is unannotated in the chicken and the duck. The newly identified RNF39L sequence has 72% GC content overall in the mallard duck, making the contig harder to assemble and place in the genome.

Our identification of two closely related PML homologues in ducks and chickens prompted us to search available avian and reptilian genomes, which indicate that birds and reptiles have two paralogs of PML. In mammals TRIM19/PML is a key component of PML nuclear bodies (Lallemand-Breitenbach et al., 2018). PML nuclear bodies regulate many important processes in mammals, such as the DNA damage response, apoptosis and gene expression (Hsu et al., 2016; Hoischen et al., 2018; Kurihara et al., 2020). PML is also involved in immune responses to viruses, by regulating important signaling pathways during infection (El Bougrini et al., 2011; Yan et al., 2021) and thus are targeted by viruses to shut down this signaling (El McHichi et al., 2010; Scherer et al., 2017). PML has not been annotated in any published fish (van der Aa et al., 2009; Boudinot et al., 2011) or amphibian lineages (NCBI). It is unknown if either of PML paralog forms PML nuclear bodies in birds or is an antiviral protein. Birds also appear to be missing TRIM20/PYRIN, a C-V TRIM with a PYRIN domain in the N-terminus. TRIM20 has a proinflammatory role in mammals due it its interactions with the inflammasome component apoptosis-associated speck-like protein containing caspase recruitment domain (ASC) (Yu et al., 2006; Samukawa et al., 2021). We could not find a TRIM20 homolog in ducks in our transcriptome, nor in the available genomes of other birds. Interestingly, a search of our transcriptome and NCBI also did not find an avian ASC protein either, suggesting the “pyrin-inflammasome” may be absent in birds.

Our phylogenetic trees showed most TRIM subfamilies segregate to separate clades. The MHC-linked TRIM proteins all shared a clade, however two avian specific TRIM proteins also clustered in that clade, diaTRIM58 and TRIM11L. These TRIMs potentially translocated to or from the MHC region earlier in vertebrate evolution. The C-IV subfamily, the largest and most diverse of the subfamilies has two minor clades, the first containing mainly the MHC-linked TRIM proteins. The MHC region has rapidly duplicated and expanded throughout vertebrate evolution and many genes in this region are involved in adaptive or innate immune responses (Abi Rached et al., 1999; Flajnik et al., 2001). In chickens, the MHC region was dubbed as the "minimal essential MHC" due to it being much more compact and simpler than MHC regions found in mammals (Kaufman et al., 1995; Kaufman et al., 1999) and our comparisons of this region between tufted duck, mallard duck and chicken demonstrate the chicken MHC region is slightly more condensed than in ducks. In humans, the MHC-linked TRIM genes attenuate innate immune signaling pathways (Jia et al., 2021), thus regulate responses to infection. As the avian MHC-linked TRIM genes are closely linked, they likely have coevolved and may also be involved in innate immunity. TRIM27L and TRIM27.1 in duck both modulate signaling downstream of constitutively active Retinoic acid-inducible gene I (RIG-I) in chicken cells, with TRIM27L increasing IFN-β promoter activity and TRIM27.1 decreasing it (Blaine et al., 2015). In humans, TRIM27 decreases IFNβ production through the inhibition of both NF-κB and IRF3 pathways (Zha et al., 2006). In birds, an ortholog of human TRIM41 was found in the MHC-B locus of chicken, with orthologues later found in the turkey (Chaves et al., 2009) and duck (Blaine et al., 2015) MHC-B locus. Human TRIM41 is not found in the MHC in humans, and instead is located on chromosome 5. The avian TRIM41 was named for sequence similarity to human TRIM41, and our phylogenetic analysis of the C-IV TRIM proteins in mammals, birds and reptiles suggest this protein was named correctly. TRIM41 in humans is known to restrict viral replication by selective targeting and ubiquitination of viral proteins (Patil et al., 2018; Patil et al., 2020) and by augmenting antiviral signaling pathways (Yu et al., 2021). It is unknown if the avian ortholog of TRIM41 is also able to restrict virus.

A second group of duck TRIM genes within C-IV subfamily having the PRY-SPRY domain, include the closely related TRIM25, 47 and 65 and RNF135. Our phylogenies support TRIM14, 25, 47, 65 and RNF135 all originating from the same common ancestral TRIM gene, which is consistent with their mammalian homologues (Kato et al., 2021). In mammals,

RNF135, TRIM14, 25 and 65 bind helicases involved in immune signaling using their PRY-SPRY domains, while TRIM47 function is unknown. RNF135 has been excluded from TRIM repertoires because it does not have the classical RBCC motif instead it has an RCC motif as it is missing the B-box domain. Previous studies performed in our lab could not locate a RING domain in RNF135, and we had suggested this protein would be largely inactive (Magor et al., 2013). However, the recent duck genome, as well as HMMer searches of the sequence obtained from our *de novo* transcriptome indicate that duck RNF135 does indeed have a RING domain. The MST places RNF135, TRIM25, and 47 on the same branch while TRIM65 is branched separately with TRIM14. Interestingly, RNF135 is linked to TRIM27L, although they are still quite distant from each other. RNF135 appears to be missing in chicken (Magor et al., 2013) and Japanese quail (*Coturnix japonica*) (Morris et al., 2020) genomes while TRIM27L is missing in chicken and turkey (Blaine et al., 2015). RIG-I, the cytoplasmic detector of single stranded RNA viruses, also appears missing in chickens (Barber et al., 2010). RIG-I is documented as being ubiquitinated by RNF135/RIPLET in mammals to increase type I interferon signaling during infection (Cadena et al., 2019; Hayman et al., 2019; Oshiumi, 2020; Kato et al., 2021; Kouwaki et al., 2021). TRIM27L can augment the RIG-I signaling pathway when cotransfected with constitutively active RIG-I in chicken cells, however a mechanism for this augmentation of the RIG-I pathway by TRIM27L is still unknown (Blaine et al., 2015). It is perhaps not coincidental that two proteins that interact with the RIG-I signaling pathway in ducks would appear to be missing from avian lineages that also appear to be missing RIG-I.

Many TRIM genes are ubiquitously expressed in all tissues sampled in these experiments; however, some do show tissue specific expression. Muscle tissues in the duck had the most specific expression of TRIM genes of any of the tissues sampled. TRIM54, 55 and 63 are also known as muscle specific ring finger (MURF) genes and as the name suggests are primarily expressed in muscle fibers in mammals (Perera et al., 2012; Uhlen et al., 2015). Similarly, duck muscle tissues highly express TRIM54, 55 and 63. TRIM9, 46 and 67 are all TRIM genes associated with neuronal development and brain tissues in humans (Berti et al., 2002b; Uhlen et al., 2015; van Beuningen et al., 2015; Boyer et al., 2018), and this pattern of higher relative expression in brain tissue is consistent with what we see in the duck. Adipose tissues had the lowest tissue specific expression of any of the duck TRIM genes with only

TRIM1, 18 and 36 showing a slight increase in relative expression compared to other tissues, and none of these genes having the highest relative expression in adipose tissue.

Immune relevant tissues had higher relative expression of the C-IV TRIM subfamily members. TRIM27.1 and TRIM14 had the highest relative expression in lung tissues, while TRIM27.2 and 39.2 had the highest relative expression both spleen and intestinal tissues. These genes are not yet functionally characterized, making them potential candidates for future immunological studies. TRIM genes which encode for immune modulating proteins had much less relative expression in immune privileged sites such as the brain and gonads. TRIM25 for example, which is known to aid in increasing RIG-I signaling during viral infection in mammals (Gack et al., 2007) and ducks (Miranzo-Navarro et al., 2014), had much less relative expression in brain, testis and ovary, where it was reduced. Likewise, the related TRIM65 and RNF135 had much less relative expression in the testis and RNF135 also had very low relative expression in the brain. As previously mentioned, all these genes are involved in regulating intracellular immune responses by way of RIG-I or MDA5 stabilization. It is possible these transcripts are much less expressed in immune privileged sites to prevent unwanted inflammatory responses. Ducks have high relative expression of TRIM14 in both lung and liver. TRIM14 has not been functionally characterized in ducks, however the TRIM14 expression in these tissues is interesting. TRIM14 is an anti-viral protein in mammals which can target multiple viruses for degradation such as hepatitis B virus (Tan et al., 2018) and influenza A virus (Wu et al., 2019). Ducks act as the natural host and reservoir to influenza A virus (Webster et al., 1978; Webster et al., 1992; Taubenberger et al., 2010), and as such usually have reduced symptoms when infected with low pathogenic strains (Webster et al., 1978; Daoust et al., 2011). Highly pathogenic strains of IAV however, replicate in the lungs of infected ducks and can cause mass die offs (Sturm-Ramirez et al., 2004; Bingham et al., 2009; Haider et al., 2017). Ducks are also susceptible to duck hepatitis A virus (DHAV), which replicates in the liver and results in liver damage and mortality in young ducks (Liu et al., 2019; Niu et al., 2019; Hisham et al., 2020). It is possible that the higher expression of TRIM14 in lung and liver is a protective mechanism that allows ducks to respond quicker to viral infections from RNA viruses such as IAV and DHV.

Some TRIM genes are notably absent in birds as they were not found in our genome searches or transcriptome. These include the cluster of PRY-SPRY genes located on chromosome 11 in humans, including TRIM6, TRIM5, 22 and 34 (Sawyer et al., 2007). Many of

these genes have direct antiviral activities, first noted in TRIM5α, shown to restrict HIV in non-human primates (Stremlau et al., 2004; Wagner et al., 2016). TRIM22 is known to restrict influenza virus in mammals (Di Pietro et al., 2013). These genes have undergone expansion and contraction in the mammalian genome, presumably in response to pathogen pressures (Sawyer et al., 2007).

We cannot rule out the possibility that more TRIM genes are present but have greatly diverged and thus were not picked up by our searches. The incompleteness of the duck genome paired with an incomplete set of tissues to use for *de novo* assembly, leaves the possibility that we have missed some genes. Notably our study is lacking tissue from the heart, stomach, pancreas and bursa. If these tissues have tissue specific expression of TRIM genes, we likely would not be able to find them in our transcriptome. Also, TRIM genes are highly regulated during development, and thus screening embryonic tissues at various stages of development might help to classify TRIM genes predominantly expressed during development that would otherwise be rare.

Throughout our analysis of avian TRIM genes, the issue of mis- or improper annotations arose continuously. Many of the avian TRIM genes are assigned locus numbers, and have computer generated descriptions rather than proper annotations. Additionally, many of these gene names are used to describe multiple independent TRIM genes (ie. TRIM39L, RFPL, etc). Care should be taken not to take pipeline assigned gene names as proper annotations, especially in lower vertebrates, as these gene names are assigned on most similar human ortholog, which often time is a TRIM gene of low similarity. When new TRIM genes in lower vertebrates are identified, phylogenetic analysis against repertoires of other species should be performed to assure the proper annotation is assigned to the TRIM. Additionally, annotating TRIM genes as "TRIM#-L" is not informative, and should only be used in automated generations not once a gene is characterized. Instead, if the TRIM gene does not have evidence of a direct ortholog already annotated in other species through phylogenies, it should be assigned a new TRIM number. As many of the TRIM100 genes have been claimed by fish TRIM genes or randomly assigned to human TRIM pseudogenes, new TRIM genes should be numbered TRIM200 and on. As genomic data becomes more readily available these annotations can be changed and adjusted to match orthologs in other species.

Our results found 57 TRIM or TRIM-like genes in the duck, with evidence that one of these genes is a TRIM-like pseudogene. We also found key differences between the duck and chicken TRIM gene repertoires that shed light on the complex and understudied mechanics of TRIM gene evolution. Most of the genes documented in this study have not been functionally characterized or cloned. Our results highlight several TRIM proteins that could be studied for antiviral function. This is the first major study in TRIM gene classification in birds, wherein species of bird were compared. As we found several differences between chicken and ducks the inclusion of additional species of birds not in the Galloanserae clade, such as songbirds or raptors, would help detail more avian-specific evolution of TRIM genes. Additionally, as more complete genomes in other vertebrates are sequenced and become publicly available, we will be able to better trace the expansions and deletions of TRIM genes in the vertebrate lineages.

4.5 References

Abi Rached, L., McDermott, M.F., and Pontarotti, P. (1999). The MHC big bang. *Immunol Rev* 167**,** 33-44. doi: 10.1111/j.1600-065x.1999.tb01380.x.

Andrews, S. (2010). "FASTQC. A quality control tool for high throughput sequence data".

Barber, M.R., Aldridge, J.R., Jr., Webster, R.G., and Magor, K.E. (2010). Association of RIG-I with innate immunity of ducks to influenza. *Proc Natl Acad Sci U S A* 107(13)**,** 5913-5918. doi: 10.1073/pnas.1001755107.

Bell, J.L., Malyukova, A., Holien, J.K., Koach, J., Parker, M.W., Kavallaris, M., et al. (2012). TRIM16 acts as an E3 ubiquitin ligase and can heterodimerize with other TRIM family members. *PLoS One* 7(5)**,** e37470.

Ben, L., and Steven, L.S. (2011). Fast gapped-read alignment with Bowtie 2. 1-24. doi: 10.1002/0471250953.bi1107s32.Aligning.

Berti, C., Messali, S., Ballabio, A., Reymond, A., and Meroni, G. (2002a). TRIM9 is specifically expressed in the embryonic and adult nervous system. *Mechanisms of Development* 113(2)**,** 159-162. doi: https://doi.org/10.1016/S0925-4773(02)00013-8.

Bingham, J., Green, D.J., Lowther, S., Klippel, J., Burggraaf, S., Anderson, D.E., et al. (2009). Infection studies with two highly pathogenic avian influenza strains (Vietnamese and Indonesian) in Pekin ducks (Anas platyrhynchos), with particular reference to clinical

disease, tissue tropism and viral shedding. *Avian Pathol* 38(4)**,** 267-278. doi: 10.1080/03079450903055371.

Birol, I., Jackman, S.D., Nielsen, C.B., Qian, J.Q., Varhol, R., Stazyk, G., et al. (2009). De novo transcriptome assembly with ABySS. *Bioinformatics* 25(21)**,** 2872-2877. doi: 10.1093/bioinformatics/btp367.

Blaine, A.H., Miranzo-Navarro, D., Campbell, L.K., Aldridge, J.R., Jr., Webster, R.G., and Magor, K.E. (2015). Duck TRIM27-L enhances MAVS signaling and is absent in chickens and turkeys. *Mol Immunol* 67(2 Pt B)**,** 607-615. doi: 10.1016/j.molimm.2015.07.011.

Bodenhofer, U., Bonatesta, E., Horejš-Kainrath, C., and Hochreiter, S. (2015). msa: an R package for multiple sequence alignment. *Bioinformatics* 31(24)**,** 3997-3999. doi: 10.1093/bioinformatics/btv494.

Bolger, A.M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30(15)**,** 2114-2120. doi: 10.1093/bioinformatics/btu170.

Boudinot, P., van der Aa, L.M., Jouneau, L., Du Pasquier, L., Pontarotti, P., Briolat, V., et al. (2011). Origin and evolution of TRIM proteins: new insights from the complete TRIM repertoire of zebrafish and pufferfish. *PloS one* 6(7)**,** e22022-e22022. doi: 10.1371/journal.pone.0022022.

Boyer, N.P., Monkiewicz, C., Menon, S., Moy, S.S., and Gupton, S.L. (2018). Mammalian TRIM67 functions in brain development and behavior. *eNeuro* 5(3). doi: 10.1523/eneuro.0186-18.2018.

Cadena, C., Ahmad, S., Xavier, A., Willemsen, J., Park, S., Park, J.W., et al. (2019). Ubiquitin-dependent and -independent roles of E3 ligase RIPLET in innate immunity. *Cell* 177(5)**,** 1187-1200.e1116. doi: 10.1016/j.cell.2019.03.017.

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., et al. (2009). BLAST+: architecture and applications. *BMC Bioinformatics* 10**,** 421. doi: 10.1186/1471-2105-10-421.

Carthagena, L., Bergamaschi, A., Luna, J.M., David, A., Uchil, P.D., Margottin-Goguet, F., et al. (2009). Human TRIM gene expression in response to interferons. *PLoS One* 4(3)**,** e4894. doi: 10.1371/journal.pone.0004894.

Charif, D., and Lobry, J.R. (2007). "SeqinR 1.0-2: A contributed package to the R project for statistical computing devoted to biological sequences retrieval and analysis," in *Structural Approaches to Sequence Evolution: Molecules, Networks, Populations,* eds. U. Bastolla, M. Porto, H.E. Roman & M. Vendruscolo. (Berlin, Heidelberg: Springer Berlin Heidelberg), 207-232.

Chaves, L.D., Krueth, S.B., and Reed, K.M. (2009). Defining the turkey MHC: sequence and genes of the B locus. *J Immunol* 183(10)**,** 6530-6537. doi: 10.4049/jimmunol.0901310.

Choudhury, N.R., Heikel, G., Trubitsyna, M., Kubik, P., Nowak, J.S., Webb, S., et al. (2017). RNA-binding activity of TRIM25 is mediated by its PRY/SPRY domain and is required for ubiquitination. *BMC Biol* 15(1)**,** 105. doi: 10.1186/s12915-017-0444-9.

Cui, H., Liu, Y., and Huang, Y. (2017). Roles of TRIM32 in corneal epithelial cells after infection with herpes simplex virus. *Cell Physiol Biochem* 43(2)**,** 801-811. doi: 10.1159/000481563.

D'Cruz, A.A., Babon, J.J., Norton, R.S., Nicola, N.A., and Nicholson, S.E. (2013). Structure and function of the SPRY/B30.2 domain proteins involved in innate immunity. *Protein Sci* 22(1)**,** 1-10. doi: 10.1002/pro.2185.

Daoust, P.Y., Kibenge, F.S., Fouchier, R.A., van de Bildt, M.W., van Riel, D., and Kuiken, T. (2011). Replication of low pathogenic avian influenza virus in naturally infected Mallard ducks (Anas platyrhynchos) causes no morphologic lesions. *J Wildl Dis* 47(2)**,** 401-409. doi: 10.7589/0090-3558-47.2.401.

Di Pietro, A., Kajaste-Rudnitski, A., Oteiza, A., Nicora, L., Towers, G.J., Mechti, N., et al. (2013). TRIM22 inhibits influenza A virus infection by targeting the viral nucleoprotein for degradation. *J Virol* 87(8)**,** 4523-4533. doi: 10.1128/JVI.02548-12.

Eisen, M.B., Spellman, P.T., Brown, P.O., and Botstein, D. (1998). Cluster analysis and display of genome-wide expression patterns. *Proceedings of the National Academy of Sciences* 95(25)**,** 14863.

El Bougrini, J., Dianoux, L., and Chelbi-Alix, M.K. (2011). PML positively regulates interferon gamma signaling. *Biochimie* 93(3)**,** 389-398. doi: 10.1016/j.biochi.2010.11.005.

El McHichi, B., Regad, T., Maroui, M.-A., Rodriguez, M.S., Aminev, A., Gerbaud, S., et al. (2010). SUMOylation promotes PML degradation during encephalomyocarditis virus infection. *Journal of virology* 84(22)**,** 11634-11645. doi: 10.1128/JVI.01321-10.

Feng, Z.Q., Cheng, Y., Yang, H.L., Zhu, Q., Yu, D., and Liu, Y.P. (2015). Molecular characterization, tissue distribution and expression analysis of TRIM25 in Gallus gallus domesticus. *Gene* 561(1)**,** 138-147. doi: 10.1016/j.gene.2015.02.025.

Fernandes, A.P., Águeda-Pinto, A., Pinheiro, A., Rebelo, H., and Esteves, P.J. (2022). Evolution of TRIM5 and TRIM22 in Bats Reveals a Complex Duplication Process. *Viruses* 14(2). doi: 10.3390/v14020345.

Finn, R.D., Clements, J., and Eddy, S.R. (2011). HMMER web server: interactive sequence similarity searching. *Nucleic Acids Res* 39(Web Server issue)**,** W29-37. doi: 10.1093/nar/gkr367.

Flajnik, M.F., and Kasahara, M. (2001). Comparative genomics of the MHC: glimpses into the evolution of the adaptive immune system. *Immunity* 15(3)**,** 351-362. doi: 10.1016/s1074-7613(01)00198-4.

Fu, B., Wang, L., Ding, H., Schwamborn, J.C., Li, S., and Dorf, M.E. (2015). TRIM32 senses and restricts influenza A virus by ubiquitination of PB1 polymerase. *PLoS Pathog* 11(6)**,** e1004960. doi: 10.1371/journal.ppat.1004960.

Gack, M.U., Shin, Y.C., Joo, C.H., Urano, T., Liang, C., Sun, L., et al. (2007). TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity. *Nature* 446(7138)**,** 916-920. doi: 10.1038/nature05732.

Gel, B., and Serra, E. (2017). karyoploteR: an R/Bioconductor package to plot customizable genomes displaying arbitrary data. *Bioinformatics* 33(19)**,** 3088-3090. doi: 10.1093/bioinformatics/btx346.

Glasauer, S.M., and Neuhauss, S.C. (2014). Whole-genome duplication in teleost fishes and its evolutionary consequences. *Mol Genet Genomics* 289(6)**,** 1045-1060. doi: 10.1007/s00438-014-0889-2.

Gregory R. Warnes, B.B., Lodewijk Bonebakker, Robert Gentleman, Wolfgang Huber, Andy Liaw, Thomas Lumley, Martin Maechler, Arni Magnusson, Steffen Moeller, Marc Schwartz and Bill Venables (2022). "gplots: Various R Programming Tools for Plotting Data"

Guo, X., He, X.X., Chen, H., Wang, Z.C., Li, H.F., Wang, J.X., et al. (2021). Revisiting the evolutionary history of domestic and wild ducks based on genomic analyses. *Zool Res* 42(1)**,** 43-50. doi: 10.24272/j.issn.2095-8137.2020.133.

Haider, N., Sturm-Ramirez, K., Khan, S.U., Rahman, M.Z., Sarkar, S., Poh, M.K., et al. (2017). Unusually high mortality in waterfowl caused by highly pathogenic avian influenza A(H5N1) in Bangladesh. *Transbound Emerg Dis* 64(1)**,** 144-156. doi: 10.1111/tbed.12354.

Han, X., Du, H., and Massiah, M.A. (2011). Detection and characterization of the in vitro e3 ligase activity of the human MID1 protein. *J Mol Biol* 407(4)**,** 505-520. doi: 10.1016/j.jmb.2011.01.048.

Hatakeyama, S. (2011). TRIM proteins and cancer. *Nat Rev Cancer* 11(11)**,** 792-804. doi: 10.1038/nrc3139.

Hatakeyama, S. (2017). TRIM family proteins: Roles in autophagy, immunity, and carcinogenesis. *Trends Biochem Sci* 42(4)**,** 297-311. doi: 10.1016/j.tibs.2017.01.002.

Hattlmann, C.J., Kelly, J.N., and Barr, S.D. (2012). TRIM22: A diverse and dynamic antiviral protein. *Mol Biol Int* 2012**,** 153415. doi: 10.1155/2012/153415.

Hayman, T.J., Hsu, A.C., Kolesnik, T.B., Dagley, L.F., Willemsen, J., Tate, M.D., et al. (2019). RIPLET, and not TRIM25, is required for endogenous RIG-I-dependent antiviral responses. *Immunology & Cell Biology* 97(9)**,** 840-852. doi: https://doi.org/10.1111/imcb.12284.

Hisham, I., Ellakany, H.F., Selim, A.A., Abdalla, M.A.M., Zain El-Abideen, M.A., Kilany, W.H., et al. (2020). Comparative pathogenicity of duck hepatitis A virus-1 isolates in experimentally infected Pekin and muscovy ducklings. *Frontiers in Veterinary Science* 7.

Hoischen, C., Monajembashi, S., Weisshart, K., and Hemmerich, P. (2018). Multimodal light microscopy approaches to reveal structural and functional properties of promyelocytic leukemia nuclear bodies. *Frontiers in Oncology* 8.

Hron, T., Pajer, P., Paces, J., Bartunek, P., and Elleder, D. (2015). Hidden genes in birds. *Genome Biol* 16(1)**,** 164. doi: 10.1186/s13059-015-0724-z.

Hsu, K.S., Guan, B.J., Cheng, X., Guan, D., Lam, M., Hatzoglou, M., et al. (2016). Translational control of PML contributes to TNFα-induced apoptosis of MCF7 breast cancer cells and decreased angiogenesis in HUVECs. *Cell Death Differ* 23(3)**,** 469-483. doi: 10.1038/cdd.2015.114.

James, L.C., Keeble, A.H., Khan, Z., Rhodes, D.A., and Trowsdale, J. (2007). Structural basis for PRYSPRY-mediated tripartite motif (TRIM) protein function. *Proceedings of the National Academy of Sciences* 104(15)**,** 6200. doi: 10.1073/pnas.0609174104.

Jia, X., Zhao, C., and Zhao, W. (2021). Emerging roles of MHC class I region-encoded E3 ubiquitin ligases in innate immunity. *Frontiers in Immunology* 12. doi: 10.3389/fimmu.2021.687102.

Joazeiro, C.A., and Weissman, A.M. (2000). RING finger proteins: mediators of ubiquitin ligase activity. *Cell* 102(5)**,** 549-552. doi: 10.1016/s0092-8674(00)00077-5.

Kaikai, H., Zhao, D., Liu, Y., Liu, Q., Huang, X., Yang, J., et al. (2021). The E3 ubiquitin ligase TRIM25 inhibits Tembusu virus replication in vitro. *Front Vet Sci* 8**,** 722113. doi: 10.3389/fvets.2021.722113.

Kalyaanamoorthy, S., Minh, B.Q., Wong, T.K.F., von Haeseler, A., and Jermiin, L.S. (2017). ModelFinder: fast model selection for accurate phylogenetic estimates. *Nature Methods* 14(6)**,** 587-589. doi: 10.1038/nmeth.4285.

Kato, K., Ahmad, S., Zhu, Z., Young, J.M., Mu, X., Park, S., et al. (2021). Structural analysis of RIG-I-like receptors reveals ancient rules of engagement between diverse RNA helicases and TRIM ubiquitin ligases. *Mol Cell* 81(3)**,** 599-613.e598. doi: 10.1016/j.molcel.2020.11.047.

Katoh, K., Rozewicki, J., and Yamada, K.D. (2019). MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. *Briefings in Bioinformatics* 20(4)**,** 1160-1166. doi: 10.1093/bib/bbx108.

Kaufman, J. (2022). Innate immune genes of the chicken MHC and related regions. *Immunogenetics* 74(1)**,** 167-177. doi: 10.1007/s00251-021-01229-2.

Kaufman, J., Milne, S., Göbel, T.W.F., Walker, B.A., Jacob, J.P., Auffray, C., et al. (1999). The chicken B locus is a minimal essential major histocompatibility complex. *Nature* 401(6756)**,** 923-925. doi: 10.1038/44856.

Kaufman, J., Völk, H., and Wallny, H.J. (1995). A "minimal essential Mhc" and an "unrecognized Mhc": two extremes in selection for polymorphism. *Immunol Rev* 143**,** 63-88. doi: 10.1111/j.1600-065x.1995.tb00670.x.

Keiser, M.J., Roth, B.L., Armbruster, B.N., Ernsberger, P., Irwin, J.J., and Shoichet, B.K. (2007). Relating protein pharmacology by ligand chemistry. *Nature Biotechnology* 25(2)**,** 197-206. doi: 10.1038/nbt1284.

Kim, J.-K., Negovetich, N.J., Forrest, H.L., and Webster, R.G. (2009). Ducks: the "Trojan horses" of H5N1 influenza. *Influenza and other respiratory viruses* 3(4)**,** 121-128. doi: 10.1111/j.1750-2659.2009.00084.x.

Kouwaki, T., Nishimura, T., Wang, G., Nakagawa, R., and Oshiumi, H. (2021). Ubiquitin ligase RIPLET mediates polyubiquitination of RIG-I and LGP2 and regulates the innate immune responses to SARS-CoV-2 infection. *bioRxiv***,** 2021.2001.2025.428042. doi: 10.1101/2021.01.25.428042.

Kurihara, M., Kato, K., Sanbo, C., Shigenobu, S., Ohkawa, Y., Fuchigami, T., et al. (2020). Genomic profiling by ALaP-Seq reveals transcriptional regulation by PML bodies through DNMT3A exclusion. *Mol Cell* 78(3)**,** 493-505.e498. doi: 10.1016/j.molcel.2020.04.004.

Lallemand-Breitenbach, V., and de Thé, H. (2018). PML nuclear bodies: from architecture to function. *Curr Opin Cell Biol* 52**,** 154-161. doi: 10.1016/j.ceb.2018.03.011.

Letunic, I., Doerks, T., and Bork, P. (2015). SMART: recent updates, new developments and status in 2015. *Nucleic Acids Res* 43(Database issue)**,** D257-260. doi: 10.1093/nar/gku949.

Li, H.-F., Zhu, W.-Q., Song, W.-T., Shu, J.-T., Han, W., and Chen, K.-W. (2010). Origin and genetic diversity of Chinese domestic ducks. *Molecular Phylogenetics and Evolution* 57(2)**,** 634-640. doi: https://doi.org/10.1016/j.ympev.2010.07.011.

Li, L., Feng, W., Cheng, Z., Yang, J., Bi, J., Wang, X., et al. (2019a). TRIM62-mediated restriction of avian leukosis virus subgroup J replication is dependent on the SPRY domain. *Poult Sci* 98(11)**,** 6019-6025. doi: 10.3382/ps/pez408.

Li, L., Niu, D., Yang, J., Bi, J., Zhang, L., Cheng, Z., et al. (2020). TRIM62 From chicken as a negative regulator of reticuloendotheliosis virus replication. *Frontiers in Veterinary Science* 7.

Li, T., Hu, X., Zhang, T., Song, X., Zhang, H., Dai, N., et al. (2021). Cloning, analysis, and anti-duck Tembusu virus innate immune response of Cherry Valley duck tripartite motif-containing 32. *Poult Sci* 100(5)**,** 101048. doi: 10.1016/j.psj.2021.101048.

Li, Y., Ma, X., Chen, Z., Wu, H., Wang, P., Wu, W., et al. (2019b). B1 oligomerization regulates PML nuclear body biogenesis and leukemogenesis. *Nature Communications* 10(1)**,** 3789. doi: 10.1038/s41467-019-11746-0.

Liao, Y., Smyth, G.K., and Shi, W. (2014). featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* 30(7)**,** 923-930. doi: 10.1093/bioinformatics/btt656.

Liu, R., Shi, S., Huang, Y., Chen, Z., Chen, C., Cheng, L., et al. (2019). Comparative pathogenicity of different subtypes of duck hepatitis A virus in Pekin ducklings. *Vet Microbiol* 228**,** 181-187. doi: 10.1016/j.vetmic.2018.11.030.

Ma, X., Yang, T., Luo, Y., Wu, L., Jiang, Y., Song, Z., et al. (2019). TRIM28 promotes HIV-1 latency by SUMOylating CDK9 and inhibiting P-TEFb. *Elife* 8. doi: 10.7554/eLife.42426.

Magor, K.E., Miranzo Navarro, D., Barber, M.R., Petkau, K., Fleming-Canepa, X., Blyth, G.A., et al. (2013). Defense genes missing from the flight division. *Dev Comp Immunol* 41(3)**,** 377-388. doi: 10.1016/j.dci.2013.04.010.

Marín, I. (2012). Origin and diversification of TRIM ubiquitin ligases. *PLOS ONE* 7(11)**,** e50030. doi: 10.1371/journal.pone.0050030.

Massiah, M.A. (2019). *Zinc-Binding B-Box Domains with RING Folds Serve Critical Roles in the Protein Ubiquitination Pathways in Plants and Animals.* London: IntechOpen.

Massiah, M.A., Simmons, B.N., Short, K.M., and Cox, T.C. (2006). Solution structure of the RBCC/TRIM B-box1 domain of human MID1: B-box with a RING. *J Mol Biol* 358(2)**,** 532-545. doi: 10.1016/j.jmb.2006.02.009.

Meroni, G. (2012). Genomics and evolution of the TRIM gene family. *Adv Exp Med Biol* 770**,** 1-9. doi: 10.1007/978-1-4614-5398-7_1.

Metzger, M.B., Pruneda, J.N., Klevit, R.E., and Weissman, A.M. (2014). RING-type E3 ligases: master manipulators of E2 ubiquitin-conjugating enzymes and ubiquitination. *Biochim Biophys Acta* 1843(1)**,** 47-60. doi: 10.1016/j.bbamcr.2013.05.026.

Meyer, M., Gaudieri, S., Rhodes, D.A., and Trowsdale, J. (2003). Cluster of TRIM genes in the human MHC class I region sharing the B30.2 domain. *Tissue Antigens* 61(1)**,** 63-71. doi: 10.1034/j.1399-0039.2003.610105.x.

Minh, B.Q., Nguyen, M.A.T., and von Haeseler, A. (2013).). Ultrafast approximation for phylogenetic bootstrap. *Molecular Biology and Evolution* 30(5)**,** 1188-1195. doi: 10.1093/molbev/mst024.

Miranzo-Navarro, D., and Magor, K.E. (2014). Activation of duck RIG-I by TRIM25 is independent of anchored ubiquitin. *PLoS One* 9(1)**,** e86968. doi: 10.1371/journal.pone.0086968.

Morris, K.M., Hindle, M.M., Boitard, S., Burt, D.W., Danner, A.F., Eory, L., et al. (2020). The quail genome: insights into social behaviour, seasonal biology and infectious disease response. *BMC Biol* 18(1)**,** 14. doi: 10.1186/s12915-020-0743-4.

Nguyen, L.-T., Schmidt, H.A., von Haeseler, A., and Minh, B.Q. (2014). IQ-TREE: A Fast and Effective Stochastic Algorithm for Estimating Maximum-Likelihood Phylogenies. *Molecular Biology and Evolution* 32(1)**,** 268-274. doi: 10.1093/molbev/msu300.

Nisole, S., Stoye, J.P., and Saib, A. (2005). TRIM family proteins: retroviral restriction and antiviral defence. *Nat Rev Microbiol* 3(10)**,** 799-808. doi: 10.1038/nrmicro1248.

Niu, Y., Ma, H., Ding, Y., Li, Z., Sun, Y., Li, M., et al. (2019). The pathogenicity of duck hepatitis A virus types 1 and 3 on ducklings. *Poultry Science* 98(12)**,** 6333-6339. doi: https://doi.org/10.3382/ps/pez455.

Oshiumi, H. (2020). Recent advances and contradictions in the study of the individual roles of ubiquitin ligases that regulate RIG-I-like receptor-mediated antiviral innate immune responses. *Front Immunol* 11**,** 1296. doi: 10.3389/fimmu.2020.01296.

Oshiumi, H., Miyashita, M., Inoue, N., Okabe, M., Matsumoto, M., and Seya, T. (2010). The ubiquitin ligase Riplet is essential for RIG-I-dependent innate immune responses to RNA virus infection. *Cell Host Microbe* 8(6)**,** 496-509. doi: 10.1016/j.chom.2010.11.008.

Ozato, K., Shin, D.M., Chang, T.H., and Morse, H.C., 3rd (2008). TRIM family proteins and their emerging roles in innate immunity. *Nat Rev Immunol* 8(11)**,** 849-860. doi: 10.1038/nri2413.

Pan, C., Zhao, H., Shen, L., and Sheng, J. (2011). Molecular characterization and expression pattern of tripartite motif protein 39 in Gallus gallus with a complete PRY/SPRY domain. *Int J Mol Sci* 12(6)**,** 3797-3809. doi: 10.3390/ijms12063797.

Patil, G., Xu, L., Wu, Y., Song, K., Hao, W., Hua, F., et al. (2020). TRIM41-mediated ubiquitination of nucleoprotein limits vesicular stomatitis virus infection. *Viruses* 12(2). doi: 10.3390/v12020131.

Patil, G., Zhao, M., Song, K., Hao, W., Bouchereau, D., Wang, L., et al. (2018). TRIM41-mediated ubiquitination of nucleoprotein limits influenza A virus infection. *J Virol* 92(16). doi: 10.1128/JVI.00905-18.

Perera, S., Mankoo, B., and Gautel, M. (2012). Developmental regulation of MURF E3 ubiquitin ligases in skeletal muscle. *J Muscle Res Cell Motil* 33(2)**,** 107-122. doi: 10.1007/s10974-012-9288-7.

Qin, B., Xiao, T., Ding, C., Deng, Y., Lv, Z., and Su, J. (2021). Genome-wide identification and expression analysis of potential antiviral tripartite motif proteins (TRIMs) in grass carp (*Ctenopharyngodon idella*).. *Biology (Basel)* 10(12). doi: 10.3390/biology10121252.

Rajsbaum, R., Garcia-Sastre, A., and Versteeg, G.A. (2014). TRIMmunity: the roles of the TRIM E3-ubiquitin ligase family in innate antiviral immunity. *J Mol Biol* 426(6)**,** 1265-1284. doi: 10.1016/j.jmb.2013.12.005.

Reymond, A., Meroni, G., Fantozzi, A., Merla, G., Cairo, S., Luzi, L., et al. (2001). Reymond et al. (2001). The tripartite motif family identifies cell compartments. *EMBO*, 20: 2140-2151. https://doi.org/10.1093/emboj/20.9.2140

Rice, P., Longden, I., and Bleasby, A. (2000). EMBOSS: the European Molecular Biology Open Software Suite. *Trends Genet* 16(6)**,** 276-277. doi: 10.1016/s0168-9525(00)02024-2.

Robinson, M.D., McCarthy, D.J., and Smyth, G.K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26(1)**,** 139-140. doi: 10.1093/bioinformatics/btp616.

Rose, R., Golosova, O., Sukhomlinov, D., Tiunov, A., and Prosperi, M. (2019). Flexible design of multiple metagenomics classification pipelines with UGENE. *Bioinformatics* 35(11)**,** 1963-1965. doi: 10.1093/bioinformatics/bty901.

RStudio Team (2020). "RStudio: Integrated Development Environment for R". (Boston, MA: RStudio, Inc.).

Ruby, T., Bed'Hom, B., Wittzell, H., Morin, V., Oudin, A., and Zoorob, R. (2005). Characterisation of a cluster of TRIM-B30.2 genes in the chicken MHC B locus. *Immunogenetics* 57(1-2)**,** 116-128.

Samukawa, S., Yoshimi, R., Kirino, Y., and Nakajima, H. (2021). The PRY/SPRY domain of pyrin/TRIM20 interacts with β(2)-microglobulin to promote inflammasome formation. *Sci Rep* 11(1)**,** 23613. doi: 10.1038/s41598-021-03073-6.

Sanchez, J.G., Okreglicka, K., Chandrasekaran, V., Welker, J.M., Sundquist, W.I., and Pornillos, O. (2014). The tripartite motif coiled-coil is an elongated antiparallel hairpin dimer. *Proc Natl Acad Sci U S A* 111(7)**,** 2494-2499. doi: 10.1073/pnas.1318962111.

Sardiello, M., Cairo, S., Fontanella, B., Ballabio, A., and Meroni, G. (2008). Genomic analysis of the TRIM family reveals two groups of genes with distinct evolutionary properties. *BMC Evol Biol* 8(1)**,** 225. doi: 10.1186/1471-2148-8-225.

Sawyer, S.L., Emerman, M., and Malik, H.S. (2007). Discordant evolution of the adjacent antiretroviral genes TRIM22 and TRIM5 in mammals. *PLOS Pathogens* 3(12)**,** e197. doi: 10.1371/journal.ppat.0030197.

Sawyer, S.L., Wu, L.I., Emerman, M., and Malik, H.S. (2005). Positive selection of primate TRIM5alpha identifies a critical species-specific retroviral restriction domain. *Proc Natl Acad Sci U S A* 102(8)**,** 2832-2837. doi: 10.1073/pnas.0409853102.

Scherer, M., Schilling, E.M., and Stamminger, T. (2017). The Human CMV IE1 protein: An offender of PML nuclear bodies. *Adv Anat Embryol Cell Biol* 223**,** 77-94. doi: 10.1007/978-3-319-53168-7_4.

Shiina, T., Briles, W.E., Goto, R.M., Hosomichi, K., Yanagiya, K., Shimizu, S., et al. (2007). Extended gene map reveals tripartite motif, C-type lectin, and Ig superfamily type genes within a subregion of the chicken MHC-B affecting infectious disease. *J Immunol* 178(11)**,** 7162-7172. doi: 10.4049/jimmunol.178.11.7162.

Short, K.M., and Cox, T.C. (2006). Subclassification of the RBCC/TRIM superfamily reveals a novel motif necessary for microtubule binding. *Journal of Biological Chemistry* 281(13)**,** 8970-8980. doi: https://doi.org/10.1074/jbc.M512755200.

Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., et al. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular systems biology* 7**,** 539-539. doi: 10.1038/msb.2011.75.

Simao, F.A., Waterhouse, R.M., Ioannidis, P., Kriventseva, E.V., and Zdobnov, E.M. (2015). BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics* 31(19)**,** 3210-3212. doi: 10.1093/bioinformatics/btv351.

Smith, J., Smith, N., Yu, L., Paton, I.R., Gutowska, M.W., Forrest, H.L., et al. (2015). A comparative analysis of host responses to avian influenza infection in ducks and chickens highlights a role for the interferon-induced transmembrane proteins in viral resistance. *BMC Genomics* 16(1)**,** 574. doi: 10.1186/s12864-015-1778-8.

Song, B., Javanbakht, H., Perron, M., Park, D.H., Stremlau, M., and Sodroski, J. (2005). Retrovirus restriction by TRIM5alpha variants from Old World and New World primates. *Journal of virology* 79(7)**,** 3930-3937. doi: 10.1128/JVI.79.7.3930-3937.2005.

Stremlau, M., Owens, C.M., Perron, M.J., Kiessling, M., Autissier, P., and Sodroski, J. (2004). The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. *Nature* 427(6977)**,** 848-853. doi: 10.1038/nature02343.

Sturm-Ramirez, K.M., Ellis, T., Bousfield, B., Bissett, L., Dyrting, K., Rehg, J.E., et al. (2004). Reemerging H5N1 influenza viruses in Hong Kong in 2002 are highly pathogenic to ducks. *Journal of virology* 78(9)**,** 4892-4901. doi: 10.1128/jvi.78.9.4892-4901.2004.

Tan, G., Xu, F., Song, H., Yuan, Y., Xiao, Q., Ma, F., et al. (2018). Identification of TRIM14 as a type I IFN-stimulated gene controlling hepatitis B virus replication by targeting HBx. *Front Immunol* 9**,** 1872. doi: 10.3389/fimmu.2018.01872.

Taubenberger, J.K., and Kash, J.C. (2010). Influenza virus evolution, host adaptation, and pandemic formation. *Cell host & microbe* 7(6)**,** 440-451. doi: 10.1016/j.chom.2010.05.009.

Uhlen, M., Fagerberg, L., Hallstrom, B.M., Lindskog, C., Oksvold, P., Mardinoglu, A., et al. (2015). Proteomics. Tissue-based map of the human proteome. *Science* 347(6220)**,** 1260419. doi: 10.1126/science.1260419.

UniProt Consortium, T. (2018). UniProt: the universal protein knowledgebase. *Nucleic Acids Research* 46(5)**,** 2699-2699. doi: 10.1093/nar/gky092.

van Beuningen, S.F.B., Will, L., Harterink, M., Chazeau, A., van Battum, E.Y., Frias, C.P., et al. (2015). TRIM46 controls neuronal polarity and axon specification by driving the formation of parallel microtubule arrays. *Neuron* 88(6)**,** 1208-1226. doi: 10.1016/j.neuron.2015.11.012.

van der Aa, L.M., Levraud, J.-P., Yahmi, M., Lauret, E., Briolat, V., Herbomel, P., et al. (2009). A large new subset of TRIM genes highly diversified by duplication and positive selection in teleost fish. *BMC Biology* 7(1)**,** 7. doi: 10.1186/1741-7007-7-7.

Venuto, S., and Merla, G. (2019). E3 ubiquitin ligase TRIM proteins, cell cycle and mitosis. *Cells* 8(5). doi: 10.3390/cells8050510.

Versteeg, G.A., Rajsbaum, R., Sánchez-Aparicio, M.T., Maestre, A.M., Valdiviezo, J., Shi, M., et al. (2013). The E3-ligase TRIM family of proteins regulates signaling pathways triggered by innate immune pattern-recognition receptors. *Immunity* 38(2)**,** 384-398. doi: 10.1016/j.immuni.2012.11.013.

Vince Caery, L.L.a.R.G. (2021). "RBGL: An interface to the BOOST graph library". (http://www.bioconductor.org).

Wagner, J.M., Roganowicz, M.D., Skorupka, K., Alam, S.L., Christensen, D., Doss, G., et al. (2016). Mechanism of B-box 2 domain-mediated higher-order assembly of the retroviral restriction factor TRIM5α. *Elife* 5. doi: 10.7554/eLife.16309.

Wang, S., Yu, M., Liu, A., Bao, Y., Qi, X., Gao, L., et al. (2021). TRIM25 inhibits infectious bursal disease virus replication by targeting VP3 for ubiquitination and degradation. *PLoS Pathog* 17(9)**,** e1009900. doi: 10.1371/journal.ppat.1009900.

Watanabe, M., and Hatakeyama, S. (2017). TRIM proteins and diseases. *J Biochem* 161(2)**,** 135-144. doi: 10.1093/jb/mvw087.

Webster, R.G., Bean, W.J., Gorman, O.T., Chambers, T.M., and Kawaoka, Y. (1992). Evolution and ecology of influenza A viruses. *Microbiol Rev* 56(1)**,** 152-179.

Webster, R.G., Yakhno, M., Hinshaw, V.S., Bean, W.J., and Murti, K.G. (1978). Intestinal influenza: replication and characterization of influenza viruses in ducks. *Virology* 84(2)**,** 268-278. doi: 10.1016/0042-6822(78)90247-7.

Wei, Y., Zhou, H., Wang, A., Sun, L., Wang, M., Jia, R., et al. (2016). TRIM25 identification in the Chinese goose: Gene structure, tissue expression profiles, and antiviral immune responses in vivo and in vitro. *Biomed Res Int* 2016**,** 1403984. doi: 10.1155/2016/1403984.

Weinert, C., Morger, D., Djekic, A., Grütter, M.G., and Mittl, P.R.E. (2015). Crystal structure of TRIM20 C-terminal coiled-coil/B30.2 fragment: implications for the recognition of higher order oligomers. *Scientific Reports* 5(1)**,** 10819. doi: 10.1038/srep10819.

Wickham, H. (2016). "ggplot2: Elegant Graphics for Data Analysis. ". (Singer-Verlag New York).

Wu, S., Zhang, J., Xue, Q., Liu, J., Huang, B., He, Z., et al. (2020). Duck TRIM32 functions in IFN-β signaling against the Infection of H5N6 highly pathogenic avian influenza virus. *Front Immunol* 11**,** 377. doi: 10.3389/fimmu.2020.00377.

Wu, X., Wang, J., Wang, S., Wu, F., Chen, Z., Li, C., et al. (2019). Inhibition of influenza A virus replication by TRIM14 via its multifaceted protein-protein interaction with NP. *Front Microbiol* 10(FEB)**,** 344. doi: 10.3389/fmicb.2019.00344.

Yan, H.-Y., Wang, H.-Q., Zhong, M., Wu, S., Yang, L., Li, K., et al. (2021). PML suppresses influenza virus replication by promoting FBXW7 expression. *Virologica Sinica*. doi: 10.1007/s12250-021-00399-3.

Yu, J.W., Wu, J., Zhang, Z., Datta, P., Ibrahimi, I., Taniguchi, S., et al. (2006). Cryopyrin and pyrin activate caspase-1, but not NF-κB, via ASC oligomerization. *Cell Death & Differentiation* 13(2)**,** 236-249. doi: 10.1038/sj.cdd.4401734.

Yu, Z., Li, X., Yang, M., Huang, J., Fang, Q., Jia, J., et al. (2021). TRIM41 is required to innate antiviral response by polyubiquitinating BCL10 and recruiting NEMO. *Signal Transduction and Targeted Therapy* 6(1)**,** 90. doi: 10.1038/s41392-021-00477-8.

Zha, J., Han, K.J., Xu, L.G., He, W., Zhou, Q., Chen, D., et al. (2006). The Ret finger protein inhibits signaling mediated by the noncanonical and canonical IkappaB kinase family members. *J Immunol* 176(2)**,** 1072-1080. doi: 10.4049/jimmunol.176.2.1072.

Zhang, J., Hu, M.M., Wang, Y.Y., and Shu, H.B. (2012). TRIM32 protein modulates type I interferon induction and cellular antiviral response by targeting MITA/STING protein for K63-linked ubiquitination. *J Biol Chem* 287(34)**,** 28646-28655. doi: 10.1074/jbc.M112.362608.

Zheng, Q., Hou, J., Zhou, Y., Yang, Y., Xie, B., and Cao, X. (2015). Siglec1 suppresses antiviral innate immune response by inducing TBK1 degradation via the ubiquitin ligase TRIM27. *Cell Res* 25(10)**,** 1121-1136. doi: 10.1038/cr.2015.108.

Zhou, J.R., Liu, J.H., Li, H.M., Zhao, Y., Cheng, Z., Hou, Y.M., et al. (2020). Regulatory effects of chicken TRIM25 on the replication of ALV-A and the MDA5-mediated type I interferon response. *Vet Res* 51(1)**,** 145. doi: 10.1186/s13567-020-00870-1.

Zhou, Z., Li, M., Cheng, H., Fan, W., Yuan, Z., Gao, Q., et al. (2018). An intercross population study reveals genes associated with body size and plumage color in ducks. *Nature Communications* 9(1)**,** 2648. doi: 10.1038/s41467-018-04868-4.

Table 4.1: TRIM and TRIM-like genes present in the domestic mallard duck (*Anas platyrhynchos*)

Name	Annotation(description) (NCBI)	GeneID (NCBI)	Chromosome location	TRIM Subfamily	SMART predicted Domains
TRIM1	MID2	101802046	Chr10	C-I	R-BB-BB-CC-FN3-PS
TRIM2	TRIM2	101790291	Chr4	C-VII	R-BB-BB-FIL-NHL-NHL-NHL
TRIM3	TRIM3	119716941	Chr1	C-VII	R-BB-BB-FIL-NHL-NHL
TRIM7.1	TRIM7	101789720	Chr17	C-IV	R-BB-CC-PS
TRIM7.2	LOC119712990 (TRIM7-L)	119712990	Chr17	C-IV	R-BB-CC-PS
TRIM8	TRIM8	101804612	Chr6	C-V	R-CC
TRIM9	TRIM9	101803111	Chr5	C-I	R-BB-BB-CC-FN3-S
TRIM10	LOC101791922 (TRIM10)	101791922	Chr17	C-IV	UC-PS
TRIM10L	LOC110354628 (TRIM10-L)	110354628	Chr17	C-IV	UC-PS
TRIM11L	LOC113845682 (RFP-L)	113845682	Chr29	C-IV	R-CC-PS
TRIM13	TRIM13	101802794	Chr1	C-XI	R-BB-CC
TRIM14	TRIM14	101793362	Z	C-IV	BB-CC-CC-PS
avTRIM15L	RNF39L	119718713	Chr17	C-IV	R-CC-PS
TRIM18	MID1	101796423	Chr1	C-I	R-BB-BB-CC-FN3-PS

TRIM19.1	PML	113844857	Chr11	C-V	R-BB-DUF3583
TRIM19.2	LOC101796105 (Protein PML)	101796105	Chr11	C-V	R-BB-BB-DUF3583-EXOIII
TRIM23	TRIM23	101794054	Z	C-IX	R-BB-BB-CC-ARF
TRIM24	TRIM24	101802679	Chr1	C-VI	R-BB-BB-CC-PHD-BROMO
TRIM25	TRIM25	101791574	Chr19	C-IV	R-BB-BB-CC-PS
TRIM25L	PPARGC1B	101790707	Ch14	C-IV	NA
TRIM27.1	LOC101791103 (Zinc protein RFP)	101791103	Chr17	C-IV	R-BB-CC-PS
TRIM27.2	LOC101791534 (Zinc protein RFP)	101791534	Chr17	C-IV	R-BB-CC-PS
TRIM27L	LOC101790919 (Zinc protein RFP)	101790919	Chr17	C-IV	R-BB-CC-PS
TRIM28	N/A	N/A	Chr32•	C-VI	BB-BB-CC-PHD-BROMO
TRIM29	TRIM29	101802683	Chr25	C-V	BB-BB-CC
TRIM32	TRIM32	101799144	Chr18	C-VII	R-BB-CC-NHL-NHL*-NHL-NHL*-NHL
TRIM33	TRIM33	101795192	Chr27	C-VI	PHD-R-BB-CC-PHD-BROMO
TRIM35	TRIM35	101790163	Chr3	C-IV	R-BB-CC-PS
TRIM36	TRIM36	101791978	Z	C-I	R-BB-BB-CC-FN3-S*
TRIM37	TRIM37	101795200	Chr20	C-VIII	R-BB-CC-MATH-CC-CC
TRIM39	N/A	N/A	N/A	C-IV	R-BB-CC-PS

TRIM39.2	LOC101805457 (TRIM39-L)	101805457	Chr17	C-IV	R-BB-CC-PS
TRIM41	TRIM41	101792635	Chr17	C-IV	R-BB-CC-PS
TRIM42	TRIM42	101790093	Chr9	C-III	R-R-BB-CC-FN3
TRIM45	TRIM45	101801381	Chr1	C-X	R-BB-BB-CC-FIL
TRIM46	TRIM46	119713752	Chr26	C-I	R-BB-CC-FN3-S*
TRIM47	TRIM47	113845515	Chr19	C-IV	R-BB-BB-CC-PS
TRIM50	TRIM50	119713291	Chr20	C-IV	R-BB-CC-PS
TRIM54	TRIM54	119716393	Chr3	C-II	R-BB-CC
TRIM55	TRIM55	101799926	Chr2	C-II	R-BB-CC
diaTRIM58	LOC101804875 (TRIM39-L)	101804875	Chr20	C-IV	R-BB-CC*-PS
TRIM59	TRIM59	101803860	Chr9	C-XI	R-BB-CC-TM
TRIM59L	LOC101789404 (Serine rich adhesin for platelets)	101789404	Chr4	C-XI	R-BB-CC
TRIM62	TRIM62	101795644	Chr22	C-IV	R-BB-CC-PS
TRIM63	TRIM63	119713599	Chr24	C-II	R-BB-CC
TRIM65	TRIM65	101798054	Chr19	C-IV	R-BB-CC-PS
TRIM66	TRIM66	101801868	Chr5	C-VI	R-R-BB-CC-PHD-BROMO
TRIM67	TRIM67	101804091	Chr3	C-I	R-BB-BB-CC-FN3-PS
TRIM71	TRIM71	101796941	Chr2	C-VII	R-BB-BB-CC-FIL-NHL-NHL-NHL-NHL-NHL-NHL-NHL
BSPRY	BSPRY	101793618	Chr18	UC	PS
FSD1	N/A	N/A	N/A	C-I	BB-FN3-PS

FSD1L	FSD1L	101795640	Z	C-I	BB-FN3-PS
FSD2	FSD2	101796456	11	C-I	FN3-S
NHLRC1	NHLRC1	101796822	Chr2	C-VII	R-NHL
RNF39R	N/A	N/A	N/A	C-IV	R
RNF135	RNF135	106014788	Chr19	C-IV	R-CC-PS
RNF207	RNF207	101790007	Chr22	C-V	R-BB-BB-CC

Footnotes:

1 – Abbreviations are described as: R (RING), BB (B-box), CC (coiled coil), P (PRY), S(SPRY), FIL (Filamin domain), FN3 (fibronectin type III domain), PHD (Plant homeo Domain), BR (Bromodomain.), NHL (NCL-1, HT2A and Lin-41 repeats), ARF (ADP ribosylation factor-like), MATH (Meprin and TRAF homology domain), DUF3583 (Domain of unknown function 3583), EXOIII(Exonuclease III) and TM (Transmembrane)

2 – Asterix (*) denotes domain was below threshold of prediction by SMART. Black dot (●) denotes location found in related species but not mallard duck *(Anas platyrhynchos).*

Table 4.2. TRIM and TRIM-like genes present in the chicken (*Gallus gallus*)

Name (Duck)	Annotation(description) (NCBI)	GeneID (NCBI)	ProteinID (NCBI)	Identity to duck (%)
TRIM1	TRIM1	422131	XP_004940642.3	97.53
N/A	LOC416146 (MID2-L)	416146	NP_001376669.2	N/A
TRIM2	TRIM2	100857562	NP_001244243.1	99.04
TRIM3	TRIM3	425131	XP_004939021.1	95.81
TRIM7.1	TRIM7.1	417040	NP_001385147.1	95.17
TRIM7.2	TRIM7.2	425772	NP_001092824.2	96.69
TRIM8	TRIM8	423864	NP_001026404.2	98.91
TRIM9	TRIM9	423579	XP_015132237.1	100
TRIM10	BTN1	768783	XP_015150487.1	82.64
TRIM10-L	TRIM10	100858749	XP_040504620.1	68.8
TRIM11-L	N/A	N/A	N/A	N/A
TRIM13	TRIM13	418872	XP_015131811.2	94.61

TRIM14	TRIM14	427282	NP_001026602.2	81.99
avTRIM15L	N/A	N/A	N/A	N/A
TRIM18	MID1	373920	NP_001384590.1	99.55
TRIM19.1	PML	415302	XP_015134517.2	74.54
TRIM19.2	PMLL	100857563	XP_040562550.1	54.72
TRIM23	TRIM23	427164	XP_424752.3	96.91
TRIM24	TRIM24	418106	XP_015143032.2	91.67
TRIM25	TRIM25	417401	NP_001305387.1	70.54
TRIM25L	LOC416147 (TRIM25-L)	416147	NP_001376668.2	N/A
TRIM27.1	TRIM27.1	417042	NP_001025842.2	79.88
TRIM27.2	TRIM27.2	430359	NP_001092829.1	80.88
TRIM27L	N/A	N/A	N/A	N/A
TRIM28	TRIM28	107050474	NP_001305930.2	69.52
TRIM29	TRIM29	419754	XP_015153673.1	97.63
TRIM32	TRIM32	101748429	XP_040505171.1	95.53
TRIM33	TRIM33	419883	NP_001366055.1	97.49
TRIM35	TRIM35	771180	XP_004935921.1	78.03
TRIM36	TRIM36	426811	XP_040512100.1	90.38
TRIM37	TRIM37	417628	NP_001006224.2	96.04
TRIM39	N/A	N/A	N/A	N/A
N/A	TRIM39.1	101747534	NP_001299812.1	70.3
TRIM39.2	TRIM39.2	417041	NP_001006196.3	78.76
TRIM41	TRIM41	417043	NP_001025843.1	88.1
TRIM42	TRIM42	424818	XP_422632.5	81.02
TRIM45	TRIM45	769776	XP_040516325.1	82.78
TRIM46	N/A		N/A	N/A
TRIM47	TRIM47	770100	XP_040542204.1	80.88
TRIM50	TRIM50	417461	XP_015151390.2	90.49
TRIM54	TRIM54	107053153	XP_015140486.2	93.68
TRIM55	TRIM55	420166	NP_001186333.1	94.9

diaTRIM58	TRIM39	770718	XP_001232970.5	81.3
TRIM59	TRIM59	425013	NP_001026491.1	80.15
TRIM59L	LOC422426	422426	XP_015140854.2	67.29
TRIM62	TRIM62	429807	XP_015152721.2	99.16
TRIM63	TRIM63	426754	XP_015153240.2	87.6
TRIM65	TRIM65	417367	XP_415625.3	68.98
TRIM66	TRIM66	423056	XP_040529288.1	84.42
TRIM67	TRIM67	100857188	XP_025004963.1	90.5
TRIM71	TRIM71	428445	NP_001032352.2	91.51
BSPRY	BSPRY	417272	XP_415546.4	85.63
FSD1L	FSD1L	427302	XP_004949409.1	73.61
FSD2	FSD2	427503	XP_046755109.1	87.72
FSD1	LOC107051263 (FSD1L)	107051263	XP_025000152.1	91.62
NHLRC1	NHLRC1	428477	XP_040519561.1	79.16
RNF39R	N/A	N/A	N/A	N/A
RNF135	N/A	N/A	N/A	N/A
RNF207	RNF207	419374	XP_417536.2	91.32

Supplementary file S4.1. Project numbers, tissue and data included from the NCBI SRA database

SRA accession	**Project#**	**sample name**	**type**	**Tissue or description**
SRR064714	SRP003365	duck_spleen_lane_1	control	spleen
SRR064716	SRP003365	duck_spleen_lane_2	control	spleen
SRR064718	SRP003365	duck_liver_lane_1	control	liver
SRR064720	SRP003365	duck_liver_lane_2	control	liver
SRR11261962	PRJNA610706	Sample_60h_C	control	fibroblast

SRR11261963	PRJNA610706	Sample_48h_C	control	fibroblast
SRR11261964	PRJNA610706	Sample_36h_C	control	fibroblast
SRR11261965	PRJNA610706	Sample_24h_C	control	fibroblast
SRR11261966	PRJNA610706	Sample_12h_C	control	fibroblast
SRR11516784	PRJNA624183	mock_3	control	fibroblast
SRR11516785	PRJNA624183	mock_2	control	fibroblast
SRR11516786	PRJNA624183	mock_1	control	fibroblast
SRR1295590	PRJNA248056	PD3	pekin	pekin adipose
SRR1295593	PRJNA248056	PD4	pekin	pekin adipose
SRR1295594	PRJNA248056	PD5	pekin	pekin adipose
SRR1610922	PRJNA263681	JB1	B strain	Jejunum tissue of B strain of Pekin duck
SRR1610923	PRJNA263681	JB2	B stain	Jejunum tissue of B strain of Pekin duck
SRR1610924	PRJNA263681	JF1	F strain	Jejunum tissue of F

				strain of Pekin duck
SRR1610925	PRJNA263681	JF2	F strain	Jejunum tissue of F strain of Pekin duck
SRR1610926	PRJNA263681	JB1	B strain	Breast Muscle tissue of B strain of Pekin duck
SRR1610927	PRJNA263681	JB2	B strain	Breast Muscle tissue of B strain of Pekin duck
SRR1610928	PRJNA263681	JF1	F strain	Breast Muscle tissue of F strain of Pekin duck
SRR1610929	PRJNA263681	JF2	F strain	Breast Muscle tissue of F strain of Pekin duck
SRR1796022	PRJNA271731	APLFG1	female	ovary
SRR1796023	PRJNA271731	APLFG2	female	ovary
SRR1796024	PRJNA271731	APLFG3	female	ovary
SRR1796025	PRJNA271731	APLFG4	female	ovary
SRR1796026	PRJNA271731	APLFG5	female	ovary

SRR1796027	PRJNA271731	APLFS1	female	spleen
SRR1796028	PRJNA271731	APLFS2	female	spleen
SRR1796029	PRJNA271731	APLFS3	female	spleen
SRR1796030	PRJNA271731	APLFS4	female	spleen
SRR1796031	PRJNA271731	APLFS5	female	spleen
SRR1796032	PRJNA271731	APLMG1	male	testis
SRR1796033	PRJNA271731	APLMG2	male	testis
SRR1796034	PRJNA271731	APLMG3	male	testis
SRR1796035	PRJNA271731	APLMG4	male	testis
SRR1796036	PRJNA271731	APLMG5	male	testis
SRR1796037	PRJNA271731	APLMS1	male	spleen
SRR1796038	PRJNA271731	APLMS2	male	spleen
SRR1796039	PRJNA271731	APLMS3	male	spleen
SRR1796040	PRJNA271731	APLMS4	male	spleen
SRR1796041	PRJNA271731	APLMS5	male	spleen
SRR6318552	PRJNA419583	SMM1_brain	SM	brain
SRR6318553	PRJNA419583	JDM1_brain	JD	brain
SRR6318554	PRJNA419583	GYM1_brain	GY	brain
SRR6318555	PRJNA419583	SXM1_brain	SX	brain
SRR6318556	PRJNA419583	MLM1_breast_muscle	MLK	muscle
SRR6318557	PRJNA419583	CVM1_breast_muscle	CV	muscle
SRR6318558	PRJNA419583	PKM1_breast_muscle	PK	muscle
SRR6318564	PRJNA419583	SMM1_breast_muscle	SM	muscle
SRR6318565	PRJNA419583	JDM1_breast_muscle	JD	muscle
SRR6318566	PRJNA419583	SXM1_breast_muscle	SX	muscle
SRR6318567	PRJNA419583	GYM1_breast_muscle	GY	muscle
SRR6318569	PRJNA419583	PKM1_liver	PK	liver
SRR6318570	PRJNA419583	CVM1_liver	CV	liver
SRR6318571	PRJNA419583	MLM1_liver	ML	liver

SRR6318572	PRJNA419583	JDM1_liver	JD	liver
SRR6318573	PRJNA419583	SMM1_liver	SM	liver
SRR6318574	PRJNA419583	SXM1_liver	SX	liver
SRR6318575	PRJNA419583	GYM1_liver	GY	liver
SRR6318578	PRJNA419583	PKM1_brain	PK	brain
SRR6318586	PRJNA419583	MLM1_brain	ML	brain
SRR6318587	PRJNA419583	CVM1_brain	CV	brain
SRR6760943	PRJNA434353	duck_replicate2	duck cell line for assembly	fibroblast
SRR6760944	PRJNA434353	duck_replicate1	duck cell line for assembly	fibroblast
SRR6760965	PRJNA434353	duck_replicate4	duck cell line for assembly	fibroblast
SRR6760966	PRJNA434353	duck_replicate3	duck cell line for assembly	fibroblast
SRR7127376	PRJNA459507	PekAL117	Pekin male fed ad libitum	liver
SRR7127377	PRJNA459507	PekAL113	Pekin male fed ad libitum	liver
SRR7127382	PRJNA459507	PekAL93	Pekin male fed ad libitum	liver
SRR7127383	PRJNA459507	PekAL89	Pekin male fed ad libitum	liver
SRR7127384	PRJNA459507	PekAL85	Pekin male fed ad libitum	liver
SRR7127385	PRJNA459507	PekAL81	Pekin male fed ad libitum	liver
SRR7127386	PRJNA459507	PekAL109	Pekin male fed ad libitum	liver

SRR7127387	PRJNA459507	PekAL105	Pekin male fed ad libitum	liver
SRR7127388	PRJNA459507	PekAL101	Pekin male fed ad libitum	liver
SRR7127389	PRJNA459507	PekAL97	Pekin male fed ad libitum	liver
SRR7811367	PRJNA489980	Cduo3	control	duodenum
SRR7811369	PRJNA489980	Cjej3	control	jejunum
SRR7811371	PRJNA489980	Cjej2	control	jejunum
SRR7811372	PRJNA489980	Cduo1	control	duodenum
SRR7811373	PRJNA489980	Cduo2	control	duodenum
SRR7811378	PRJNA489980	Cile1	control	ileum
SRR7811379	PRJNA489980	Cile2	control	ileum
SRR7811381	PRJNA489980	Cile3	control	ileum
SRR7811382	PRJNA489980	Cjej1	control	jejunum
SRR797835	PRJNA194464	control-3C307-lung	control	lung
SRR8053813	PRJNA496401	small YR-ovary 3	small yolk ratio - shaoxin	ovary
SRR8053814	PRJNA496401	big YR-ovary 3	big yolk ratio - shaoxin	ovary
SRR8053815	PRJNA496401	small YR-ovary 1	small yolk ratio - shaoxin	ovary
SRR8053816	PRJNA496401	big YR-ovary 1	big yolk ratio - shaoxin	ovary
SRR8053817	PRJNA496401	small YR-liver 1	small yolk ratio - shaoxin	liver
SRR8053818	PRJNA496401	big YR-liver 1	big yolk ratio - shaoxin	liver
SRR8053819	PRJNA496401	small YR-liver 2	small yolk ratio - shaoxin	liver

SRR8053820	PRJNA496401	big YR-liver 2	big yolk ratio - shaoxin	liver
SRR8053821	PRJNA496401	big YR-liver 3	big yolk ratio - shaoxin	liver
SRR8053822	PRJNA496401	small YR-ovary 2	small yolk ratio - shaoxin	ovary
SRR8053823	PRJNA496401	big YR-ovary 2	big yolk ratio - shaoxin	ovary
SRR8053824	PRJNA496401	small YR-liver 3	small yolk ratio - shaoxin	liver
SRR16103914	PRJNA767080	Int_mock_1_1	mock	intestine
SRR16103930	PRJNA767080	Spleen_mock_3_3	mock	spleen
SRR16103931	PRJNA767080	Spleen_mock_3_2	mock	spleen
SRR16103932	PRJNA767080	Spleen_mock_3_1	mock	spleen
SRR16103933	PRJNA767080	Spleen_mock_2_2	mock	spleen
SRR16103934	PRJNA767080	Spleen_mock_1_3	mock	spleen
SRR16103935	PRJNA767080	Spleen_mock_1_2	mock	spleen
SRR16103936	PRJNA767080	Spleen_mock_1_1	mock	spleen
SRR16103947	PRJNA767080	Lung_mock_3_3	mock	lung
SRR16103948	PRJNA767080	Lung_mock_3_2	mock	lung

SRR16103949	PRJNA767080	Lung_mock_3_1	mock	lung
SRR16103950	PRJNA767080	Lung_mock_2_2	mock	lung
SRR16103952	PRJNA767080	Lung_mock_1_3	mock	lung
SRR16103953	PRJNA767080	Lung_mock_1_2	mock	lung
SRR16103954	PRJNA767080	Lung_mock_1_1	mock	lung
SRR16103978	PRJNA767080	Int_mock_3_3	mock	intestine
SRR16103979	PRJNA767080	Int_mock_3_2	mock	intestine
SRR16103980	PRJNA767080	Int_mock_3_1	mock	intestine
SRR16103981	PRJNA767080	Int_mock_2_2	mock	intestine
SRR16103982	PRJNA767080	Int_mock_1_3	mock	intestine
SRR16103983	PRJNA767080	Int_mock_1_2	mock	intestine

Supplementary table S4.2. Accession numbers of C-IV TRIM proteins from representative mammals, reptiles and birds.

TRIM protein	**Species**	**Accession # (NCBI)**
diaTRIM58	Reeves Turtle	XP_039364437.1
avTRIM15L	Black swan	XP_035426390.1

RFPL	Reeves Turtle	XP_039357037.1
RFPL	Common wall lizard	XP_034962870.1
RFPL	Common wall lizard	XP_034963402.1
RFPL	Common wall lizard	XP_034963598.1
RFPL	Reeves Turtle	XP_039357050.1
RNF135	Mouse	NP_082295.1
RNF135	Human	NP_115698.3
RNF135	Soft shell turtle	XP_006125223.1
RNF135	Bearded Dragon	XP_020639259.1
RNF135	Koala	XP_020819030.1
RNF135	Emu	XP_025954624.1
RNF39	Human	AQY77078.1
RNF39	Mouse	NP_001093102.1
RNF39	Garter Snake	XP_013923654.1
RNF39	Soft shell turtle	XP_014426069.1
RNF39	Platypus	XP_028910721.1
RNF39	Budgie	XP_033929485.1
TRIM10	Human	NP_006769.2
TRIM10	Mouse	NP_035410.2
TRIM10	Wombat	XP_027732374.1
TRIM10L	Common wall lizard	XP_034957220.1
TRIM10L	Common wall lizard	XP_034963630.1
TRIM10L	Reeves Turtle	XP_039356273.1
TRIM10L	Reeves Turtle	XP_039356280.1
TRIM10L	Reeves Turtle	XP_039357044.1
TRIM11	Mouse	NP_001277917.1
TRIM11	Human	NP_660215.1
TRIM11	Koala	XP_020822438.1
TRIM11	Kiwi	XP_013797493.1
TRIM11L	Pigeon	XP_013226190.1

TRIM11L	Reeves Turtle	XP_039356684.1
TRIM14	Mouse	BAD32173.1
TRIM14	Human	NP_055603.2
TRIM14	Common lizard	XP_028568653.1
TRIM14	Koala	XP_020851210.1
TRIM14	Reeves Turtle	XP_039398751.1
TRIM15	Human	AAH38585.1
TRIM15	Koala	XP_020829853.1
TRIM15	Mouse	XP_036016670.1
TRIM16	Human	AAH53514.1
TRIM16	Mouse	NP_444399.2
TRIM16	Koala	XP_020822270.1
TRIM17	Human	NP_001020111.1
TRIM17	Mouse	NP_112449.1
TRIM21	Mouse	AAH10580.1
TRIM21	Human	NP_003132.2
TRIM21L	Common wall lizard	XP_034957290.1
TRIM22	Human	NP_006065.2
TRIM25	Human	NP_005073.2
TRIM25	Mouse	NP_033572.2
TRIM25	Reeves Turtle	XP_039357400.1
TRIM25	Fence Lizard	XP_042306860.1
TRIM25	Koala	XP_020847587.1
TRIM25L	Reeves Turtle	XP_039341926.1
TRIM26	Human	AQY77164.1
TRIM26	Mouse	NP_001020770.2
TRIM26	Wombat	XP_027732040.1
TRIM27	Human	AAH66924.1
TRIM27	Mouse	NP_033080.2
TRIM31	Human	NP_008959.3

TRIM31	Mouse	NP_666189.1
TRIM34	Mouse	AAG53512.1
TRIM34	Human	NP_001003827.1
TRIM35	Mouse	NP_084255.2
TRIM35	Human	NP_741983.2
TRIM35	Koala	XP_020839631.1
TRIM35	Common lizard	XP_034966711.1
TRIM35	Reeves Turtle	XP_039388389.1
TRIM38	Mouse	NP_001025106.1
TRIM38	Human	NP_006346.1
TRIM39	Human	NP_067076.2
TRIM39	Mouse	NP_077788.2
TRIM39	Koala	XP_020829830.1
TRIM39	Komodo	XP_044307822.1
TRIM39.1	Tufted duck	XP_032061034.1
TRIM39L	Common wall lizard	XP_034963559.1
TRIM39L	Reeves Turtle	XP_039357073.1
TRIM4	Human	NP_148977.2
TRIM4	Meerkat	XP_029802732.1
TRIM40	Mouse	NP_001028407.1
TRIM40	Human	NP_001273562.1
TRIM40	Koala	XP_020829846.1
TRIM41	Human	AAH18765.2
TRIM41	Mouse	NP_663352.2
TRIM41	Koala	XP_020834861.1
TRIM41	Reeves Turtle	XP_039358136.1
TRIM41	Bearded Dragon	XP_020633185.1
TRIM43	Mouse	NP_001171329.1
TRIM43	Human	NP_620155.1
TRIM47	Mouse	NP_001192010.1

TRIM47	Human	XP_005257844.1
TRIM47	Wombat	XP_027728789.1
TRIM47	Wall lizard	XP_034954978.1
TRIM47	Reeves Turtle	XP_039357784.1
TRIM48	Human	AAO14946.1
TRIM48	Woodchuck	XP_046295335.1
TRIM49	Human	NP_065091.1
TRIM5	Human	NP_149023.2
TRIM5	Grassrat	XP_034362060.1
TRIM50	Mouse	AAI30023.1
TRIM50	Human	NP_001268380.1
TRIM50	Bearded Dragon	XP_020657306.1
TRIM50	Koala	XP_020861517.1
TRIM50	Reeves Turtle	XP_039364251.1
TRIM51	Human	NP_116070.2
TRIM51	Gray squirrel	XP_047374956.1
TRIM58	Mouse	NP_001034136.1
TRIM58	Human	NP_056246.3
TRIM58	Koala	XP_020822486.1
TRIM58L	Reeves Turtle	XP_039357095.1
TRIM6	Human	AAH65575.1
TRIM6	Mouse	NP_001013637.1
TRIM60	Human	NP_001244954.1
TRIM60	Rabbit	XP_002720615.1
TRIM60	Reeves Turtle	XP_039395527.1
TRIM61	Human	NP_001012414.1
TRIM61	Pika	XP_040827292.1
TRIM62	Mouse	NP_001357685.1
TRIM62	Human	NP_060677.2
TRIM62	Koala	XP_020839045.1

TRIM62	Common wall lizard	XP_028597330.1
TRIM62	Reeves Turtle	XP_039364566.1
TRIM64	Human	NP_001129958.1
TRIM64	Cow	NP_001179468.2
TRIM65	Human	NP_775818.2
TRIM65	Koala	XP_020858271.1
TRIM65	Mouse	XP_036012675.1
TRIM65	Reeves Turtle	XP_039356207.1
TRIM65	Fence lizard	XP_042308310.1
TRIM68	Human	NP_060543.5
TRIM68	Mouse	NP_932129.2
TRIM69	Mouse	NP_536771.2
TRIM69	Human	NP_892030.3
TRIM7	Human	NP_976038.1
TRIM7	Bearded Dragon	XP_020665743.1
TRIM7	Platypus	XP_028910464.1
TRIM7	Reeves Turtle	XP_039356619.1
TRIM7	Mouse	XP_006534631.1
TRIM72	Human	NP_001008275.2
TRIM72	Mouse	NP_001073401.1
TRIM72	Koala	XP_020860234.1
TRIM75	Mouse	NP_001028601.1
TRIM75	Human	NP_001382999.1
TRIM7L	Common wall lizard	XP_034962867.1
TRIM7L	Reeves Turtle	XP_039374781.1
TRIML1	human	AAH15684.1
TRIML1	Koala	XP_020853539.1
TRIML1	Mouse	AAI41203.1
TRIML2	Human	AAI43710.1
TRIML2	Mouse	NP_001153884.1

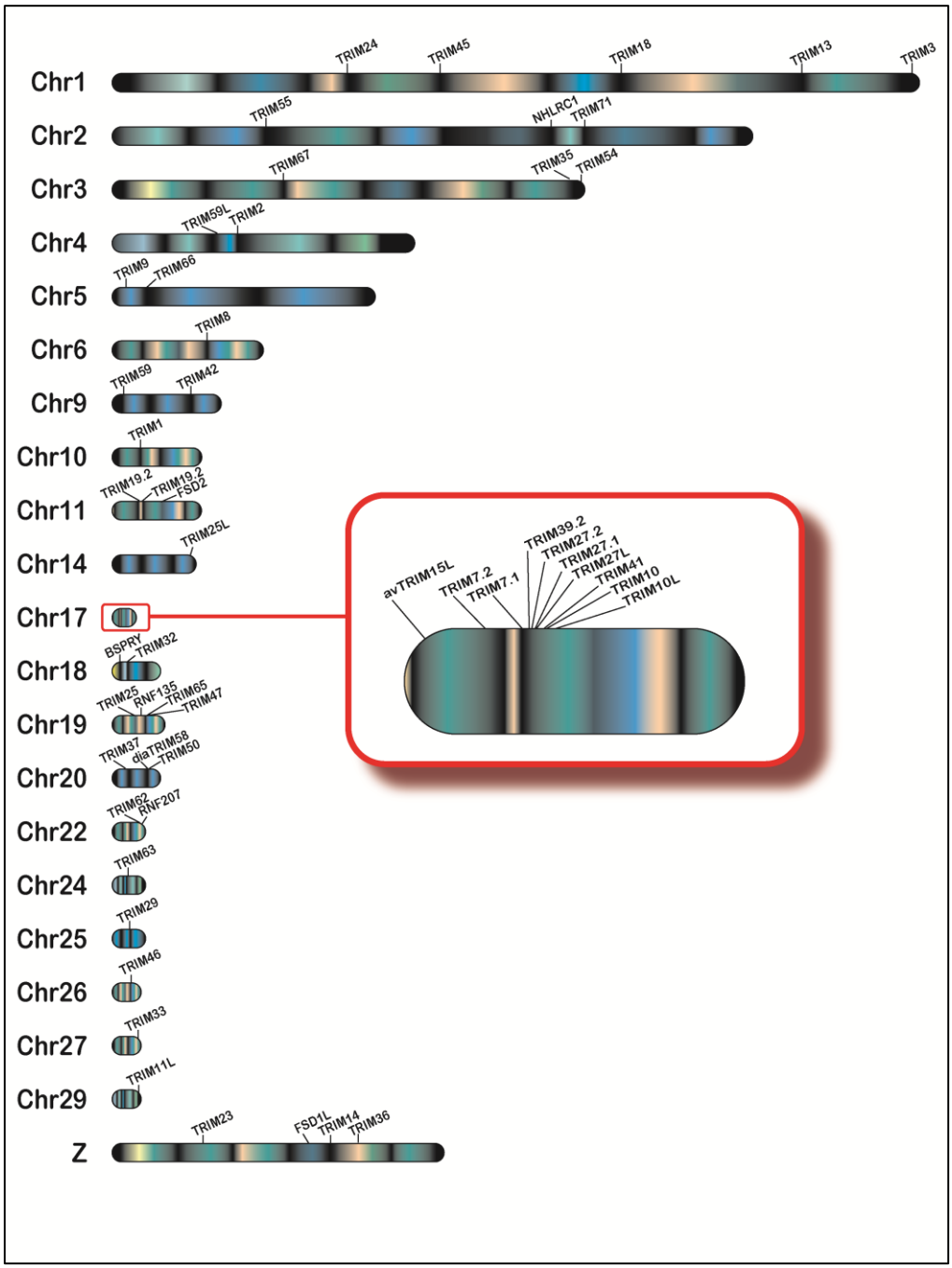


Figure 4.1. Genomic locations of TRIM or TRIM-like genes in the duck. TRIM and TRIM-like genes in the duck were submitted to NCBI BLAST and locations in the duck genome were mapped using KaryoplottR in the R studio environment. Chromosome 17 is magnified to allow for visualization of the expansion of the TRIM and TRIM-like genes in the duck MHC (B) locus

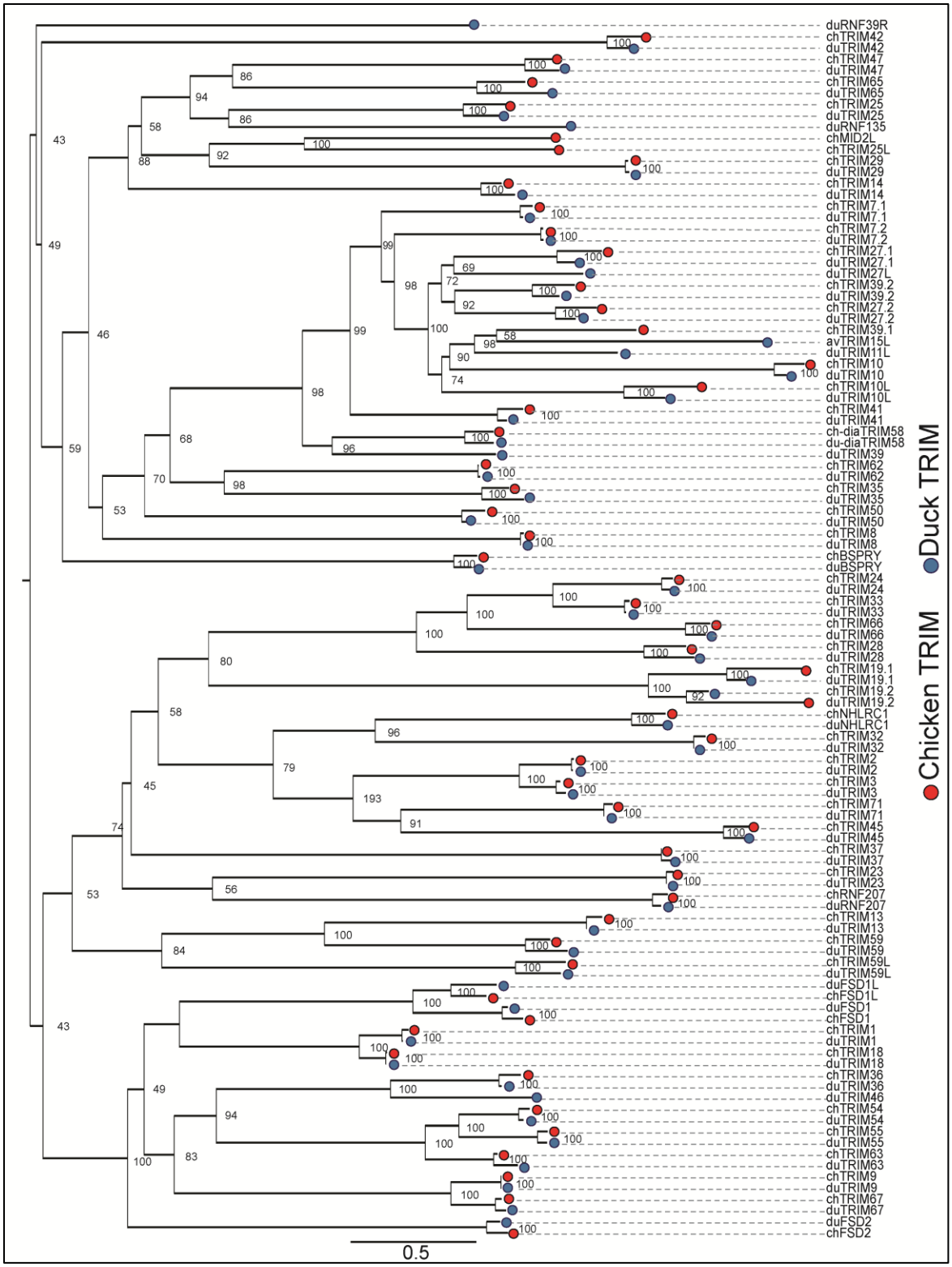


Figure 4.2. Duck and chicken TRIM proteins share homology. Phylogenetic relationships between the duck and chicken TRIM protein sequences were investigated using maximum likelihood (ML) trees with 10000 Ultrafast bootstrap replications. External nodes were colour-

coded to indicate species of origin for each TRIM protein. Initial ML tree was inferred using IQTree and visualized using FigTree. Resulting trees were edited for clarity using Adobe Illustrator.

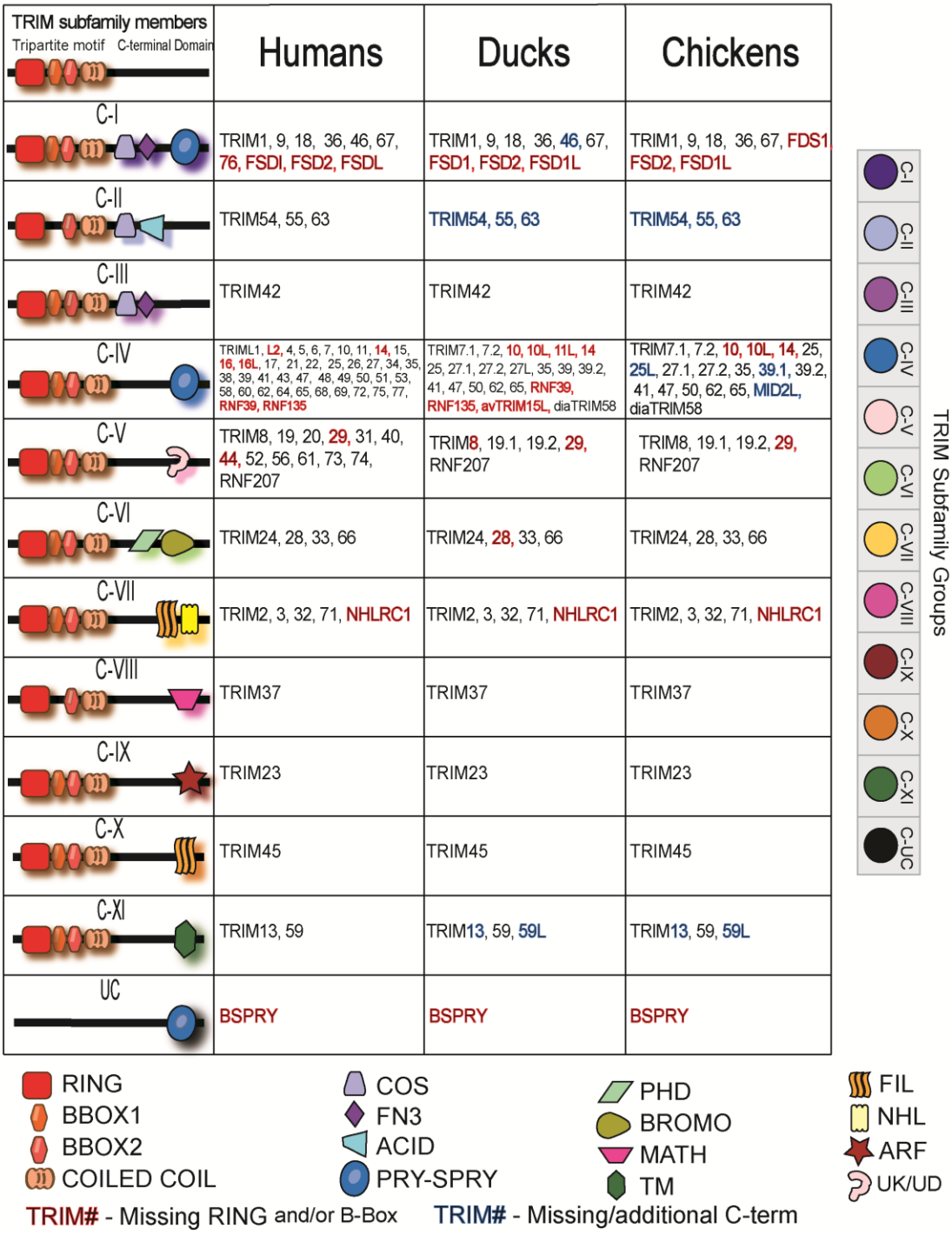


Figure 4.3. Classification of TRIM proteins by their C-terminal domains for human, duck, and chicken. RING: Really interesting new gene. COS: C-terminal subgroup one signature. FN3: Fibronectin, type III. PHD: Plant Homeo Domain. BROMO: Bromodomian. MATH: meprin and TRAF homology domain. TM: Transmembrane domain. FIL: Filamin domain. NHL: NCL-1, HT2A and Lin-41 repeats. ARF: ADP ribosylation factor-like. UC: Uncharacterized.

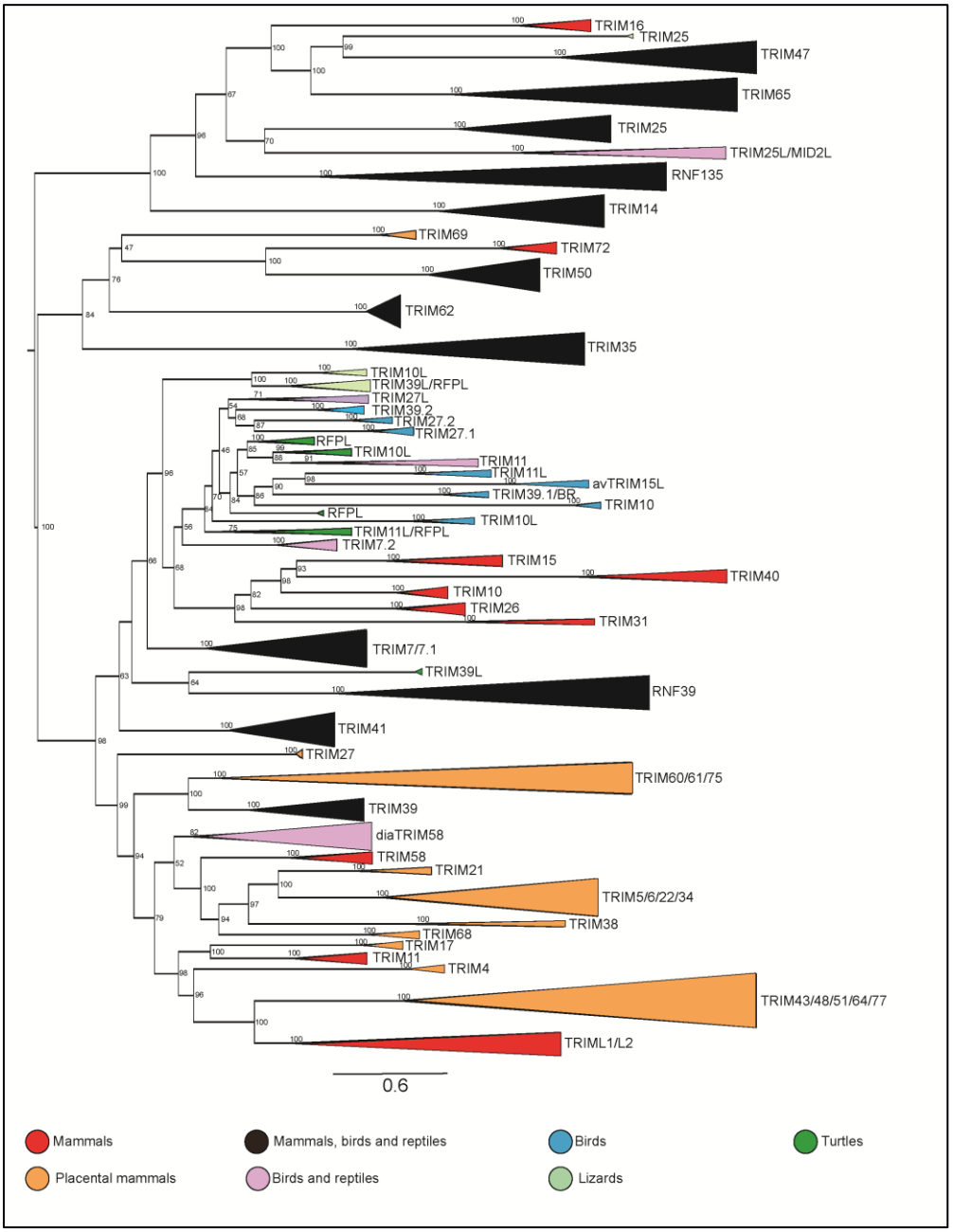


Figure 4.4. TRIM protein subfamily C-IV has direct orthologs between species and unique expansions within species. Phylogenetic relationships between C-IV subfamily members from human, non-primate placental mammal, marsupial, bird, lizard and turtle protein sequences were investigated using maximum likelihood (ML) tree with 1000 Ultrafast bootstrap replications. External nodes were combined when direct homology was inferred between species. Initial ML tree was inferred using IQTree and visualized using FigTree. Resulting trees were edited for clarity using Adobe illustrator.

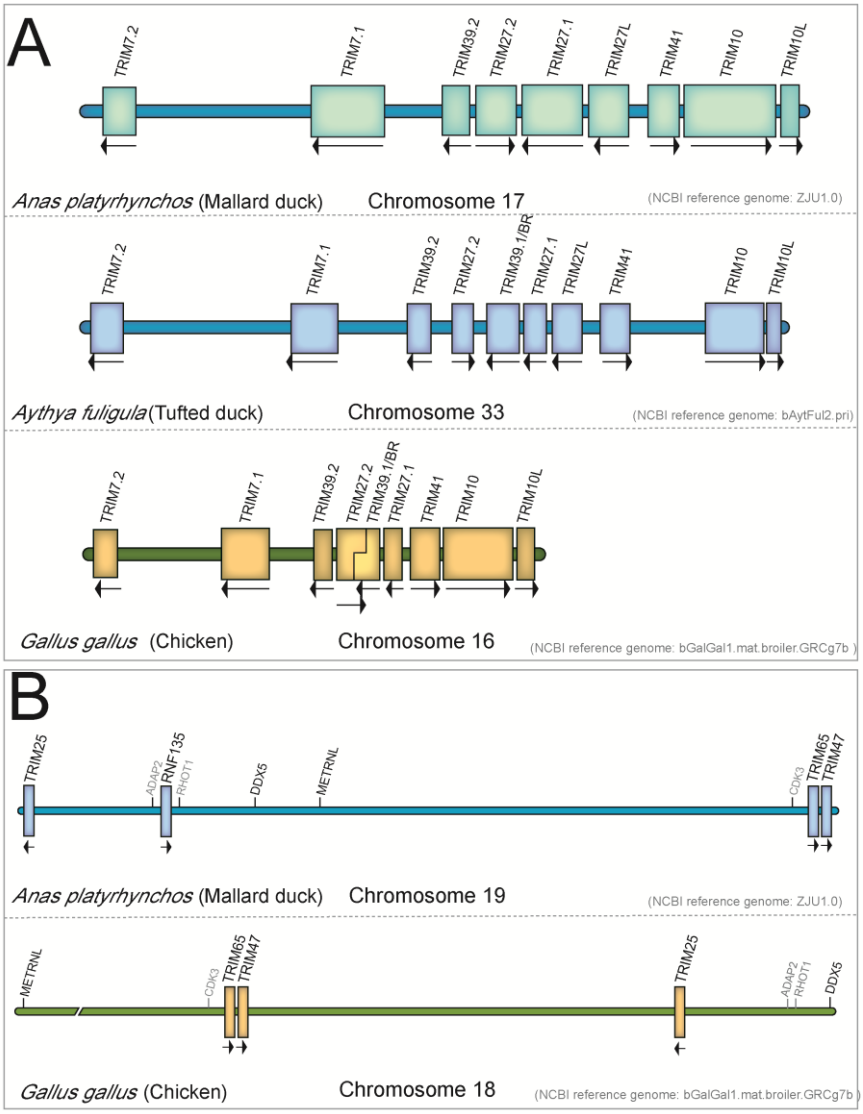


Figure 4.5. Genomic organization of the MHC-linked TRIM genes and the TRIM25 locus. Genomic locations and direction of transcription of the MHC-linked TRIM genes from mallard duck, tufted duck and chicken were compared (A). The TRIM25 region of chromosome 19 was

compared to the same syntenic region on chicken chromosome 18 (B). All sizes of genes and chromosome length in (A) were normalized to the size of mallard duck gene TRIM10L. All sizes of genes and chromosome length in (B) were normalized to duck TRIM25. The white break in the chicken chromosome 18 indicates an unshown region of approximately 74kb in length.

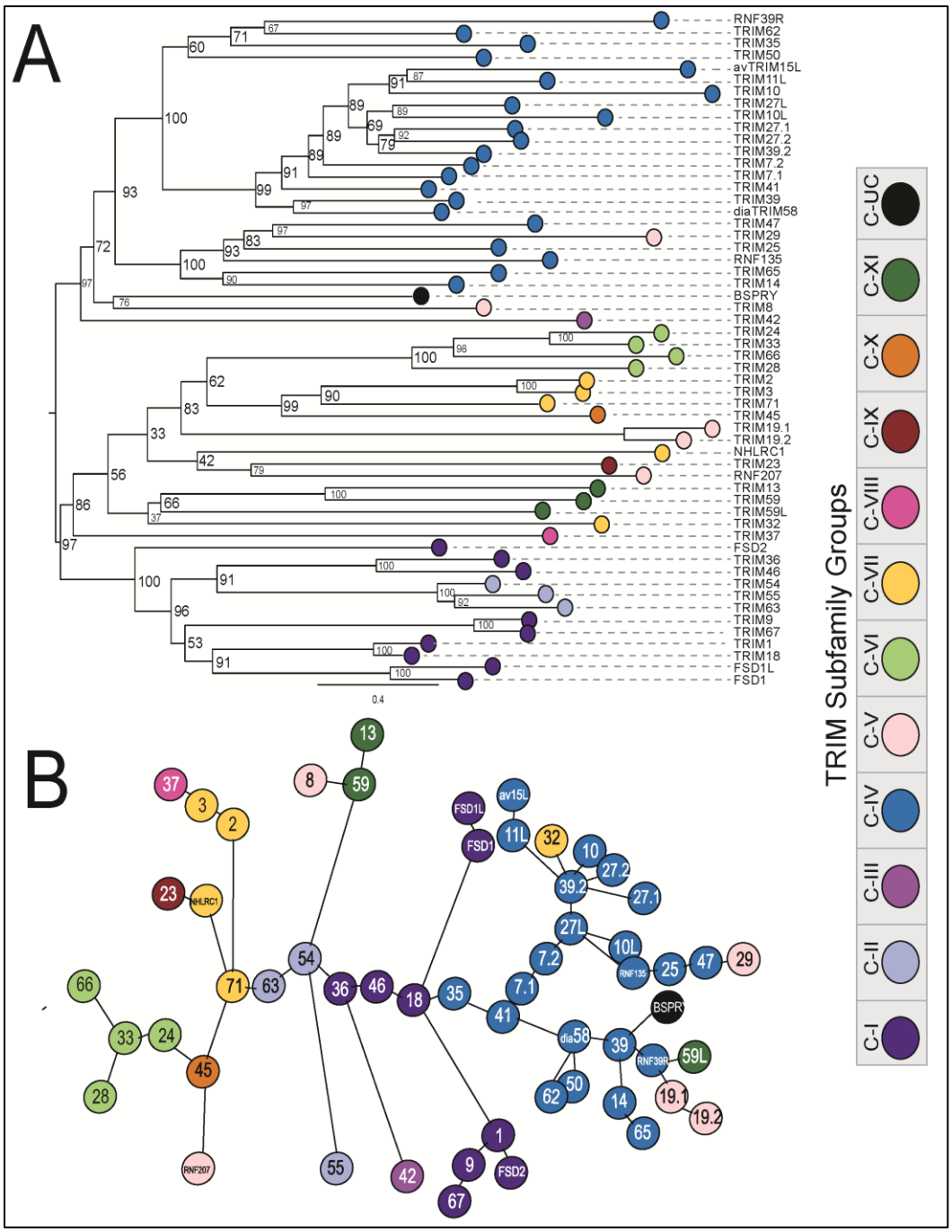


Figure 4.6. Duck TRIM and TRIM-like proteins cluster with other members of TRIM subfamilies. Phylogenetic relationships between the duck TRIM protein sequences were investigated using maximum likelihood (ML) trees with 10000 Ultrafast bootstrap replications (A). The distances between protein sequences were also investigated using a minimum spanning network to infer similarity and function (B). Each external node was colour-coded according to TRIM subfamily designation. All trees were made using IQTree and visualized using FigTree.

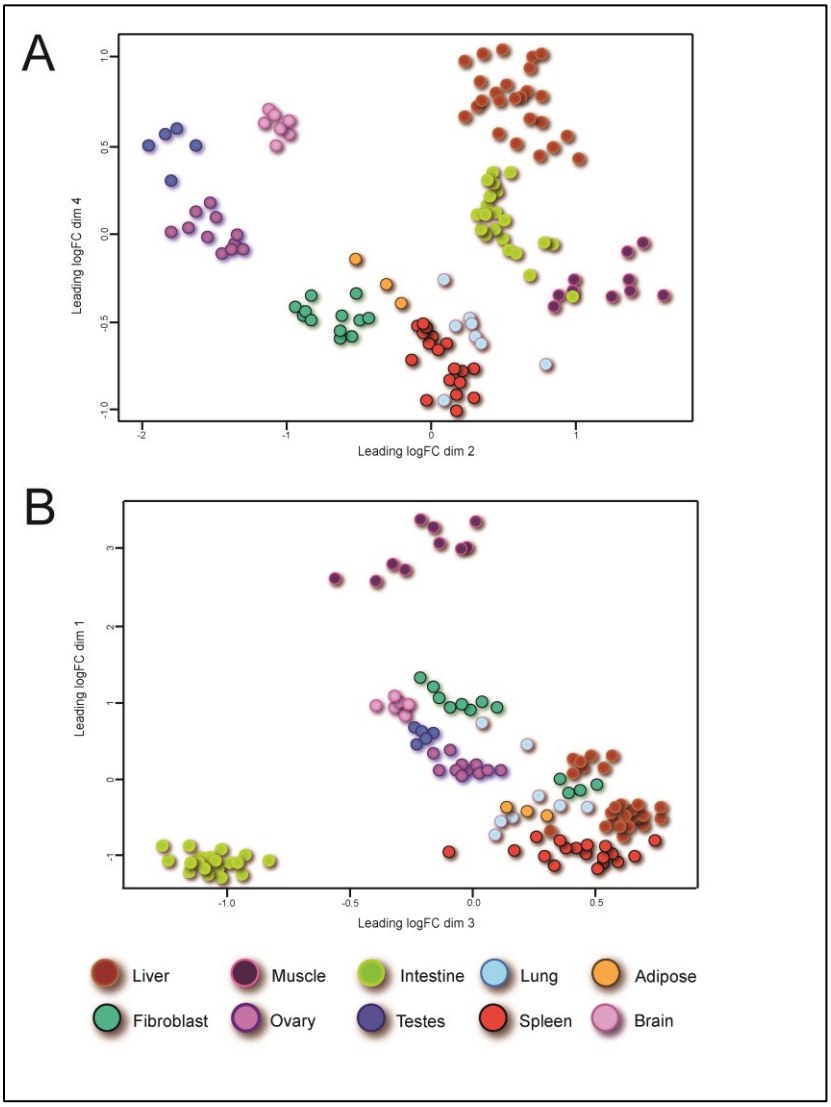


Figure 4.7. Multidimensional scaling plot (MDS) of duck TRIM and TRIM-like gene expression in tissues sampled. All RNA-seq libraries were accessed from the NCBI short sequence read archive (SRA), normalized by library size, and compared using the TMM method in EdgeR in the RStudio environment and edited for clarity in Adobe Illustrator. Individual

libraries were compared using dimensions 4 and 2 (A) and dimensions 1 and 3 (B). Individual samples were colour coded by tissue that library was sequenced from.

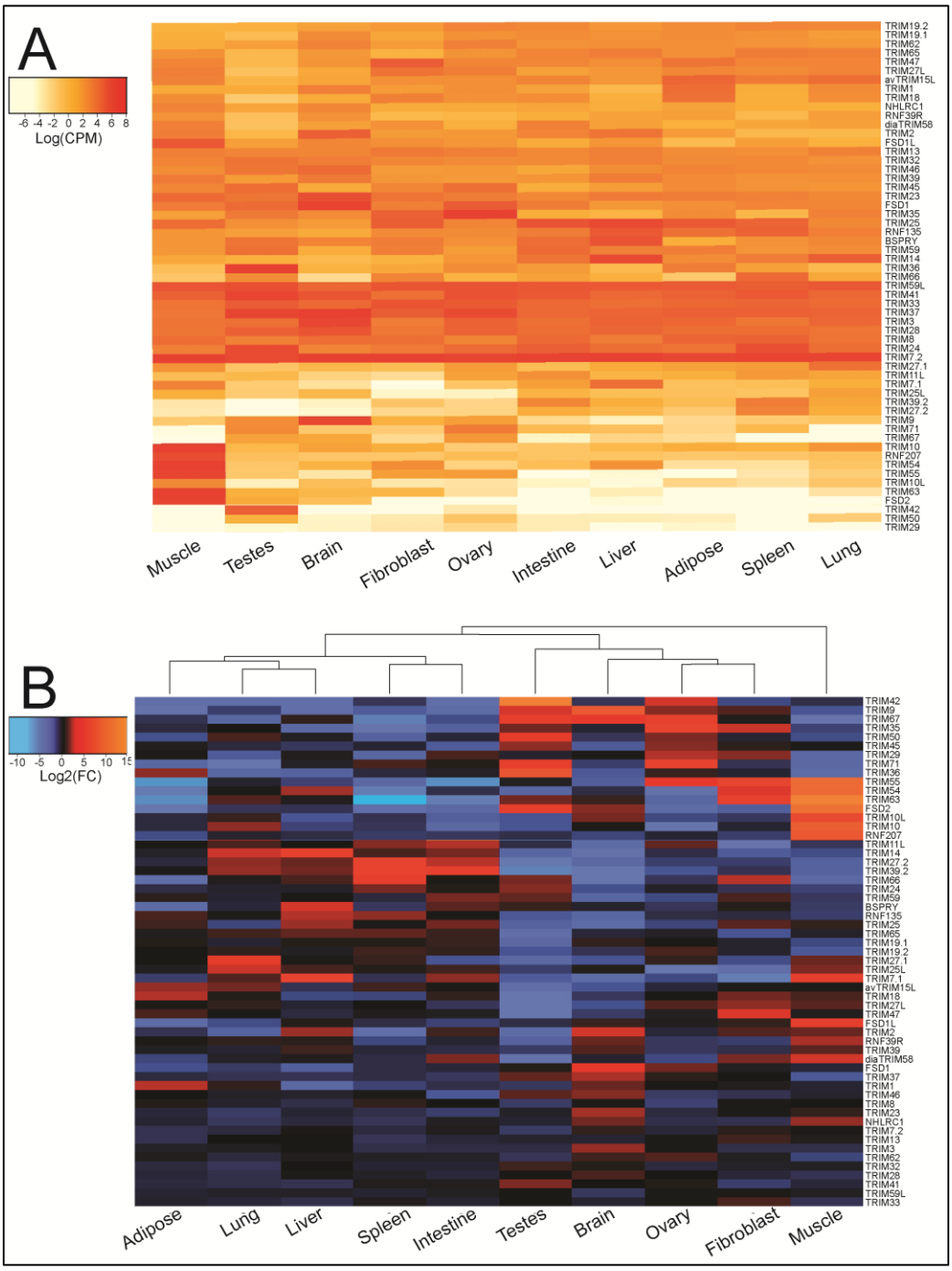


Figure 4.8. Many duck TRIM genes are ubiquitously expressed and abundant while some demonstrate tissue specific expression. The 57 duck TRIM or TRIM-like genes were used as a reference to align RNA-seq reads from muscle, testes, brain, fibroblast, ovary, intestine, liver, adipose, spleen and lung tissues collected from domestic mallard (*Anas platyrhynchos*). Mapped

TRIM gene reads were normalized to library size and averages of log read counts per million (CPM) were visualized in a heatmap (A). Reads mapped to each tissue were then compared to the average read count across all tissues to determine relative tissue expression (B). All analyses were conducted by EdgeR in the Rstudio environment. Heatmaps were created using ggplot2 in Rstudio, and all heatmaps were edited in Adobe Illustrator for clarity.

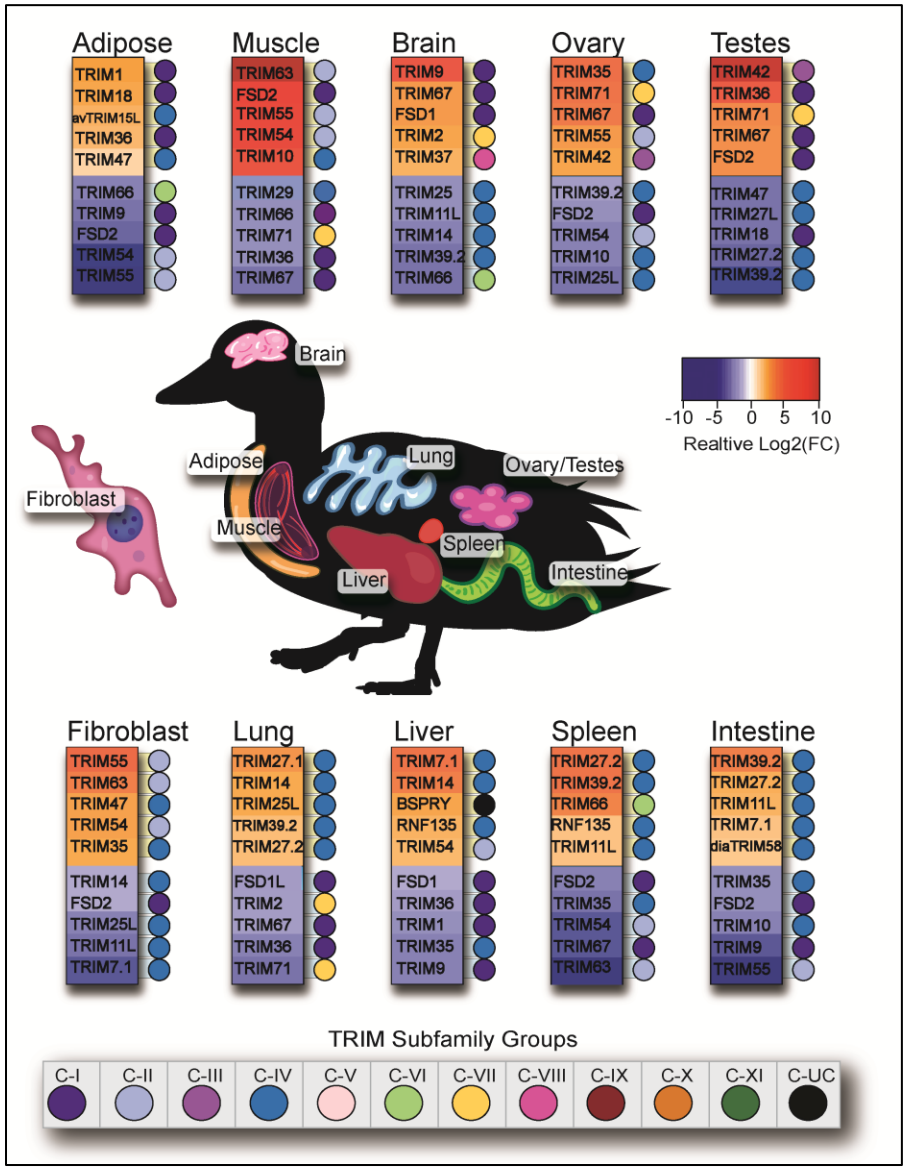
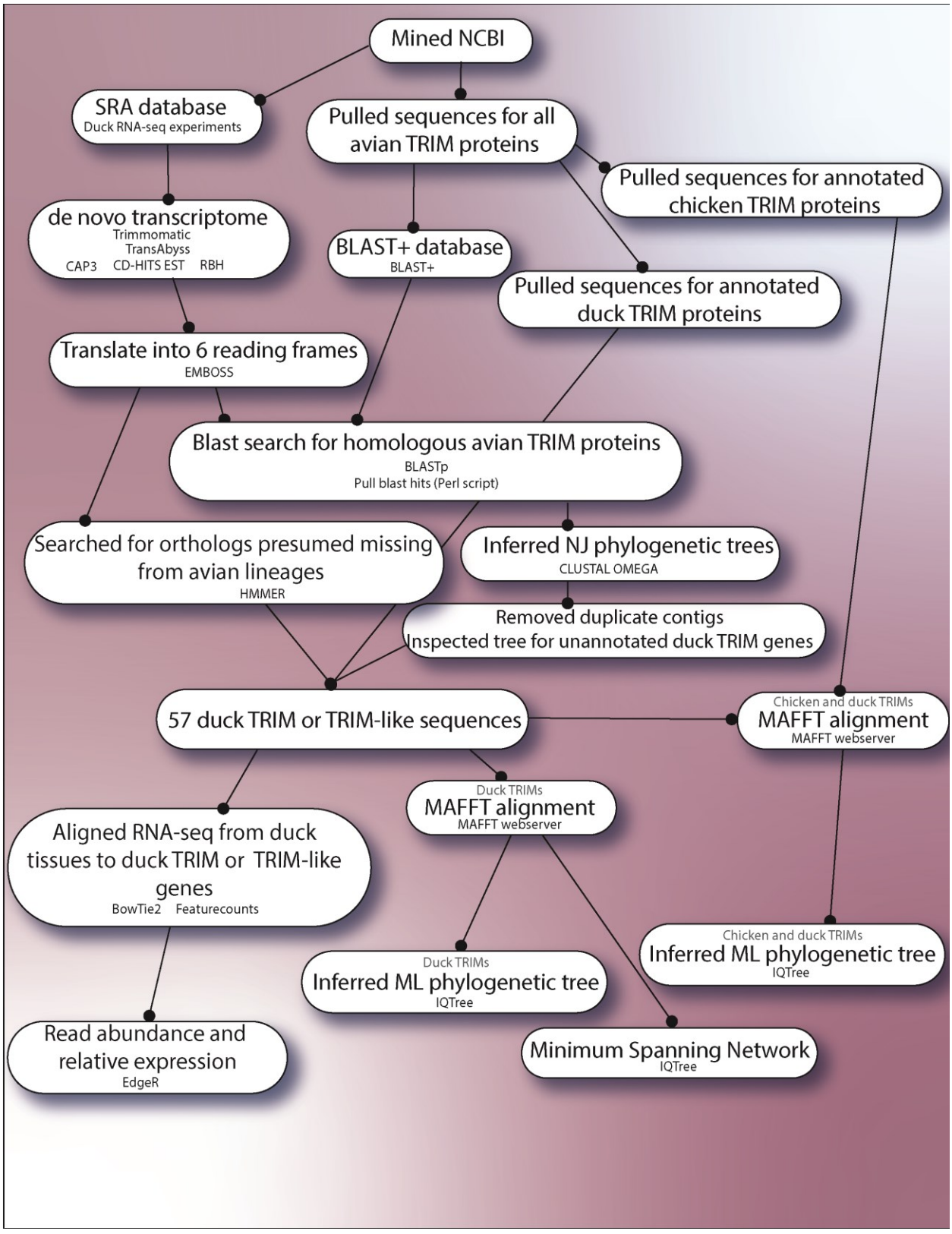


Figure 4.9. Immune relevant tissues have higher relative expression of C-IV TRIM family members. The 57 duck TRIM or TRIM-like genes expressed in muscle, testes, brain, fibroblast, ovary, intestine, liver, adipose, spleen and lung were sorted by statistical significance

(FDR<0.05) and then organized into top 5 highest or lowest relatively expressed TRIM gene for each tissue. Each TRIM gene was colour-coded according to TRIM subfamily designation.



Supplementary figure 4.1. Flow chart documenting workflow used to generate *de novo* transcriptome and find *Anas platyrhynchos* TRIM genes.

CHAPTER 5

The duck TRIM gene repertoire is upregulated in response to highly pathogenic avian influenza virus

5.1 Introduction

Tripartite motif (TRIM) proteins are an ancient family of proteins, with orthologous TRIM genes found in all eukaryotes (Marín, 2012). TRIM proteins are defined by their conserved tripartite motif, which consists of a RING, B-box and coiled-coil domain. TRIM proteins also have a variable C-terminal domains which distinguishes TRIM proteins into 11 different subfamilies, designated subfamily C-I to C-XI (Short et al., 2006; Ozato et al., 2008; Watanabe et al., 2017). Many of these proteins are involved in cellular functions such as cell cycle regulation (Hatakeyama, 2011; Hatakeyama, 2017), transcriptional regulation (Watanabe et al., 2017), autophagy (Hatakeyama, 2017) and development (Reymond et al., 2001; Berti et al., 2002). TRIM proteins also restrict pathogens, usually through E3 ubiquitin ligase activity, and can do this through pathway modulation or directly targeting pathogen components (Nisole et al., 2005; Ozato et al., 2008; Rajsbaum et al., 2014).

TRIM proteins were identified as a target of interest in species-specific antiviral protection with the discovery of the antiviral activities of TRIM5α. TRIM5α is a species-specific restriction factor against HIV, as in humans it does not effectively restrict HIV, however in monkeys TRIM5α is a very effective anti-HIV mediator (Stremlau et al., 2004; Song et al., 2005). It is possible that many more TRIM proteins act as species-specific antiviral effectors in other vertebrates, but have yet to be discovered, as very little research has been done of TRIM proteins in non-mammalian species.

Dabbling ducks (ducks belonging to the genus *Anas*) and other waterfowl are the natural host and reservoir of influenza A viruses (Webster et al., 1992; Taubenberger et al., 2010). As such, ducks can contract the virus, allow the virus to replicate to high titres, yet quickly clear the virus without exhibiting the severe symptoms and even mortality that other species, such as

humans and chickens, exhibit. However, there are some strains of virus that are lethal to ducks, such as the H5N1 circulating in Europe and Asia (Sturm-Ramirez et al., 2004; Bingham et al., 2009; Haider et al., 2017). Additionally, North America has had an H5N1 highly pathogenic avian influenzas (HPAI) strain circulating that has also killed geese, raptors and even some small carrion feeding mammals (2022b; 2022a). As ducks generally are asymptomatic when infected with IAV, they have been given the moniker "The Trojan horse of infection", as seemingly healthy birds can introduce the virus to other species (Kim et al., 2009). Low-pathogenic avian influenza (LPAI) replicates in the intestines of infected ducks (Webster et al., 1978), however due to receptor specificity of these viruses, it replicates in the lungs of infected chickens (Kuchipudi et al., 2009; Costa et al., 2012). Chickens are also more susceptible to IAV replication, which we hypothesized was due to the apparent lack of the cytoplasmic RNA-virus pattern recognition receptor (PRR) retinoic acid-inducible gene I (RIG-I) (Barber et al., 2010). RIG-I is an essential PRR in IAV viral RNA detection (Kato et al., 2006; Coch et al., 2017). We documented that RIG-I and essential components of the RIG-I signaling pathway are upregulated in ducks in response to both HPAI and LPAI strains of IAV (Campbell et al., 2021). Ducks have tight regulation of the RIG-I signaling pathway, having a quick and strong upregulation of this pathway on days corresponding to high viral titres, with an equally fast return to basal expression levels.

Many TRIM proteins modulate the RIG-I signaling pathway (as summarized in Chapter 2) both increasing and decreasing RIG-I signaling. Several TRIM proteins increase IFN production downstream of RIG-I through ubiquitination, including TRIM25 (Gack et al., 2007; Miranzo-Navarro et al., 2014), RNF135 (Hayman et al., 2019), TRIM13 (Narayan et al., 2014) and TRIM4 (Yan et al., 2014). Conversely, TRIM40 decreases IFN production downstream of RIG-I by ubiquitinating and causing the subsequent degradation of RIG-I (Zhao et al., 2017)

Ducks have orthologs to some TRIM proteins that exert anti-IAV effects in mammals. In mammals, TRIM32 directly binds and degrades the IAV protein PB1 in the nucleus (Fu et al., 2015) and has anti-IAV activities against HPAI IAV in duck cells (Wu et al., 2020). The IAV nucleoprotein (NP) is targeted for degradation by TRIM14 (Wu et al., 2019) and TRIM41 (Patil et al., 2018). Ducks have TRIM14 and TRIM41 (Chapter 4) but neither of these proteins has been functionally characterized in the duck. TRIM35 targets PB2 for degradation which prevents PB2 from inhibiting antiviral signaling pathways (Sun et al., 2020). TRIM25 targets IAV

ribonucleoprotein in the nucleus to stop viral synthesis in mammals (Meyerson et al., 2017). TRIM25 also augments RIG-I signaling through catalyzing the addition of K63-linked polyubiquitin chains to RIG-I, stabilizing RIG-I and increasing the interaction between RIG-I and MAVS in both mammals and ducks (Gack et al., 2007; Miranzo-Navarro et al., 2014) however the duck TRIM25 can also add unlinked ubiquitin to RIG-I to stabilize it.

Many TRIM proteins are upregulated by interferon and viral infection, in fact over half the human TRIM gene repertoire is upregulated by IFNs or immune pathway stimulation (Carthagena et al., 2009; Versteeg et al., 2013). TRIM genes in non-human mammalian hosts are also upregulated in response to viral infection such as TRIM21 and 38 in pigs (Yang et al., 2022). Bovine macrophages stimulated with PAMPs upregulated 8 of the 46 TRIM genes analyzed (Toka et al., 2017). Our examination of transcriptional responses of ducks infected with IAV suggested that TRIM genes are among the genes upregulated (Campbell et al, 2021), but this was not formally investigated.

Our previous analysis of the duck TRIM gene repertoire identified several genes with potential for antiviral roles. Promyelocytic Leukemia protein (PML) or TRIM19 in humans is a known antiviral effector in mammals (Chelbi-Alix et al., 1998; Nisole et al., 2005) that can also restrict IAV (Iki et al., 2005). Interestingly, birds appear to have two paralogs of TRIM19, TRIM19.1 and TRIM19.2 (Chapter 4). TRIM27.1 and TRIM27L both belong to the C-IV subfamily and arose during a duplication event in the MHC locus of birds (Kaufman et al., 1999; Chaves et al., 2009; Blaine et al., 2015; Kaufman, 2022). While TRIM27.1 appears in all birds, TRIM27L appears to be missing in the Galliformes (Blaine et al., 2015). We also identified a gene that appears to be specific to birds and reptiles, which we have named diaTRIM58 due to its closeness in phylogenetic analysis to mammalian TRIM58 (Chapter 4).

Here we investigate the regulation of the TRIM or TRIM-like genes in ducks during infection with an HPAI H5N1 (A/Vietnam/1203/04) or an LPAI H5N6 (A/mallard/BC/500/05). We also investigate several interesting TRIM proteins for potential anti-IAV effects in both immortalized chicken cells and primary embryonic duck fibroblasts.

5.2 Materials and methods

5.2.1 Viral infection with HPAI and LPAI viruses and RNA collection

The viruses used in all experiments were the reverse genetics HPAI H5N1 strain A/Vietnam/1203/04 (VN1203) and the LPAI H5N2 strain A/mallard/BC/500/05 (BC500). Virus infections were previously performed, and methodologies used for viral infection and RNA extraction were previously published (Barber et al., 2010; Vanderven et al., 2012; Campbell et al., 2021). Briefly, 6-week old outbred Pekin ducks were infected through the natural route with 10^6 of 50 % egg infectious doses (EID) of either VN1203 or BC500 or mock infected with PBS. After 1-, 2- or 3-days post infection (dpi) ducks were humanely euthanized. From ducks infected with VN1203 lung and spleen tissues were collected from 6 individuals at each time point, but only the 4 with the best quality RNA were used. From mock infected ducks and ducks infected with BC500 lung, spleen and intestine tissues were collected from 3 ducks at each time point. RNA was extracted from tissues using TRIzol reagent (Invitrogen). Samples were DNAse treated and purified using PuroSPIN™ Total RNA Purification Kit (Luna Nanotech) and stored at -80 °C. RNA was determined to be of sufficient quality to sequence using Agilent Technologies 2100 Bioanalyzer High Sensitivity DNA Chip.

5.2.2 Library construction and sequencing

Library preparation and sequencing was performed as previously described (Campbell et al., 2021). All Library preparation and poly-adenylated RNA sequencing were performed by LC Sciences (https://www.lcsciences.com/). A total of 72 libraries were created for sequencing. Libraries were not made for tissues (spleen, lung and intestine) from two individual mock treated ducks at the 2 dpi timepoint due to insufficient RNA quality.

All sequences used in these experiments can be retrieved from the NCBI short sequence read archive (SRA) repository under the BioProject ID PRJNA767080.

5.2.3 Differential expression analysis

RNA sequences were initially trimmed for adaptors and low-quality bases by LC Sciences using CutAdapt (Martin, 2011). All RNA-seq libraries were verified for quality using FastQC (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/). We removed any unpaired reads and further separated these reads into pairs using Trimmomatic (Bolger et al., 2014). RNA-seq libraries were aligned to the 57 TRIM or TRIM-like genes previously identified by our group (Chapter 4) using Bowtie2 v2.3.4.3 (Ben et al., 2011). We counted, summarized and assigned reads from the Bowtie2 alignment using FeatureCounts (Liao et al., 2014). The output from FeatureCounts was loaded into EdgeR (Robinson et al., 2010) in the RStudio v4.1.3 (RStudio

Team, 2020) environment. All libraries were normalized using the trimmed mean of M values (TMM) method. We analyzed differential expression of TRIM genes across all time points (1, 2 and 3 dpi) in tissues from VN1203 or BC500 infected ducks compared to mock using EdgeR makecontrast function and used a general linearized model (GLM) to estimate the variance of TRIM gene expression across time points for each tissue, when compared to mock for each tissue. We filtered results by false discovery rate (FDR) < 0.05 and used the hits from each tissue to make heatmaps using gplots heatmap.2 function (Gregory R. Warnes, 2022). All heatmaps were modified in Adobe Illustrator for clarity.

5.2.4 Cloning and expression plasmid construction

To amplify TRIM32 and diaTRIM58 coding sequences from duck, TRIM32 and diaTRIM58 sequences were extracted from Ensembl and primers were designed in the 5′- and 3′-untranslated regions (UTRs). PCR amplification was performed on cDNA synthesized from RNA collected from a lung from a duck infected with VN1203 on 1 dpi. cDNA was synthesized using SuperScript III (Invitrogen) and oligo dT primers. Primers used to amplify genes are presented in Table 5.1. Resulting amplification fragments were inserted into a pCR2.1 TOPO cloning vector (Invitrogen). Using either pCR2.1-TRIM32 or pCR2.1-diaTRIM58 as a template, primers were designed to add a 5′-NheI restriction site and a 5′-2xFLAG epitope tag to each template. Reverse primers were designed to add a 3′-KpnI restriction site. 2xFLAG-TRIM32 and 2xFLAG-diaTRIM58 were then cloned into the pcDNA3.1+(hyg) cloning vector. All PCR reactions were performed using Phusion polymerase (NEB)**.**

TRIM19.1 and TRIM19.2 could not be amplified in one piece due to sequence complexity (Supplementary figure 5.1) and were instead amplified in overlapping fragments. TRIM19.1 was amplified from cDNA from the lung of a 4-week-old Pekin duck donated by Duckscetera (Bonnyville, Alberta). cDNA was made using Superscript IV with a mix of oligo dTs and random hexamers. The second fragment was amplified using nested PCR, while the first and third fragments were amplified using standard PCR. All PCRs were performed using Phusion polymerase (NEB) with the first and third fragment amplified with the addition of 1M Betaine (ThermoFisher), and the second fragment amplified with the addition of 2.5 % DMSO (Thermofisher). TRIM19.2 was amplified from cDNA made from the brain of a 3-week-old Pekin duck donated from Duckscetera (Bonnyville, Alberta). cDNA was made using SuperScript IV with gene specific primers designed to amplify from a region in the 3′-UTR. Both TRIM19.2

fragments were amplified using nested PCR, using Phusion (NEB) and Betaine (ThermoFisher) reagent. Sequences for both TRIM19.1 and TRIM19.2 were verified and compared to the annotated duck TRIM19 genes on NCBI. Expression constructs were synthesized by Genscript (https://www.genscript.com/). Both genes were synthesized into a pcDNA3.1+(hyg) vector. TRIM19.1 was synthesized with a 5′-2xFLAG epitope tag while TRIM19.2 was synthesized with a 5′-Myc epitope tag. The 5′ end of both TRIM19 paralogs was chosen for epitope tag location due to mammalian TRIM19 genes retaining their function with 5′ FLAG epitope tags (Quimby et al., 2006; Jul-Larsen et al., 2010).

Cloning and expression construct design and amplification were previously reported for TRIM25 (Miranzo-Navarro et al., 2014), TRIM27.1 and TRIM27L (Blaine et al., 2015). Briefly, both TRIM25 TRIM27.1 and TRIM27L sequences were amplified from Pekin duck cDNA, had 3′ V5-epitope tags added by PCR amplification and were inserted into pcDNA3.1(+)hyg expression vectors. TRIM25-V5 was amplified and cloned by Domingo Miranzo-Navarro and kindly provided by Danyel Evseev. TRIM27.1-V5 and TRIM27L-V5 were amplified, cloned and provided by Alysson Blaine.

5.2.5 DEF extraction and cell culture conditions

Pekin duck eggs were acquired from Duckscetera (Bonnyville, Alberta) and were incubated at 37 °C in a humid environment for 12 days before harvest. Embryos were removed from eggs, organs and heads were removed, and embryonic trunks were rinsed with Hank's balanced salt solution (Gibco). Trunks were minced with a scalpel, then homogenized in Hank's balanced salt solution using a Power Gen 125 homogenizer (FisherScientific). Trunks were then incubated in 2.5 % trypsin (Gibco) for 30 minutes on a rotator at room temperature. The resulting mixture was strained using a 100 µm cell strainer (Corning) and then spun down at 2500 rpm at 8 °C. The supernatant was carefully removed, then DMEM + 20 % fetal bovine serum (FBS) (Gibco) was added to the pellet before it was spun down again for 5 minutes (1500 rpm at 8 °C). The supernatant was removed from the pellet, and DMEM without added FBS was added to resuspend the pellet. The cell mixture was spun down again (1500 rpm at 8 °C). The supernatant was removed and the pellet was resuspended in DMEM + 10 % FBS and strained using a 40 µm cell strainer. Cells were allowed to grow to 90 % confluency in T75 flasks and then were stored in liquid nitrogen at a density of $1.5x10^6$ cells/mL in freezing medium (DMEM + 10 % FBS + 10 % DMSO). All primary duck embryonic fibroblast (DEF) cells were passed 3 times before

transfecting. Both DF-1 and DEF cells were grown at 39 °C + 5 % CO_2 in DMEM + 10 % FBS (Gibco).

5.2.6 Transfection of cells

To determine antiviral ability of TRIM19.1, TRIM19.2, TRIM25, TRIM27.1, TRIM27L, TRIM32 and diaTRIM58, expression plasmids containing these TRIM gene were transfected into either immortalized chicken DF-1 cells or DEF cells. To transfect DF-1 cells, cells were seeded at a density of $1x10^6$ cells per well 24 hours post transfection in 6-well plates. Using 2 µg of plasmid DNA per well, we followed the Lipofectamine™ 2000 (Invitrogen) manufacturer's protocol to transfect cells. Cells were transfected for 18 hours before proceeding with infection. To transfect DEF cells, we used a combination of JetOPTIMUS® transfection reagent (PolyPlus Transfection), FuGENE® HD transfection reagent (Promega) and CombiMag magnetofection reagent (OZ BioSciences). DEF cells were seeded at a density of $1.5x10^6$ cells per well in a 6-well plate and allowed to grow for 24 hours. To magnetofect the DEF cells, we used 2 µg plasmid DNA per well at a ratio of 1µg:1µL plasmid DNA: JetOPTIMUS® and FuGENE® and 1µg:1µL plasmid DNA: CombiMag reagent ratios. Briefly, plasmid DNA was mixed thoroughly with JetOPTIMUS reagent in Opti-MEM™ buffer (Gibco) and in a separate tube with FuGENE reagent in Opti-MEM™ buffer. The mixtures were incubated for 5 minutes at room temperature before being added to a new tube containing CombiMag. This mixture was incubated at room temperature for 20 minutes. Once the incubation was complete, the cells were given fresh media (DMEM + 10% FBS) and each mixture (JetOPTIMUS + CombiMag and FuGENE + CombiMag) was pipetted onto the cells drop-wise. The 6-well plate was then incubated on a super magnetic plate (Oz Biosciences) for 20 minutes. Fresh media was added to cells after the magnetofection reaction was complete.

5.2.7 Confocal microscopy

DF-1 cells were transfected for 24-hours, then fixed in 2 % paraformaldehyde (Sigma) for 20 minutes. Cells were permeabilized using 0.25 % Triton-X (Sigma). Cells were blocked for 1 hour in 4 % bovine serum albumin (BSA, HyClone). V5 and FLAG epitope tags were stained with anti-V5 Alexa Fluor (647451098, ThermoFisher) and anti-FLAG iFluor 647 (A01811100, ThermoFisher). Nuclei were stained using Hoechst 33342. Cells were visualised using a WaveFx confocal microscope at the Cell Imaging Centre located at the University of Alberta.

5.2.8 DEF and DF-1 infections with IAV

Both DF-1 and DEF cells were infected 18 hours post transfection. DF-1 or DEF cells were infected with either the H6N2 avian strain A/Ck/Ca/431/2000 (CA431) or the mouse-adapted H1N1strain A/Puerto Rico/PR8/1934 (PR8). The IAV strains used for the transcriptome study, VN1203 and BC500, were not used for these infections as we are unable to import these viruses into Canada. Prior to infection, cells were washed 3x with PBS. Virus was added at a multiplicity of infection (MOI) of 1 in infection media (DMEM + 0.3 % BSA) plus 0.1 µg/mL of TPCK trypsin. Cells and virus were incubated at 39 °C for 1 hour and rocked every 20 minutes. After the incubation cells were washed 3x with PBS and fresh infection media plus TPCK trypsin was added. Infections were allowed to progress for 24 hours, then cells were washed, trypsinized in 0.25 % Trypsin + EDTA (Gibco) for 5 minutes. Fresh media was added to wells to stop trypsinization, and cells were spun down at 400 x g for 5 minutes. Supernatant was removed from cells and cells were washed in 1 mL PBS. Cells were spun down again at 400 x g for 5 minutes, supernatant was removed, and 2 % paraformaldehyde (PFA) (pH 7.4 in PBS) was added to the cells. Cells were incubated in PFA for 20 minutes at room temperature.

5.2.9 Flow cytometry staining and analysis

Fixed DF-1 and DEF cells were permeabilized by incubating cells in 0.25 % Tween-20 (Sigma) for 15 minutes. Cells were washed 3 times in wash buffer (1x PBS + 0.5 % BSA). Cells were then blocked in 4 % BSA for 1 hour with rocking. All antibodies and stains were diluted in 4 % BSA. Anti-nucleoprotein (NP) conjugated to FITC MA-7322 (Invitrogen) was used to stain cells infected with CA431 while anti-NP-FITC ab210526 (Abcam) was used to stain cells infected with PR8. To visualize epitope tags, fixed cells were incubated with anti-V5 Alexa-Fluor 647 (451098, ThermoFisher) and anti-FLAG iFluor 647 (A01811100, ThermoFisher). Cells were incubated with antibodies for 1 hour then washed 3 times in PBS + 0.5 % BSA. Compensation controls consisted of anti-NP FITC only, Anti-V5 and Anti-FLAG iFluor 647 only, Hoechst only and unstained cells.

Flow cytometry analysis was performed on stained cells in the University of Alberta Faculty of Medicine and Dentistry Flow Cytometry Facility, with grant support from Canadian Institutes of Health Research (CIHR) and financial support from the Faculty of Medicine and Dentistry.

5.3 Results

5.3.1 VN1203 infection differentially regulates 36 TRIM genes in lungs of infected ducks

To determine the differential expression response of TRIM genes in lungs of ducks infected with VN1203 or BC500, we aligned our RNA-seq libraries to 57 TRIM or TRIM-like genes. The 57 TRIM or TRIM-like genes used for alignment were previously identified by us through data mining of NCBI and *de novo* transcriptome assembly (Chapter 4). Genes were subjected to a GLM test of variance and filtered for statistical significance (FDR < 0.05). Infection with VN1203 induced a robust DE response in lungs of infected birds, with 36 TRIM genes DE (Figure 5.1A). In comparison, infection with BC500 only induced 9 genes to be DE in the lung (Figure 5.1B). RNF135, TRIM25, TRIM19.1 and TRIM19.2 are all strongly upregulated in lungs of ducks infected with VN1203 and BC500. Likewise, TRM13 and the suspected pseudogene TRIM25L are both strongly downregulated in lungs of ducks infected with VN1203 and BC500. Some of the MHC-linked TRIM genes were also upregulated, including TRIM27L and TRIM27.1, agreeing with our previous qPCR analysis of these genes in infected tissues (Blaine et al., 2015). Additionally, the MHC-linked genes TRIM7.2, TRIM27.2 and TRIM39.2 showed differential regulation over all 3 dpi, however TRIM27.2 and TRIM39.2 were upregulated on 1 dpi while TRIM7.2 was downregulated. The Diapsid lineage specific TRIM diaTRIM58 was also upregulated on 1 dpi in lungs of ducks infected with VN1203.

5.3.2 VN1203 infection differentially regulates 28 TRIM genes in spleen of infected ducks

To determine the differential expression response of TRIM genes in spleen of ducks infected with VN1203 or BC500, we aligned our RNA-seq libraries to 57 TRIM or TRIM-like genes. Mapped reads were subjected to GLM test of variance and filtered for statistical significance (FDR < 0.05). VN1203 infection induced much more differential expression of TRIM genes in spleens (28 genes) when compared to spleens from ducks infected with BC500 (7 genes) (Figure 5.2). RNF135, TRIM13, TRIM19.1, TRIM19.2, TRIM35 and diaTRIM58 were similarly upregulated in spleen of ducks infected with either VN1203 or BC500. TRIM25L was slightly upregulated on 1 dpi then downregulated on 2 and 3 dpi in spleens of ducks infected with BC500, however in spleens of ducks infected with VN1203 TRIM25L was downregulated on all 3 dpi. Similar to of ducks infected with VN1203, many of the MHC-linked genes were DE in the spleen in response to VN1203 infection (Figure 5.2A). TRIM10, TRIM10L, TRIM27,1 and TRIM27L are duck MHC-linked TRIM genes which were all upregulated in response to VN1203 infection in the spleen. Interestingly, TRIM10 is downregulated in lungs of VN1203

infected ducks, however it is upregulated in spleens of these same ducks.

5.3.3 11 TRIM genes are differentially expressed in intestines of BC500 infected ducks

To determine the differential expression response of TRIM genes in intestines of ducks infected with BC500, we aligned our RNA-seq libraries to 57 TRIM or TRIM-like genes. Mapped reads were subjected to a GLM test of variance and filtered for statistical significance (FDR < 0.05). Surprisingly, there were only 11 TRIM genes DE over the 3 days post infection in intestines of ducks infected with BC500 (Figure 5.3). RNF135, TRIM19.1 and TRIM19.2 were upregulated in intestines of ducks infected with BC500. Most of the genes which were highlighted as being DE were upregulated on 2 dpi, except for TRIM8, TRIM41 and TRIM62. These genes were downregulated on 2 dpi.

5.3.4 Comparison of TRIM genes between tissues infected with either VN1203 or BC500

To determine which TRIM genes were similarly upregulated or had unique expression specific to their tissue, we used diVenn to compare expression between tissues. Genes were considered up or downregulated depending on the predominance of differential expression over all 3 dpi and coded accordingly. The resulting Venn diagram shows 20 genes that are similarly DE in both lung and spleen from ducks infected with VN1203 (Figure 5.4A). RNF135, TRIM13, 19.1, 19.2, 25, 27.1, 27L and diaTRIM58 are upregulated in both lung and spleen of ducks infected with VN1203. Genes downregulated in both lung and spleen in ducks infected with VN1203 were BSPRY, RNF39R, TRIM3, 8, 25L, 59L and 62. There were 5 genes that were upregulated in one tissue but downregulated in the other in lung and spleen. TRIM10 is upregulated in spleen but downregulated in lung. TRIM23, 24, 33 and 37 are upregulated in lung but downregulated in spleen. In ducks infected with VN1203 there were 15 TRIM genes that were uniquely DE in lung, and 7 that were uniquely DE in spleen.

As there were many fewer TRIM genes DE in ducks infected with BC500, there were only 3 genes that were upregulated in lung, spleen and intestine: RNF135, TRIM19.1 and TRIM19.2 (Figure 5.4B). There were no TRIM genes that were downregulated in all tissues in BC500 infected ducks. TRIM25L was downregulated in both lung and spleen, while TRIM13

was upregulated in both spleen and intestine. There were 5, 2 and 7 genes uniquely expressed in lung, spleen and intestine (respectively) of BC500 infected ducks.

5.3.5 Overexpressed TRIM proteins localize to distinct subcellular locations

We selected several candidate TRIM genes to test for anti-IAV activity. We amplified TRIM coding sequences for TRIM19.1, TRIM19.2, TRIM25, TRIM27.1, TRIM27L, TRIM32 and diaTRIM58. Once amplified, an epitope tag of either V5, c-Myc or 2xFLAG was cloned onto either the 5′ end of TRIM19.1 and 19.2. TRIM27.1, 27L, 25, 32 and diaTRIM58 had epitope tags added to 3′ end of the gene (Figure 5.5A). The resulting fragment was placed in a pcDNA3.1+(hyg) expression vector. We confirmed TRIM expression constructs for protein expression by transfecting vector and insert into chicken DF-1 cells for 24 hours, staining for relevant epitope tags and visualizing using confocal microscopy (Figure 5.5). TRIM19.1 localized partially to the nucleus of transfected cells, however we observed a large cytoplasmic localization of protein (Figure 5.5B). We were unable to visualize TRIM19.2 using confocal microscopy, however PCR targeting a fragment of TRIM19.2 including the added epitope tag could be amplified from cDNA, and a western blot against the Myc epitope could detect protein, albeit at a low amount compared to V5 and FLAG controls (Supplementary figure 5.2). TRIM27.1 localized to the cytoplasm of both cell types and formed hollow tube-like structures (Figure 5.5C). TRIM27L localized to the cytoplasm and formed dense aggregates, however some protein was detected in the nucleus (Figure 5.5D). TRIM25 localized diffusely in the cytoplasm of transfected cells (Figure 5.5E). Both TRIM32 and diaTRIM58 form similar cytoplasmic aggregates (Figure 5.5F and 5.5G).

5.3.6 TRIM27, TRIM32 and diaTRIM58 can restrict both PR8 and CA431in DEF cells

To determine if TRIM19.1, 19.2, 25, 27.1, 27L, 32 and diaTRIM58 overexpression can restrict IAV, TRIM expression plasmids were transfected into either chicken DF-1 or DEF cells for 18 hours. Cells were then infected for 24 hours with an MOI=1 with either a LPAI avian H6N2 A/Ck/CA/431/2000 (CA431) or a mouse adapted H1N1 A/Puerto Rico/8/1934 (PR8) (Figure 5.6). We then analyzed cells for percent infection using flow cytometry. Overexpression of TRIM19.1, 19.2, 27.1 and 25 did not reduce overall percent infection of avian CA431 in DF-1 or DEF cells (Figure 5.6A). TRIM27L did not reduce the percent infection of CA431 in DF-1 cells but did reduce overall percent infection in DEF cells. Overexpression of TRIM32 reduced the overall percent infection of CA431 by approximately 20% in DF-1 cells and 40% in DEF cells.

Likewise, overexpression of diaTRIM58 reduced the percent infection in DF-1 cells by 20% and in DEF cells by 40%. Surprisingly TRIM25 overexpression did not significantly reduce CA431 infection in either DEF or DF-1 cells. Cells were stained with anti-epitope tag antibodies to confirm transfection, however after 24 hours the overall percent infected cell populations remained low for both CA431 infected DF-1 and DEF cells (Table 5.2) and PR8 infected DF-1 and DEF cells (Table 5.3). Previous experiments found that TRIM protein expression peaked at 24 hours post transfection (Data not shown). It is unknown if the low transfection rates are due to viral interference or cells rapidly reducing expression of transiently transfected genes after 24 hours.

To ensure that any antiviral or lack of antiviral effect was not due to the strain of virus being able to target or utilize the TRIM proteins, we repeated these infection experiments using the mouse adapted PR8 (Figure 5.6B). Surprisingly, TRIM25 was still unable to reduce PR8 in either DF-1 or DEF cells. As with CA431, diaTRIM58 was able to restrict PR8 replication in both DF-1 and DEF cells. TRIM32 was not able to restrict PR8 in DF-1 cells but did significantly reduce percent infection in DEF cells. Surprisingly, TRIM19.1 showed a statistically significant restriction of PR8 in DEF cells only, although this only reduced overall percent infection by approximately 10% in these cells. TRIM27L was still unable to restrict PR8 in DF-1 cells but could significantly restrict PR8 replication in DEF cells. This result suggests that TRIM27L is interacting with signaling components present only in DEF cells and is likely not restricting through direct mechanisms. TRIM19.2 and TRIM27.1 did not significantly affect the overall relative infection in PR8 infected DF-1 or DEF cells.

5.4 Discussion

Here we continue our previous work on characterizing the duck TRIM gene repertoire by investigating transcriptional regulation of these genes in response to IAV. We demonstrated that the HPAI VN1203 induced much stronger differential expression (DE) of TRIM genes in lungs and spleen of infected ducks than the LPAI BC500 IAV strain did. Sixteen TRIM genes were similarly expressed (up or downregulated) by VN1203 in lung and spleen and 5 of the duck TRIM genes had opposite DE in lung compared to spleen (upregulated in one tissue, downregulated in the other). Only 5 TRIM genes were similarly DE in lung, spleen and intestine of BC500 infected ducks. From the lists of TRIM genes DE in ducks infected with BC500 or

VN1203, we chose TRIM19.1, TRIM19.2, TRIM25, TRIM27.1, TRIM27L, and diaTRIM58 to study as potential antiviral effectors. TRIM19.1 and TRIM19.2 were upregulated by both VN1203 and BC500 in all tissues sampled. TRIM25, TRIM27.1, TRIM27L and diaTRIM58 are upregulated during VN1203. TRIM32 is upregulated slightly in lung of ducks infected with VN1203 and has previously documented antiviral activity in duck (Wu et al., 2020). We found that TRIM32, diaTRIM58 and TRIM27L reduced IAV infection in duck cells, while TRIM19.1, TRIM19.2, TRIM25 and TRIM27.1 did not reduce virus titre. Interestingly, while TRIM32 and diaTRIM58 also reduced IAV virus in chicken DF-1 cells, TRIM27L did not.

Over half the TRIM repertoire is DE in response to VN1203 infection in lung and spleen of infected ducks. Out of the 57 TRIM or TRIM-like genes we previously documented in ducks (Chapter 4) 36 TRIM genes were DE in lungs of infected ducks and 28 TRIM genes were DE in spleen of infected ducks. Surprisingly, BC500 induced very few TRIM genes to be DE in lung, spleen or intestine of infected ducks. We previously documented tissue specific transcriptional responses of all genes in ducks infected with both VN1203 and BC500 and demonstrated that while VN1203 induced much more differential expression in lung and spleen, BC500 also induced a strong amount of differential expression of transcripts in the intestines (Campbell et al., 2021). A study looking at TRIM differential expression in response to both type I and type II IFNs in human macrophages and leukocytes found 27 of the 75 human TRIM genes sampled changed expression (Carthagena et al., 2009). Reporter assays using the IFN-β, ISRE or NK-κB reporter promoters found that over half of the 75 cloned human TRIM genes activated these important immune signaling pathways (Versteeg et al., 2013).

Overall, we found 18 TRIM genes upregulated lungs of ducks infected with VN1203 and 15 genes upregulated in spleens on all 3-dpi. BC500 infection only caused upregulation of 5 TRIM genes in the lung and 7 in the spleen. RNF135, TRIM19.1, TRIM19.2 and TRIM25 were upregulated in lung in response to VN1203 and BC500. RNF135, TRIM13, TRIM19.1, TRIM19.2, diaTRIM58 and TRIM35 were similarly upregulated by BC500 and VN1203 infection in the spleen. TRIM29 was uniquely upregulated in the lung in response to BC500. It is worth noting that inspection of the aligned reads reveals TRIM29 has very low read counts, and this increase across all 3 dpi may be a sequencing artifact, and indeed in our previous analysis of TRIM gene expression in healthy duck tissue, TRIM29 has negligible expression in all tissues, with very low expression in fibroblast, ovary and intestine (Chapter 4). In humans TRIM29 is

highly expressed in airway epithelial cells and negatively regulates IFN-β production through inhibition of STING (Xing et al., 2018). TRIM29 upregulation in lungs of BC500 but not VN1203 infected may be a protective response, as VN1203 but not BC500 replicates in lung, and upregulating TRIM29 during VN1203 could aid viral replication. RNF135, TRIM19.1, and TRIM19.2 are upregulated in lung and spleen by both VN1203 and BC500 infection. TRIM25 is upregulated in both lung and spleen in ducks infected with VN1203, and only in lung of ducks infected with BC500. RNF135 and TRIM25 both stabilize RIG-I to increase downstream IFN-β signaling (Gack et al., 2007; Oshiumi et al., 2010; Hayman et al., 2019). RIPLET catalyzes the addition of K63 linked ubiquitin to K788 of RIG-I C-terminal domain (Oshiumi et al., 2009; Oshiumi et al., 2013). This causes structural changes in RIG-I which allows TRIM25 to access the CARD domains of RIG-I. TRIM25 facilitates the addition of K63-linked ubiquitin to the second CARD domain of RIG-I (Gack et al., 2007), which causes the tetramerization of RIG-I CARD domains and subsequent activation of MAVS (Peisley et al, 2014). We previously demonstrated that duck TRIM25 mutated at both lysine residues used for TRIM25 attachment of ubiquitin chains is still active, suggesting unattached K63-linked polyubiquitin chains can activate duck RIG-I CARD domains (Miranzo-Navarro et al., 2014). It has recently been suggested that RNF135 is more important than TRIM25 for activation of full-length RIG-I through C-terminal stabilization (Hayman et al., 2019). RIPLET has not been functionally characterized in ducks.

The muscle specific ring finger (MuRF) TRIM genes TRIM54 and TRIM63 (Perera et al., 2012) both are downregulated in lung during infection with either VN1203 or BC500. TRIM10 is also downregulated in lung, and we have previously documented duck TRIM10 as being highly expressed in muscle of healthy ducks. Interestingly, when we performed a global transcriptional analysis of lungs from ducks infected with VN1203 we saw subsets of many genes involved in muscle function downregulated (Campbell et al., 2021).

Curiously, very few TRIM genes are differentially expressed in intestines of BC500 infected ducks. BC500 preferentially replicates in intestines of infected ducks. Indeed, cloacal swabs of ducks infected with BC500 found incredibly high titres of this virus, in fact these titres were much higher than those found in trachea of VN1203 infected ducks (Vanderven et al., 2012). This is in stark contrast to the overall number of genes DE in intestines of BC500 infected ducks (Campbell et al., 2021). Eight genes were upregulated on all 3 dpi in intestines, with 3

genes downregulated on days all 3 dpi. Many of these upregulated genes are also upregulated in lung or spleen of VN1203 or BC500 infected ducks, such as RNF135, TRIM19.1, TRIM19.2, TRIM13, TRIM32 and TRIM47. TRIM9 and TRIM18 are genes which only show differential expression in intestines of BC500 infected ducks and are upregulated in response to BC500 infection. TRIM9 is enriched in neuronal cells in humans (Berti et al., 2002; Uhlen et al., 2015) and inhibits NF-κB signaling in neuronal tissues to decrease inflammation in the brain (Shi et al., 2014). There are two orthologous TRIM9 genes in shrimp, both of these genes appear to interact with the NF-κB signaling pathway in shrimp, with TRIM9 increasing NF-κB signaling, and TRIM9-1 decreasing it (Sun et al., 2019; Sun et al., 2022). In humans, TRIM13 inhibits MDA5 activation, while also positively regulating RIG-I (Versteeg et al., 2013; Narayan et al., 2014), suggesting the upregulation of TRIM13 in intestines of infected ducks could be tailoring the antiviral response to influenza.

Several TRIM genes were upregulated in one tissue and downregulated in the other in spleen and lung in ducks infected with VN1203, TRIM23 is upregulated on 2 and 3 dpi in lung and downregulated on all 3 days post infection in spleen in ducks infected with VN1203. In mice, TRIM23 can restrict IAV but not HSV in a selective autophagic manner (Sparrer et al., 2017). As TRIM23 is highly conserved across vertebrates, it is possible duck TRIM23 is also restricting IAV, and is upregulated to contain viral replication later in the infection cycle. TRIM33 is upregulated on 2 and 3 dpi in lung but downregulated in spleen of VN1203 infected ducks. This may be a protective response. TRIM33 is a transcription factor that inhibits late stage *ifnb* promoter activity in mouse macrophages but not fibroblasts (Ferri et al., 2015). This transcriptional repression likely helps prevent tissue damage from out-of-control inflammatory responses. We investigated transcriptional responses of proinflammatory genes in ducks infected with VN1203, many of them were upregulated on 1 dpi, and then rapidly dropped back down to basal levels on 2 and 3 dpi (Saito et al., 2018; Fleming-Canepa et al., 2019; Campbell et al., 2021). If duck TRIM33 is functionally analogous to mammalian TRIM33, it could be reducing inflammation, however TRIM33 has not yet been functionally characterized in birds. The opposite regulation of select TRIM genes in lung and spleen in VN1203 infected ducks may also be due to differences in tissues or migration of cells expressing them. TRIM genes have cell specific function and expressions. Our transcriptome data comes from samples of whole tissues.

Further analysis using purified cell types from different tissues could determine which specific cells the transcriptional changes are coming from.

TRIM27L but not TRIM27.1 restricted viral replication in DEF but not DF-1 cells. TRIM27L and TRIM27.1 are located in the MHC region, and TRIM27L appears to have been lost in the galliform lineage (Blaine et al., 2015). We have demonstrated that TRIM27L could activate chicken IFN-β promoter activity in DF-1 cells when cotransfected with constitutively active RIG-I, suggesting that TRIM27L may be interacting with RIG-I. As chickens and other Galliformes appear to be missing RIG-I, TRIM27L might be unable to activate IFN signaling pathways in DF-1 cells, consistent with our results. TRIM27.1 slightly downregulated IFN-β promoter activity downstream of RIG-I in DF-1 cells, however overexpression of TRIM27.1 did not make DF-1 or DEF cells more susceptible to infection. These results also suggest that neither TRIM27.1 or TRIM27L can directly restrict IAV, as that effect would like likely be seen as a reduction of IAV in DF-1 cells.

TRIM32 inhibits LPAI H6N2 avian strain of IAV in both DF-1 and DEF cells but could only restrict the mouse adapted PR8 in DEF cells. TRIM32 can restrict IAV in humans by K48-linked polyubiquitination of the IAV protein PB1 (Fu et al., 2015) and has demonstrated anti-IAV capabilities against a HPAI H5N6 strain of IAV in ducks (Wu et al., 2020). In our study, TRIM32 was only slightly upregulated in lungs of ducks infected with VN1203, and in intestines of ducks infected with BC500. Other studies found that TRIM32 was upregulated on 3 dpi by a HPAI H5N6 virus in lung, brain and spleen of infected ducks (Wu et al., 2020). Duck TRIM32 also helps induce IFN-β through direct interactions with STING. Our experiments suggest that duck TRIM32 can restrict mouse adapted IAV in DEF but not DF-1 cells. However, 2 out of 3 independent experiments show that overexpression of duck TRIM32 in DF-1 cells did restrict PR8 in DF-1 cells, and 1 out of 3 times it did not. More replications of this experiment are likely to show that TRIM32 can restrict PR8 in DF-1 cells. To our knowledge, no studies have been performed to date investigating if duck TRIM32 is also targeting PB1 of IAV for degradation.

The reptile and bird specific TRIM, diaTRIM58 significantly restricted IAV replication of CA431 and PR8 in both DEF and DF-1 cells. Previously, we reported that diaTRIM58 was specific to avian and reptilian lineages and appeared ancestral to the expansion of MHC-linked genes in these species (Chapter 4). As this gene was upregulated by VN1203 in all tissues sampled, and upregulated by BC500 in spleens of infected ducks, we wanted to test it for

antiviral ability against IAV. It is unknown if this protein can directly restrict IAV or is augmenting a signaling pathway that is conserved between chicken and duck. As we were the first to characterize this gene and map its lineage specifically to Diapsids (Chapter 4), there is little known about this protein. With evidence of restriction against IAV, it would be interesting to study the antiviral potential of diaTRIM58 against more avian specific RNA-viruses such as Newcastle disease, Tembusu virus or infectious bursal disease. The mechanism of diaTRIM58 inhibition of IAV should be further investigated to determine the specific means of inhibition as this could potentially lead to new drug targets for IAV therapeutics.

Neither TRIM19.1 or TRIM19.2 restricted IAV replication in DF-1 or DEF cells. Birds and reptiles have two recent paralogs of TRIM19, which we named TRIM19.1 and TRIM19.2 (Chapter 4). TRIM19 only appears in later vertebrate lineages, with direct orthologs in birds and mammals, but is not documented in fish (Boudinot et al., 2011). As TRIM19.1 and TRIM19.2 were strongly upregulated by both VN1203 and BC500 in all tissues sampled, perhaps the most surprising result of this study is that neither protein was able to restrict IAV when over expressed. In mammals, TRIM19 is the main component of promyelocytic leukemia (PML) nuclear bodies (Lallemand-Breitenbach et al., 2010; 2018). TRIM19 also restricts a vast array of viruses such as retroviruses (Dutrieux et al., 2015; Kahle et al., 2015; Masroori et al., 2016) and RNA viruses such as VSV (Chelbi-Alix et al., 1998), rabies virus (Blondel et al., 2010) and IAV (Chelbi-Alix et al., 1998; Li et al., 2009; Yan et al., 2021). Many DNA viruses have evolved to inhibit TRIM19 (El McHichi et al., 2010; Scherer et al., 2017) or utilize PML nuclear bodies for replication (Guion et al., 2019). It may be that duck TRIM19.1 and TRIM19.2 are only involved in cellular processes and not immune function, as antiviral ability could have arisen later in mammals. It is also possible that, as with mammals, the antiviral activities of TRIM19 in duck are isoform dependent. Human TRIM19 has 7 dominant isoforms, named PML I-VII (Condemine et al., 2006; Nisole et al., 2013). Isoforms PML-III, IV and VI all confer resistance to IAV (Chelbi-Alix et al., 1998; Iki et al., 2005), however this resistance appears to be strain specific, as not all isoforms can successfully inhibit all strains of IAV (Li et al., 2009). Duck TRIM19.1 and TRIM19.2 appear to be closest in length and structure to human PML isoforms PML-II and PML-I, respectively. As duck TRIM19.1 and TRIM19.2 are some of the highest and most consistently upregulated TRIM genes in response to IAV infection in the duck, determining

isoform sequences and isoform expression of these genes would be informative and could lead to identification of antiviral isoforms of duck TRIM19.1 and TRIM19.2.

Our results suggest duck TRIM25 cannot successfully inhibit either PR8 or CA431 in either DF-1 or DEF cells. This result is unexpected. TRIM25 in mammals is an E3 ubiquitin ligase that directly restricts IAV through targeting of viral RNA in the nucleus (Meyerson et al., 2017). TRIM25 can also stabilize RIG-I CARD domains to increase downstream signaling through addition of polyubiquitin of to RIG-I CARD domains, resulting in increased IFN-β production in cells in both mammals and birds (Gack et al., 2007; Miranzo-Navarro et al., 2014). Additionally, TRIM25 has been characterized for antiviral activity in birds, and can control replication of Tembusu virus in duck cells (Kaikai et al., 2021), infectious bursal disease in chicken cells (Wang et al., 2021) and avian leukosis virus in chicken cells (Zhou et al., 2020). This suggests that due to the long evolutionary history of IAV and ducks, the virus has evolved evasion mechanisms against host TRIM25. The NS1 protein of IAV targets TRIM25 by interacting with its coiled-coil domain preventing RIG-I ubiquitination by TRIM25 (Gack et al., 2009). This inhibition appears to be species-dependent, as NS1 derived from avian sources preferentially bound chicken TRIM25, NS1 from both mammalian and avian strains all bound human TRIM25, and NS1 from all strains tested, including the mouse adapted PR8, did not bind mouse TRIM25 (Rajsbaum et al., 2012). A recent study comparing NS1 from both avian and mammalian strains of IAV demonstrated that NS1 bound duck TRIM25 but did not inhibit IFN induction or RIG-I ubiquitination (Evseev et al, 2022). It is possible that the DEF cells used in this experiment were not expressing RIG-I, or it was not becoming activated in time for antiviral effect to be observed in the cell culture. To determine if RIG-I is expression is responsible for TRIM25 antiviral activity, DEF cells should be analyzed after IAV infection to see if expression of RIG-I is induced.

The duck TRIM proteins tested for antiviral activity may not have shown antiviral activity *in vitro* due to artifacts from construct design and transient transfection. While TRIM25, TRIM27.1, TRIM27L, TRIM32 and diaTRIM58 all had C-terminal epitope tags, TRIM19.1 and TRIM19.2 were designed with N-terminal epitope tags. The design of TRIM19.1 and TRIM19.2 was based on epitope tag placement from other groups (Quimby et al., 2006; Jul-Larsen et al., 2010). It is of note though, that if either TRIM19.1 or TRIM19.2 are utilizing the E3 ligase activity of their respective RING domains, this placement could have inhibited their function.

Likewise, PRYSPRY domains are often associated with substrate recognition and placement of epitope tags at the C-terminal end could be preventing this bidning. Our transient transfection results show very little expression of our TRIM genes 42 hours post transfection. We have previously observed that TRIM protein expression peaks at 24 hours, and then rapidly decreases (data not shown). We have attempted to make stable DF-1 cell lines expressing TRIM27.1, TRIM27L, diaTRIM58 and TRIM32. While diaTRIM58 and TRIM32 stable cell lines appear feasble, multiple attempts at creating TRIM27.1 and TRIM27L have proven unsuccessful, with cells maintaining antibiotic resistance markers, but gradually losing expression of TRIM27.1 or TRIM27L. The vector used, pcDNA3.1+(Hyg), uses the CMV promoter, which has been documented as being susceptible to silencing through methylation (Brooks et al, 2004). If TRIM27.1 and TRIM27L overexpression were resulting in cytotoxicity or other negative effects on the cells, it is likely the surviving cells could have silenced expression of these genes. Further attempts at creating stable cell lines with these genes should involve utilizing a constitutivly active promoter, such as the human ubiquitin C promoter, which would reuslt in more physiological relevent expression levels of TRIM proteins.

Our results are the first to look at the differential expression of the duck TRIM gene repertoire in response to both HPAI and LPAI IAV. This study also highlighted many genes upregulated in response to infection, which had not been previously characterized in ducks, such as TRIM19.1, TRIM19.2 and diaTRIM58. We also investigated the antiviral activities of two duck MHC-linked TRIM proteins, TRIM27.1 and TRIM27L in both duck and chicken cells. Here we report that diaTRIM58 is a novel anti-IAV protein that can restrict IAV in both duck and chicken cells, while TRIM27L only has anti-IAV activities in DEF cells. While neither TRIM19.1 or TRIM19.2 had anti-IAV activities in this study, their differential expression patterns suggest these two proteins may warrant further functional studies. Only a select few of TRIM proteins in non-mammalian species have been functionally characterized. It is likely many of these proteins have co-evolved with a vast array of pathogens and many more yet serve as species specific anti-pathogen effectors. Over half of the duck TRIM protein repertoire are differentially regulated in response to HPAI IAV suggesting that, as with mammalian TRIM proteins, many of the duck TRIM protein repertoire are interferon stimulated genes and respond to the presence of virus.

5.5 References

Barber, M.R., Aldridge, J.R., Jr., Webster, R.G., and Magor, K.E. (2010). Association of RIG-I with innate immunity of ducks to influenza. *Proc Natl Acad Sci U S A* 107(13)**,** 5913-5918. doi: 10.1073/pnas.1001755107.

Ben, L., and Steven, L.S. (2011). Fast gapped-read alignment with Bowtie 2. 1-24. doi: 10.1002/0471250953.bi1107s32.

Berti, C., Messali, S., Ballabio, A., Reymond, A., and Meroni, G. (2002). TRIM9 is specifically expressed in the embryonic and adult nervous system. *Mech Dev* 113(2)**,** 159-162. doi: 10.1016/s0925-4773(02)00013-8.

Bingham, J., Green, D.J., Lowther, S., Klippel, J., Burggraaf, S., Anderson, D.E., et al. (2009). Infection studies with two highly pathogenic avian influenza strains (Vietnamese and Indonesian) in Pekin ducks (Anas platyrhynchos), with particular reference to clinical disease, tissue tropism and viral shedding. *Avian Pathol* 38(4)**,** 267-278. doi: 10.1080/03079450903055371.

Blaine, A.H., Miranzo-Navarro, D., Campbell, L.K., Aldridge, J.R., Jr., Webster, R.G., and Magor, K.E. (2015). Duck TRIM27-L enhances MAVS signaling and is absent in chickens and turkeys. *Mol Immunol* 67(2 Pt B)**,** 607-615. doi: 10.1016/j.molimm.2015.07.011.

Blondel, D., Kheddache, S., Lahaye, X., Dianoux, L., and Chelbi-Alix, M.K. (2010). Resistance to rabies virus infection conferred by the PMLIV isoform. *J Virol* 84(20)**,** 10719-10726. doi: 10.1128/jvi.01286-10.

Bolger, A.M., Lohse, M., and Usadel, B. (2014). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics (Oxford, England)* 30(15)**,** 2114-2120. doi: 10.1093/bioinformatics/btu170.

Boudinot, P., van der Aa, L.M., Jouneau, L., Du Pasquier, L., Pontarotti, P., Briolat, V., et al. (2011). Origin and evolution of TRIM proteins: new insights from the complete TRIM repertoire of zebrafish and pufferfish. *PloS one* 6(7)**,** e22022-e22022. doi: 10.1371/journal.pone.0022022.

Brooks A., Harkins R., Wang P., Qian H., Liu P. and Rubanyi G (2004). Transcriptional silencing is associated with extensive methylation of the CMV promoter following

adenoviral gene delivery to muscle. *J Gene Med*. 6(4):395-404. doi: 10.1002/jgm.516. PMID: 15079814.

Campbell, L.K., Fleming-Canepa, X., Webster, R.G., and Magor, K.E. (2021). Tissue specific transcriptome changes upon influenza A virus replication in the duck. *Frontiers in Immunology* 12(4653). doi: 10.3389/fimmu.2021.786205.

Carthagena, L., Bergamaschi, A., Luna, J.M., David, A., Uchil, P.D., Margottin-Goguet, F., et al. (2009). Human TRIM gene expression in response to interferons. *PLoS One* 4(3)**,** e4894. doi: 10.1371/journal.pone.0004894.

Chaves, L.D., Krueth, S.B., and Reed, K.M. (2009). Defining the turkey MHC: sequence and genes of the B locus. *J Immunol* 183(10)**,** 6530-6537. doi: 10.4049/jimmunol.0901310.

Chelbi-Alix, M.K., Quignon, F., Pelicano, L., Koken, M.H., and de Thé, H. (1998). Resistance to virus infection conferred by the interferon-induced promyelocytic leukemia protein. *J Virol* 72(2)**,** 1043-1051. doi: 10.1128/jvi.72.2.1043-1051.1998.

Coch, C., Stümpel, J.P., Lilien-Waldau, V., Wohlleber, D., Kümmerer, B.M., Bekeredjian-Ding, I., et al. (2017). RIG-I activation protects and rescues from lethal influenza virus infection and bacterial superinfection. *Mol Ther* 25(9)**,** 2093-2103. doi: 10.1016/j.ymthe.2017.07.003.

Condemine, W., Takahashi, Y., Zhu, J., Puvion-Dutilleul, F., Guegan, S., Janin, A., et al. (2006). Characterization of endogenous human promyelocytic leukemia isoforms. *Cancer Research* 66(12)**,** 6192-6198. doi: 10.1158/0008-5472.CAN-05-3792.

Costa, T., Chaves, A.J., Valle, R., Darji, A., van Riel, D., Kuiken, T., et al. (2012). Distribution patterns of influenza virus receptors and viral attachment patterns in the respiratory and intestinal tracts of seven avian species. *Vet Res* 43**,** 28. doi: 10.1186/1297-9716-43-28.

Dutrieux, J., Maarifi, G., Portilho, D.M., Arhel, N.J., Chelbi-Alix, M.K., and Nisole, S. (2015). PML/TRIM19-dependent inhibition of retroviral reverse-transcription by daxx. *PLOS Pathogens* 11(11)**,** e1005280. doi: 10.1371/journal.ppat.1005280.

El McHichi, B., Regad, T., Maroui, M.-A., Rodriguez, M.S., Aminev, A., Gerbaud, S., et al. (2010). SUMOylation promotes PML degradation during encephalomyocarditis virus infection. *Journal of virology* 84(22)**,** 11634-11645. doi: 10.1128/JVI.01321-10.

Evseev, D., Miranzo-Navarro, D., Fleming-Canepa, X., Webster, R. and Magor, K. (2022) Avian influenza NS1 proteins inhibit human, bt not duck, RIG-I ubiquitination and interferon

signaling. Journal of virology. doi: 10.1128/jvi.00776-22. Epub ahead of print. PMID: 36069546

Ferri, F., Parcelier, A., Petit, V., Gallouet, A.-S., Lewandowski, D., Dalloz, M., et al. (2015). TRIM33 switches off Ifnb1 gene transcription during the late phase of macrophage activation. *Nature Communications* 6(1)**,** 8900. doi: 10.1038/ncomms9900.

Fleming-Canepa, X., Aldridge, J.R., Jr., Canniff, L., Kobewka, M., Jax, E., Webster, R.G., et al. (2019). Duck innate immune responses to high and low pathogenicity H5 avian influenza viruses. *Vet Microbiol* 228**,** 101-111. doi: 10.1016/j.vetmic.2018.11.018.

Fu, B., Wang, L., Ding, H., Schwamborn, J.C., Li, S., and Dorf, M.E. (2015). TRIM32 senses and restricts influenza A virus by ubiquitination of PB1 polymerase. *PLoS Pathog* 11(6)**,** e1004960. doi: 10.1371/journal.ppat.1004960.

Gack, M.U., Albrecht, R.A., Urano, T., Inn, K.S., Huang, I.C., Carnero, E., et al. (2009). Influenza A virus NS1 targets the ubiquitin ligase TRIM25 to evade recognition by the host viral RNA sensor RIG-I. *Cell Host Microbe* 5(5)**,** 439-449. doi: 10.1016/j.chom.2009.04.006.

Gack, M.U., Shin, Y.C., Joo, C.H., Urano, T., Liang, C., Sun, L., et al. (2007). TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity. *Nature* 446(7138)**,** 916-920. doi: 10.1038/nature05732.

Gregory R. Warnes, B.B., Lodewijk Bonebakker, Robert Gentleman, Wolfgang Huber, Andy Liaw, Thomas Lumley, Martin Maechler, Arni Magnusson, Steffen Moeller, Marc Schwartz and Bill Venables (2022). "gplots: Various R programming tools for plotting data"

Guion, L., Bienkowska-Haba, M., DiGiuseppe, S., Florin, L., and Sapp, M. (2019). PML nuclear body-residing proteins sequentially associate with HPV genome after infectious nuclear delivery. *PLoS Pathog* 15(2)**,** e1007590. doi: 10.1371/journal.ppat.1007590.

Haider, N., Sturm-Ramirez, K., Khan, S.U., Rahman, M.Z., Sarkar, S., Poh, M.K., et al. (2017). Unusually high mortality in waterfowl caused by highly pathogenic Avian influenza A(H5N1) in Bangladesh. *Transboundary and Emerging Diseases* 64(1)**,** 144-156. doi: 10.1111/tbed.12354.

Hatakeyama, S. (2011). TRIM proteins and cancer. *Nature Reviews Cancer* 11(11)**,** 792-804. doi: 10.1038/nrc3139.

Hatakeyama, S. (2017). TRIM family proteins: Roles in autophagy, immunity, and carcinogenesis. *Trends Biochem Sci* 42(4)**,** 297-311. doi: 10.1016/j.tibs.2017.01.002.

Hayman, T.J., Hsu, A.C., Kolesnik, T.B., Dagley, L.F., Willemsen, J., Tate, M.D., et al. (2019). RIPLET, and not TRIM25, is required for endogenous RIG-I-dependent antiviral responses. *Immunology & Cell Biology* 97(9)**,** 840-852. doi: https://doi.org/10.1111/imcb.12284.

Huang, Y., Li, Y., Burt, D.W., Chen, H., Zhang, Y., Qian, W., et al. (2013). The duck genome and transcriptome provide insight into an avian influenza virus reservoir species. *Nat Genet* 45(7)**,** 776-783. doi: 10.1038/ng.2657.

Iki, S., Yokota, S., Okabayashi, T., Yokosawa, N., Nagata, K., and Fujii, N. (2005). Serum-dependent expression of promyelocytic leukemia protein suppresses propagation of influenza virus. *Virology* 343(1)**,** 106-115. doi: 10.1016/j.virol.2005.08.010.

Jul-Larsen, Å., Grudic, A., Bjerkvig, R., and Bøe, S.O. (2010). Subcellular distribution of nuclear import-defective isoforms of the promyelocytic leukemia protein. *BMC Molecular Biology* 11(1)**,** 89. doi: 10.1186/1471-2199-11-89.

Kahle, T., Volkmann, B., Eissmann, K., Herrmann, A., Schmitt, S., Wittmann, S., et al. (2015). TRIM19/PML restricts HIV infection in a cell type-dependent manner. *Viruses* 8(1). doi: 10.3390/v8010002.

Kaikai, H., Zhao, D., Liu, Y., Liu, Q., Huang, X., Yang, J., et al. (2021). The E3 ubiquitin Ligase TRIM25 inhibits Tembusu virus replication in vitro. *Front Vet Sci* 8**,** 722113. doi: 10.3389/fvets.2021.722113.

Kato, H., Takeuchi, O., Sato, S., Yoneyama, M., Yamamoto, M., Matsui, K., et al. (2006). Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* 441(7089)**,** 101-105. doi: 10.1038/nature04734.

Kaufman, J. (2022). Innate immune genes of the chicken MHC and related regions. *Immunogenetics* 74(1)**,** 167-177. doi: 10.1007/s00251-021-01229-2.

Kaufman, J., Milne, S., Göbel, T.W.F., Walker, B.A., Jacob, J.P., Auffray, C., et al. (1999). The chicken B locus is a minimal essential major histocompatibility complex. *Nature* 401(6756)**,** 923-925. doi: 10.1038/44856.

Kim, J.-K., Negovetich, N.J., Forrest, H.L., and Webster, R.G. (2009). Ducks: the "Trojan horses" of H5N1 influenza. *Influenza and other respiratory viruses* 3(4)**,** 121-128. doi: 10.1111/j.1750-2659.2009.00084.x.

Kuchipudi, S.V., Nelli, R., White, G.A., Bain, M., Chang, K.C., and Dunham, S. (2009). Differences in influenza virus receptors in chickens and ducks: Implications for interspecies transmission. *J Mol Genet Med* 3(1)**,** 143-151. doi: 10.4172/1747-0862.1000026.

Lallemand-Breitenbach, V., and de Thé, H. (2010). PML nuclear bodies. *Cold Spring Harb Perspect Biol* 2(5)**,** a000661. doi: 10.1101/cshperspect.a000661.

Lallemand-Breitenbach, V., and de Thé, H. (2018). PML nuclear bodies: from architecture to function. *Curr Opin Cell Biol* 52**,** 154-161. doi: 10.1016/j.ceb.2018.03.011.

Li, W., Wang, G., Zhang, H., Zhang, D., Zeng, J., Chen, X., et al. (2009). Differential suppressive effect of promyelocytic leukemia protein on the replication of different subtypes/strains of influenza A virus. *Biochem Biophys Res Commun* 389(1)**,** 84-89. doi: 10.1016/j.bbrc.2009.08.091.

Liao, Y., Smyth, G.K., and Shi, W. (2014). featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* 30(7)**,** 923-930. doi: 10.1093/bioinformatics/btt656.

Marín, I. (2012). Origin and diversification of TRIM ubiquitin ligases. *PLOS ONE* 7(11)**,** e50030. doi: 10.1371/journal.pone.0050030.

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *2011* 17(1)**,** 3. doi: 10.14806/ej.17.1.200.

Masroori, N., Merindol, N., and Berthoux, L. (2016). The interferon-induced antiviral protein PML (TRIM19) promotes the restriction and transcriptional silencing of lentiviruses in a context-specific, isoform-specific fashion. *Retrovirology* 13(1)**,** 19. doi: 10.1186/s12977-016-0253-1.

Meyerson, N.R., Zhou, L., Guo, Y.R., Zhao, C., Tao, Y.J., Krug, R.M., et al. (2017). Nuclear TRIM25 specifically targets influenza virus ribonucleoproteins to block the onset of RNA chain elongation. *Cell Host Microbe* 22(5)**,** 627-638 e627. doi: 10.1016/j.chom.2017.10.003.

Miranzo-Navarro, D., and Magor, K.E. (2014). Activation of duck RIG-I by TRIM25 is independent of anchored ubiquitin. *PLoS One* 9(1)**,** e86968. doi: 10.1371/journal.pone.0086968.

Narayan, K., Waggoner, L., Pham, S.T., Hendricks, G.L., Waggoner, S.N., Conlon, J., et al. (2014). TRIM13 is a negative regulator of MDA5-mediated type I interferon production. *Journal of Virology* 88(18)**,** 10748. doi: 10.1128/JVI.02593-13.

Nenasheva, V.V., Nikitenko, N.A., Stepanenko, E.A., Makarova, I.V., Andreeva, L.E., Kovaleva, G.V., et al. (2021). Human TRIM14 protects transgenic mice from influenza A viral infection without activation of other innate immunity pathways. *Genes & Immunity* 22(1)**,** 56-63. doi: 10.1038/s41435-021-00128-6.

Nisole, S., Maroui, M.A., Mascle, X., Aubry, M., and Chelbi-Alix, M. (2013). Differential roles of PML isoforms. *Frontiers in Oncology* 3. doi: 10.3389/fonc.2013.00125.

Nisole, S., Stoye, J.P., and Saib, A. (2005). TRIM family proteins: retroviral restriction and antiviral defence. *Nat Rev Microbiol* 3(10)**,** 799-808. doi: 10.1038/nrmicro1248.

Oshiumi, H., Matsumoto, M., Hatakeyama, S., and Seya, T. (2009). Riplet/RNF135, a RING finger protein, ubiquitinates RIG-I to promote interferon-β induction during the early phase of viral infection. *Journal of Biological Chemistry* 284(2)**,** 807-817. doi: https://doi.org/10.1074/jbc.M804259200.

Oshiumi, H., Miyashita, M., Inoue, N., Okabe, M., Matsumoto, M., and Seya, T. (2010). The ubiquitin ligase Riplet is essential for RIG-I-dependent innate immune responses to RNA virus infection. *Cell Host Microbe* 8(6)**,** 496-509. doi: 10.1016/j.chom.2010.11.008.

Oshiumi, H., Miyashita, M., Matsumoto, M., and Seya, T. (2013). A distinct role of Riplet-mediated K63-Linked polyubiquitination of the RIG-I repressor domain in human antiviral innate immune responses. *PLoS Pathog* 9(8)**,** e1003533. doi: 10.1371/journal.ppat.1003533.

Ozato, K., Shin, D.M., Chang, T.H., and Morse, H.C., 3rd (2008). TRIM family proteins and their emerging roles in innate immunity. *Nat Rev Immunol* 8(11)**,** 849-860. doi: 10.1038/nri2413.

Patil, G., Zhao, M., Song, K., Hao, W., Bouchereau, D., Wang, L., et al. (2018). TRIM41-Mediated Ubiquitination of Nucleoprotein Limits Influenza A Virus Infection. *J Virol* 92(16). doi: 10.1128/JVI.00905-18.

Perera, S., Mankoo, B., and Gautel, M. (2012). Developmental regulation of MURF E3 ubiquitin ligases in skeletal muscle. *J Muscle Res Cell Motil* 33(2)**,** 107-122. doi: 10.1007/s10974-012-9288-7.

Quimby, B.B., Yong-Gonzalez, V., Anan, T., Strunnikov, A.V., and Dasso, M. (2006). The promyelocytic leukemia protein stimulates SUMO conjugation in yeast. *Oncogene* 25(21)**,** 2999-3005. doi: 10.1038/sj.onc.1209335.

Rajsbaum, R., Albrecht, R.A., Wang, M.K., Maharaj, N.P., Versteeg, G.A., Nistal-Villán, E., et al. (2012). Species-specific inhibition of RIG-I ubiquitination and IFN induction by the influenza A virus NS1 protein. *PLOS Pathogens* 8(11)**,** e1003059. doi: 10.1371/journal.ppat.1003059.

Rajsbaum, R., Garcia-Sastre, A., and Versteeg, G.A. (2014). TRIMmunity: the roles of the TRIM E3-ubiquitin ligase family in innate antiviral immunity. *J Mol Biol* 426(6)**,** 1265-1284. doi: 10.1016/j.jmb.2013.12.005.

Reymond, A., Meroni, G., Fantozzi, A., Merla, G., Cairo, S., Luzi, L., et al. (2001). Reymond et al. (2001). The tripartite motif family identifies cell compartments. 20(9)

Robinson, M.D., McCarthy, D.J., and Smyth, G.K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26(1)**,** 139-140. doi: 10.1093/bioinformatics/btp616.

RStudio Team (2020). "RStudio: Integrated Development Environment for R". (Boston, MA: RStudio, Inc.).

Saito, L.B., Diaz-Satizabal, L., Evseev, D., Fleming-Canepa, X., Mao, S., Webster, R.G., et al. (2018). IFN and cytokine responses in ducks to genetically similar H5N1 influenza A viruses of varying pathogenicity. *J Gen Virol* 99(4)**,** 464-474. doi: 10.1099/jgv.0.001015.

Scherer, M., Schilling, E.M., and Stamminger, T. (2017). The human CMV IE1 protein: An offender of PML nuclear bodies. *Adv Anat Embryol Cell Biol* 223**,** 77-94. doi: 10.1007/978-3-319-53168-7_4.

Shi, M., Cho, H., Inn, K.S., Yang, A., Zhao, Z., Liang, Q., et al. (2014). Negative regulation of NF-κB activity by brain-specific TRIpartite Motif protein 9. *Nat Commun* 5**,** 4820. doi: 10.1038/ncomms5820.

Short, K.M., and Cox, T.C. (2006). Subclassification of the RBCC/TRIM superfamily reveals a novel motif necessary for microtubule binding. *Journal of Biological Chemistry* 281(13)**,** 8970-8980. doi: https://doi.org/10.1074/jbc.M512755200.

Song, B., Javanbakht, H., Perron, M., Park, D.H., Stremlau, M., and Sodroski, J. (2005). Retrovirus restriction by TRIM5alpha variants from Old World and New World primates. *Journal of virology* 79(7)**,** 3930-3937. doi: 10.1128/JVI.79.7.3930-3937.2005.

Sparrer, K.M.J., Gableske, S., Zurenski, M.A., Parker, Z.M., Full, F., Baumgart, G.J., et al. (2017). TRIM23 mediates virus-induced autophagy via activation of TBK1. *Nat Microbiol* 2(11)**,** 1543-1557. doi: 10.1038/s41564-017-0017-2.

Stremlau, M., Owens, C.M., Perron, M.J., Kiessling, M., Autissier, P., and Sodroski, J. (2004). The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. *Nature* 427(6977)**,** 848-853. doi: 10.1038/nature02343.

Sturm-Ramirez, K.M., Ellis, T., Bousfield, B., Bissett, L., Dyrting, K., Rehg, J.E., et al. (2004). Reemerging H5N1 influenza viruses in Hong Kong in 2002 are highly pathogenic to ducks. *Journal of virology* 78(9)**,** 4892-4901. doi: 10.1128/jvi.78.9.4892-4901.2004.

Sun, M., Li, S., Jin, S., Li, X., Xiang, J., and Li, F. (2022). A novel TRIM9 protein promotes NF-κB activation through interacting with LvIMD in shrimp during WSSV infection. *Frontiers in Immunology* 13.

Sun, M., Li, S., Yu, K., Xiang, J., and Li, F. (2019). An E3 ubiquitin ligase TRIM9 is involved in WSSV infection via interaction with β-TrCP. *Developmental & Comparative Immunology* 97**,** 57-63. doi: https://doi.org/10.1016/j.dci.2019.03.014.

Sun, N., Jiang, L., Ye, M., Wang, Y., Wang, G., Wan, X., et al. (2020). TRIM35 mediates protection against influenza infection by activating TRAF3 and degrading viral PB2. *Protein Cell* 11(12)**,** 894-914. doi: 10.1007/s13238-020-00734-6.

Taubenberger, J.K., and Kash, J.C. (2010). Influenza virus evolution, host adaptation, and pandemic formation. *Cell host & microbe* 7(6)**,** 440-451. doi: 10.1016/j.chom.2010.05.009.

Toka, F.N., Dunaway, K., Mielcarska, M., Smaltz, F., and Bossowska-Nowicka, M. (2017). Expression pattern of TRIM genes in bovine macrophages stimulated with PAMPs. The Journal of Immunology 198(1 Supplement), 129.127.

Uhlen, M., Fagerberg, L., Hallstrom, B.M., Lindskog, C., Oksvold, P., Mardinoglu, A., et al. (2015). Proteomics. Tissue-based map of the human proteome. *Science* 347(6220)**,** 1260419. doi: 10.1126/science.1260419.

Vanderven, H.A., Petkau, K., Ryan-Jean, K.E., Aldridge, J.R., Jr., Webster, R.G., and Magor, K.E. (2012). Avian influenza rapidly induces antiviral genes in duck lung and intestine. *Mol Immunol* 51(3-4)**,** 316-324. doi: 10.1016/j.molimm.2012.03.034.

Varga, Z.T., Grant, A., Manicassamy, B., and Palese, P. (2012). Influenza virus protein PB1-F2 inhibits the induction of type I interferon by binding to MAVS and decreasing mitochondrial membrane potential. *J Virol* 86(16)**,** 8359-8366. doi: 10.1128/jvi.01122-12.

Versteeg, G.A., Rajsbaum, R., Sánchez-Aparicio, M.T., Maestre, A.M., Valdiviezo, J., Shi, M., et al. (2013). The E3-ligase TRIM family of proteins regulates signaling pathways triggered by innate immune pattern-recognition receptors. *Immunity* 38(2)**,** 384-398. doi: 10.1016/j.immuni.2012.11.013.

Wang, S., Yu, M., Liu, A., Bao, Y., Qi, X., Gao, L., et al. (2021). TRIM25 inhibits infectious bursal disease virus replication by targeting VP3 for ubiquitination and degradation. *PLoS Pathog* 17(9)**,** e1009900. doi: 10.1371/journal.ppat.1009900.

Watanabe, M., and Hatakeyama, S. (2017). TRIM proteins and diseases. *J Biochem* 161(2)**,** 135-144. doi: 10.1093/jb/mvw087.

Webster, R.G., Bean, W.J., Gorman, O.T., Chambers, T.M., and Kawaoka, Y. (1992). Evolution and ecology of influenza A viruses. *Microbiol Rev* 56(1)**,** 152-179.

Webster, R.G., Yakhno, M., Hinshaw, V.S., Bean, W.J., and Murti, K.G. (1978). Intestinal influenza: replication and characterization of influenza viruses in ducks. *Virology* 84(2)**,** 268-278. doi: 10.1016/0042-6822(78)90247-7.

Wu, S., Zhang, J., Xue, Q., Liu, J., Huang, B., He, Z., et al. (2020). Duck TRIM32 functions in IFN-β signaling against the infection of H5N6 highly pathogenic avian influenza virus. *Front Immunol* 11**,** 377. doi: 10.3389/fimmu.2020.00377.

Wu, X., Wang, J., Wang, S., Wu, F., Chen, Z., Li, C., et al. (2019). Inhibition of influenza A virus replication by TRIM14 via its multifaceted protein-protein interaction with NP. *Front Microbiol* 10(FEB)**,** 344. doi: 10.3389/fmicb.2019.00344.

Xiao, Y., Evseev, D., Stevens, C.A., Moghrabi, A., Miranzo-Navarro, D., Fleming-Canepa, X., et al. (2020). Influenza PB1-F2 inhibits avian MAVS signaling. *Viruses* 12(4). doi: 10.3390/v12040409.

Xing, J., Zhang, A., Minze, L.J., Li, X.C., and Zhang, Z. (2018). TRIM29 negatively regulates the Type I IFN production in response to RNA virus. *J Immunol* 201(1)**,** 183-192. doi: 10.4049/jimmunol.1701569.

Yan, J., Li, Q., Mao, A.P., Hu, M.M., and Shu, H.B. (2014). TRIM4 modulates type I interferon induction and cellular antiviral response by targeting RIG-I for K63-linked ubiquitination. *J Mol Cell Biol* 6(2)**,** 154-163. doi: 10.1093/jmcb/mju005.

Yan, H.-Y., Wang, H.-Q., Zhong, M., Wu, S., Yang, L., Li, K., et al. (2021). PML suppresses influenza virus replication by promoting FBXW7 expression. *Virologica Sinica*. doi: 10.1007/s12250-021-00399-3.

Yang, L., Liu, X., Zhang, L., Li, X., Zhang, X., Niu, G., et al. (2022). Porcine TRIM21 Enhances Porcine Circovirus 2 Infection and Host Immune Responses, But Inhibits Apoptosis of PCV2-Infected Cells. Viruses 14(1). doi: 10.3390/v14010156.

Zhao, C., Jia, M., Song, H., Yu, Z., Wang, W., Li, Q., et al. (2017). The E3 Ubiquitin Ligase TRIM40 Attenuates Antiviral Immune Responses by Targeting MDA5 and RIG-I. *Cell Rep* 21(6)**,** 1613-1623. doi: 10.1016/j.celrep.2017.10.020.

Zhou, J.R., Liu, J.H., Li, H.M., Zhao, Y., Cheng, Z., Hou, Y.M., et al. (2020). Regulatory effects of chicken TRIM25 on the replication of ALV-A and the MDA5-mediated type I interferon response. *Vet Res* 51(1)**,** 145. doi: 10.1186/s13567-020-00870-1.

Table 5.1. PCR primers used to amplify TRIM19.1, TRIM19.2, TRIM32 and diaTRIM58.

Table 1. Primers used to amplify TRIM19.1, TRIM19.2, TRIM32 and diaTRIM58.

Gene	Primer Name	Primer Sequence (5'-3')
TRIM19.1	TRIM19.1 Frag1 Fwd	CATGCCTGGCAGCACCGAA
	TRIM19.1 Frag1 Rev	CGGATGTCGCAGTAGAAAGGC
	TRIM19.1 Frag2 Fwd OuterNest*	GCCACATCACCAGCATCTACTG
	TRIM19.1 Frag2 Rev OuterNest	GCTGCATGTCCATCACCTCCT
	TRIM19.1 Frag2 Fwd InnerNest+	CTGGACGCCCAGCACTCG
	TRIM19.1 Frag2 Rev InnerNest	GCTCCGTGGCGTACAGCC
	TRIM19.1 Frag3 Fwd	GGCTGGTGGAGAAGATGAGGC
	TRIM19.1 Frag3 Rev	TCAGCAGGGAGGCAGGTC
TRIM19.2	TRIM19.2 Frag1 Fwd OuterNest	GCCTCATTATTCCTTCCTTGGGC
	TRIM19.2 Frag1 Rev OuterNest	GGCTGCATGTCCATCACCTC
	TRIM19.2 Frag1 Fwd InnerNest	GGACATGGCGGGCAAATATCC
	TRIM19.2 Frag1 Rev InnerNest	GGCGTACAGCCTCATCTTCTCC
	TRIM19.2 Frag2 Fwd OuterNest	GCATCTACTGCAAGAAGTGC
	TRIM19.2 Frag3 Rev OuterNest	GCCACCAGACAAGGAAACAATGG
	TRIM19.2 Frag2 Fwd OuterNest	CTGCATCTGCGCCCTGC
	TRIM19.2 Frag3 Rev OuterNest	GCAGCGCCAACTTTCACTTTCC
TRIM32	TRIM32 Fwd	GGCTTGGCACAGGCTAAAG
	TRIM32 Rev	GAAGGGGCTTTACCTCCAGAG
diaTRIM58	diaTRIM58 Fwd	CCTGCTTGCCAGTGTCTTCAC
	diaTRIM58 Rev	GCAGAGTCCTGGTAGAAGCAG

Footnote:

* Primers with the "Outernest" designation were used in nested PCR, and amplified the original large fragment
+ Primers with the "InnerNest" designation were used in the second nested-PCR reaction and located internally on the fragment amplified by the "OuterNest" primers.

Table 5.2. Percent transfected DEF or DF-1 cells after infection with CA431.

		DF-1			DEF	
	rep 1	**rep 2**	**rep 3**	**rep 1**	**rep 2**	**rep 3**
pcDNA	N/A	0.18	0.5	0.185	0.536	0.3
TRIM19.1	N/A	13.799	6.7	3.54	5.749	3.06
TRIM19.2	N/A	0.37	0.42	0.209	0.316	0.5
TRIM27.1	N/A	6.061	2.63	4.063	3.414	1.87
TRIM27L	N/A	5.726	2.93	0.89	4.23	2.8
TRIM25	N/A	7.948	2.249	1.533	2.073	1.032
TRIM32	N/A	7.061	1.143	4.085	2.188	1.05
diaTRIM58	N/A	3.45	1.19	3.045	2.206	1.7

Footnote: N/A indicates values not available due to staining or equipment error.

Table 5.3. Percent transfected DEF or DF-1 cells after infection with PR8.

		DF-1			DEF	
	rep 1	**rep 2**	**rep 3**	**Pek 6**	**Pek 7**	**Pek 13**
pcDNA	0.15	0.723	N/A	0.342	0.39	0.4
TRIM19.1	11.316	2.32	N/A	26.19	11.574	24.701
TRIM19.2	0.325	0.491	N/A	1	N/A	0.7
TRIM27.1	14.67	2.189	N/A	9.79	6.918	3.9
TRIM27L	6.65	1.983	N/A	10.1	8.56	6.4
TRIM25	2.265	2.013	N/A	3.76	2.278	3
TRIM32	3.12	2.31	N/A	9.76	6	5.04
diaTRIM58	13.7	1.024	N/A	5.543	5.19	2.122

Footnote: N/A indicates values not available due to staining or equipment error.

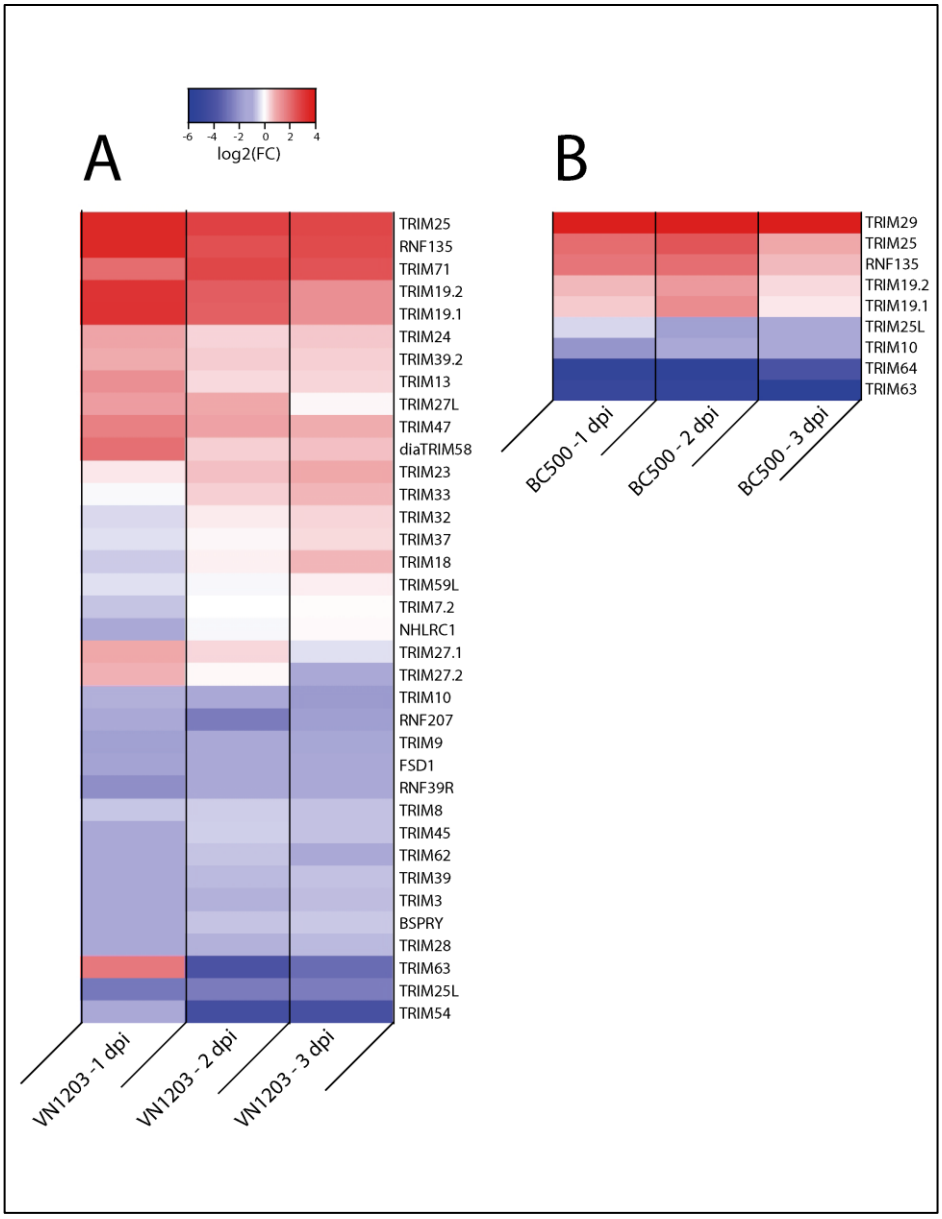


Figure 5.1. Influenza infection induced strong differential regulation of TRIM genes in the lung of infected Pekin ducks. Sequences from RNA-seq libraries from lungs of ducks infected with either VN1203 (A) or BC500 (B) strains of IAV were aligned against 57-TRIM or TRIM-like genes previously defined in ducks. Genes were analyzed using a GLM analysis of variance in EdgeR and were filtered for statistical significance (FDR<0.05).

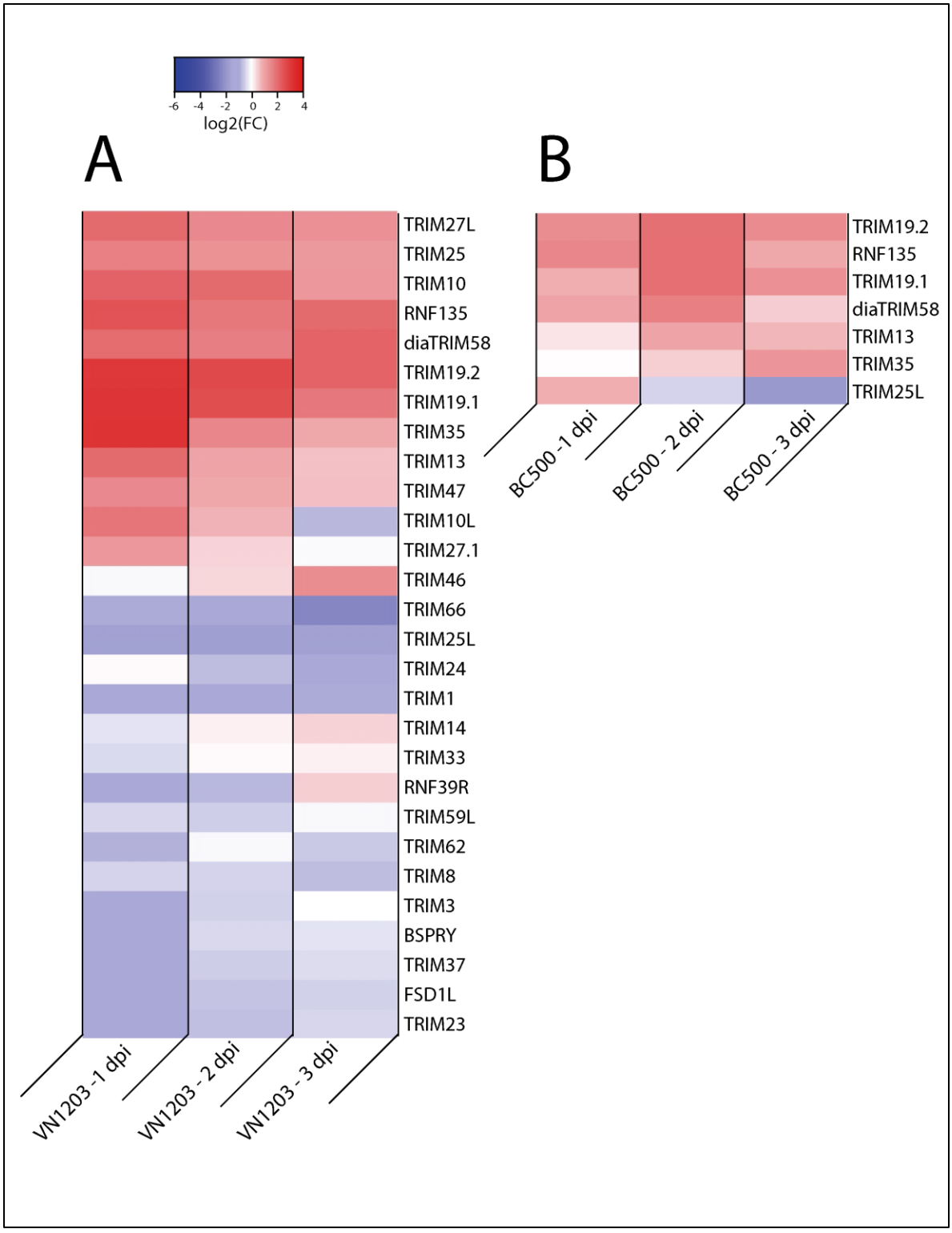


Figure 5.2. Influenza infection induced differential expression of TRIM genes in the spleen of infected Pekin ducks. Sequences from RNA-seq libraries from spleens of ducks infected with either VN1203 (A) or BC500 (B) strains of IAV were aligned against 57-TRIM or TRIM-

like genes previously defined in ducks. Genes were analyzed using a GLM analysis of variance in EdgeR and were filtered for statistical significance (FDR<0.05).

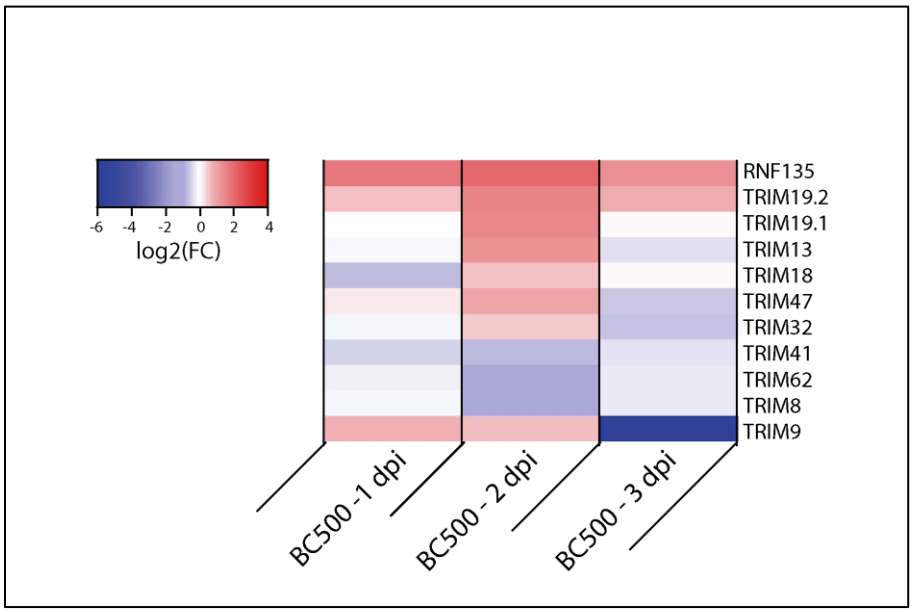


Figure 5.3. BC500 infection induced differential expression in only 11 TRIM genes in intestines of Pekin ducks. Sequence reads from RNA-seq libraries from intestines of ducks infected with the BC500 strain of IAV were aligned against 57-TRIM or TRIM-like genes previously defined in ducks. Genes were analyzed using a GLM analysis of variance in EdgeR and were filtered for statistical significance (FDR<0.05).

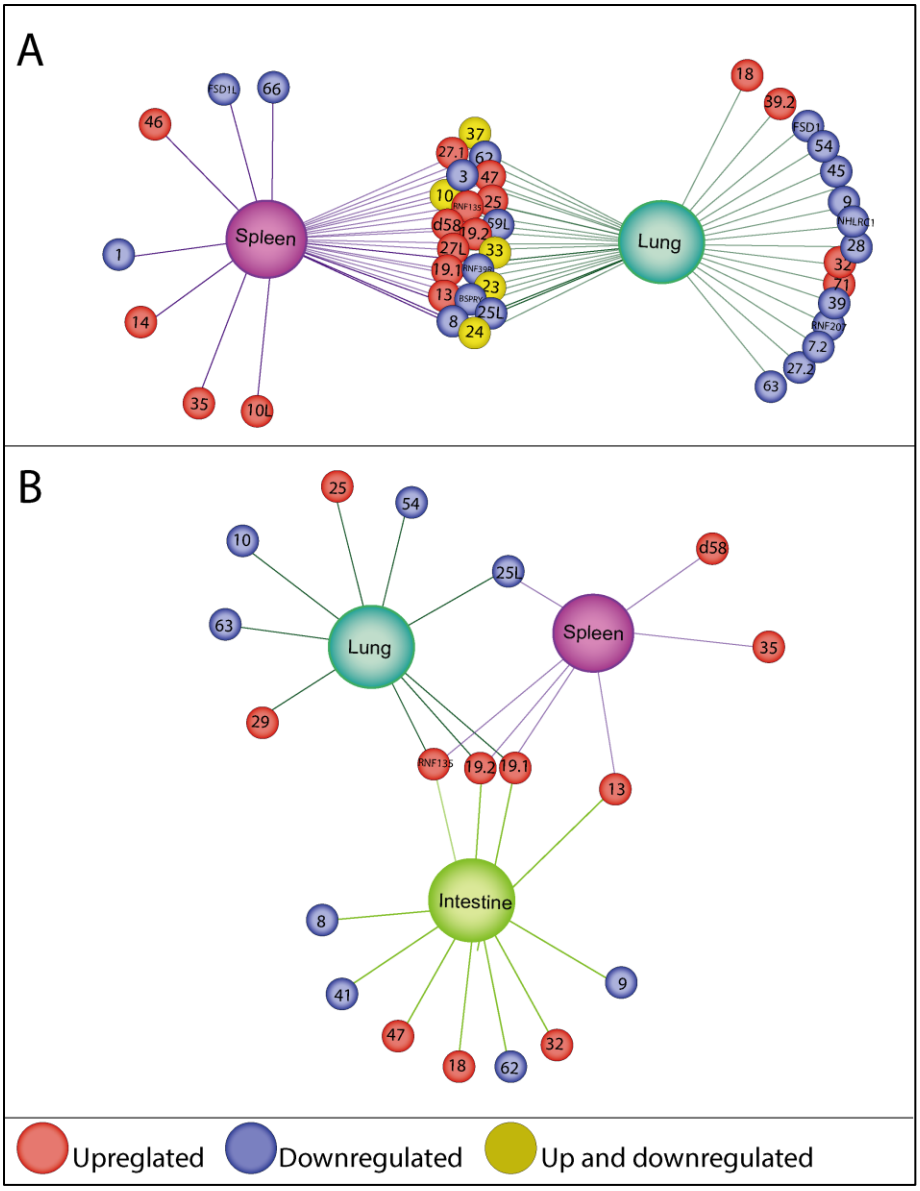


Figure 5.4. Venn diagrams demonstrating the shared regulation of TRIM genes in the tissues of ducks infected with either VN1203 or BC500. The 57 duck TRIM or TRIM-like genes were assigned up or downregulated depending on the predominance of expression over all 3 dpi. Similarities and differences of TRIM gene expression were compared between lung and spleen of ducks infected with VN1203 (A) or lung, spleen and intestines from ducks infected with BC500 (B). Venn diagrams created using the DiVenn webserver and edited for clarity using Adobe Illustrator.

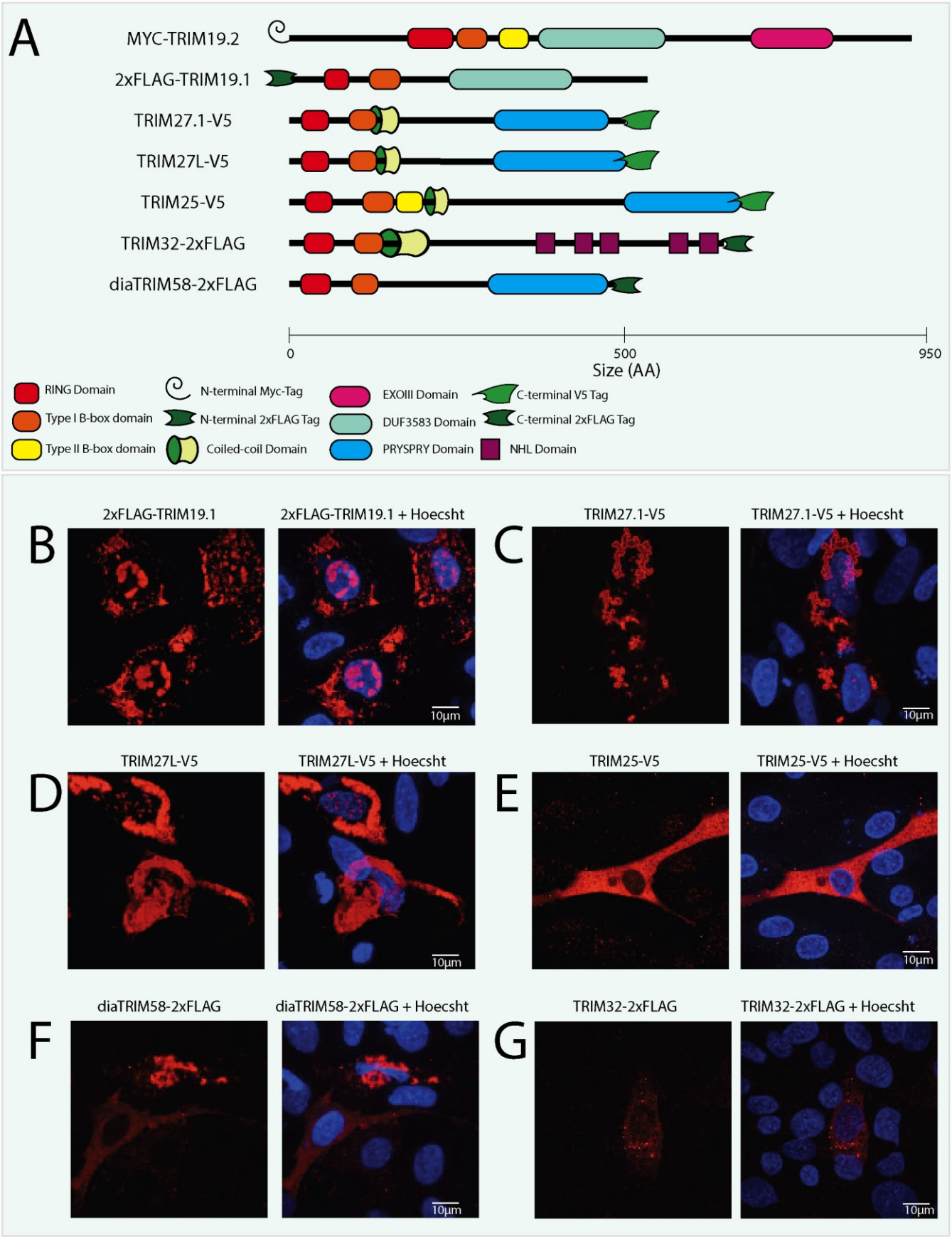

A
MYC-TRIM19.2
2xFLAG-TRIM19.1
TRIM27.1-V5
TRIM27L-V5
TRIM25-V5
TRIM32-2xFLAG
diaTRIM58-2xFLAG
0
500
950
Size (AA)
RING Domain
Type I B-box domain
Type II B-box domain
N-terminal Myc-Tag
N-terminal 2xFLAG Tag
Coiled-coil Domain
EXOIII Domain
DUF3583 Domain
PRYSPRY Domain
C-terminal V5 Tag
C-terminal 2xFLAG Tag
NHL Domain
B
2xFLAG-TRIM19.1
2xFLAG-TRIM19.1 + Hoecsht
C
TRIM27.1-V5
TRIM27.1-V5 + Hoecsht
D
TRIM27L-V5
TRIM27L-V5 + Hoecsht
E
TRIM25-V5
TRIM25-V5 + Hoecsht
F
diaTRIM58-2xFLAG
diaTRIM58-2xFLAG + Hoecsht
G
TRIM32-2xFLAG
TRIM32-2xFLAG + Hoecsht
10µm

Figure 5.5. Duck TRIM proteins localize to different subcellular compartments. Expression constructs were made of TRIM19.1, TRIM19.2, TRIM27.1, TRIM27L, TRIM25, TRIM32 or TRIM41 and were assessed for domain composition using the SMART prediction software (A). DF-1 transiently expressing 2xFLAG-TRIM19.1 (B), Myc-TRIM19.2, TRIM27.1-V5 (C), TRIM27L-V5 (D), TRIM25-V5 (E), TRIM32-2xFLAG (F) or diaTRIM58-2xFLAG (G) were analyzed using the WaveFX confocal microscope to determine subcellular localization for these proteins. Panels show epitope (red) or the epitope staining plus Hoechst 3324 (blue).

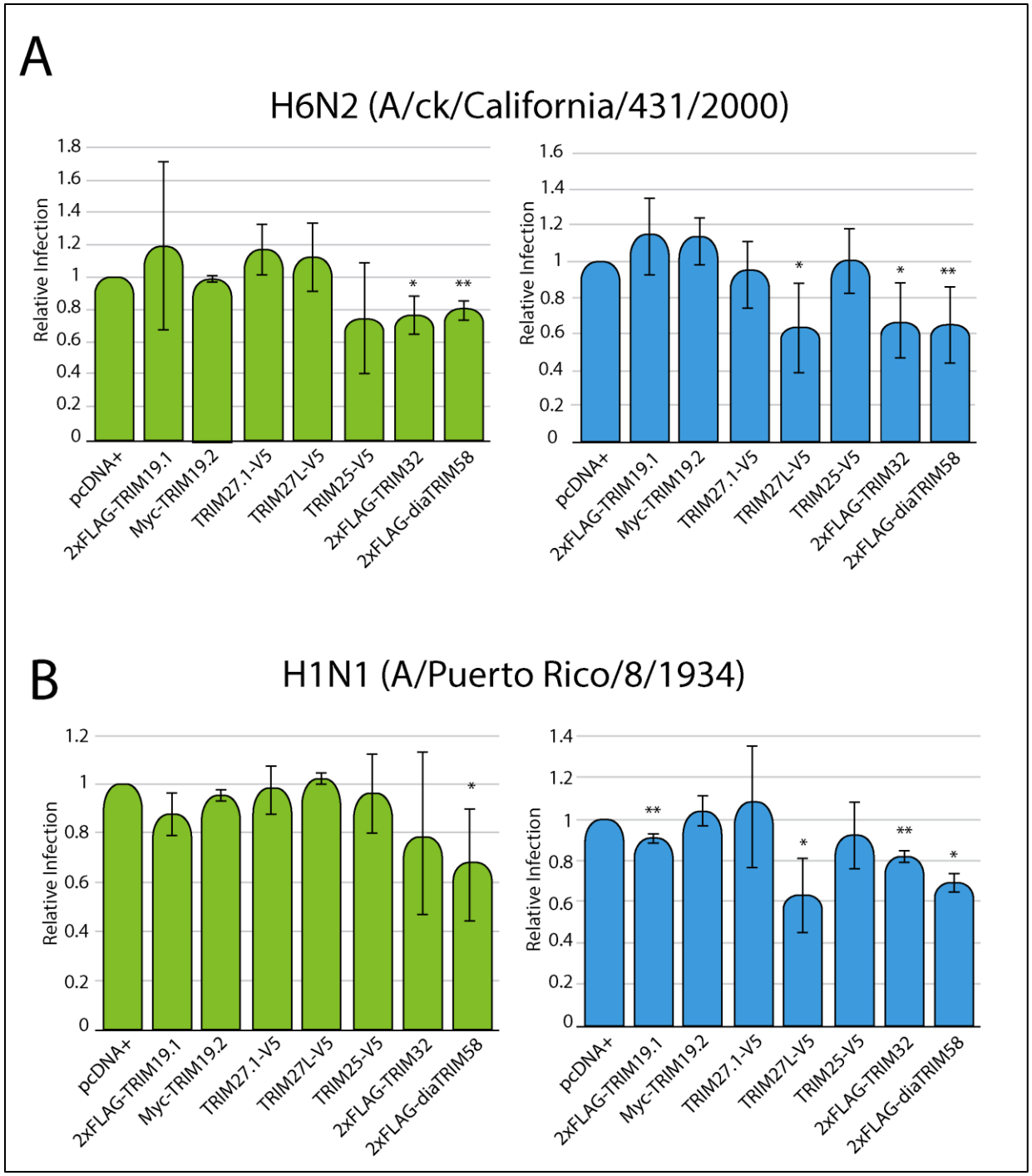
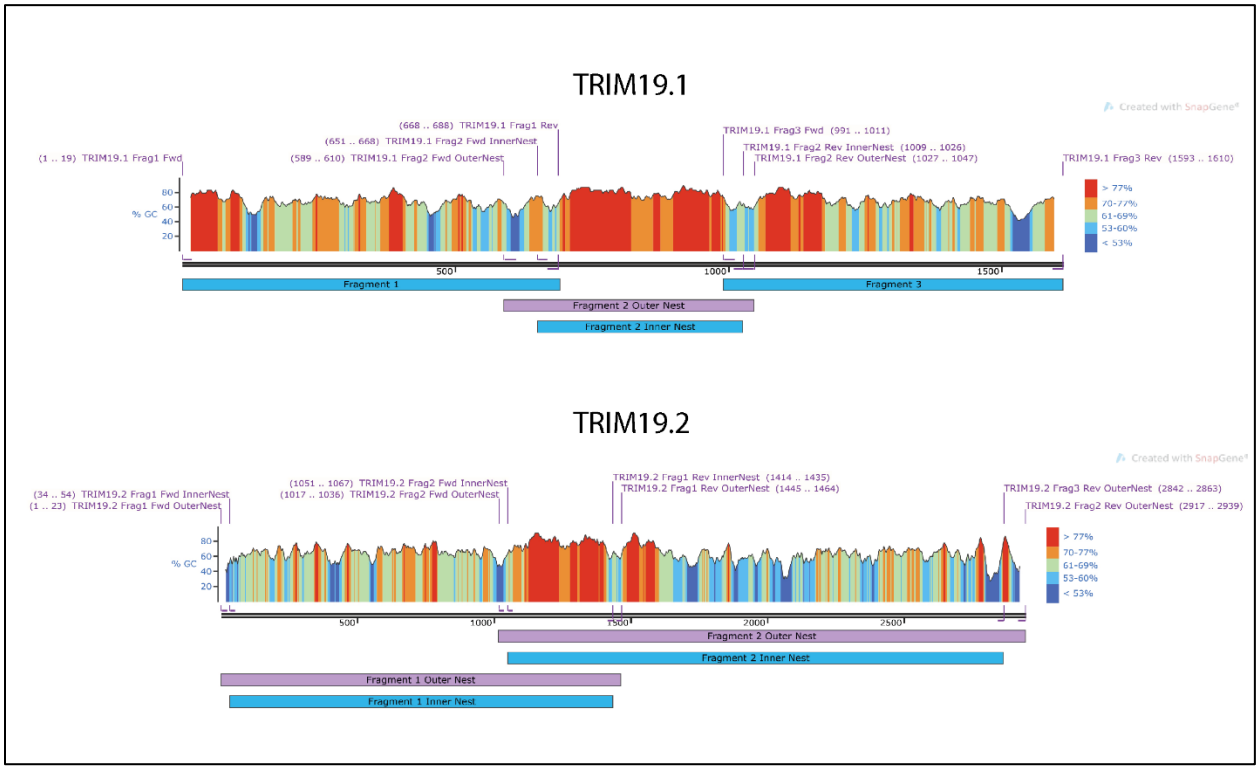
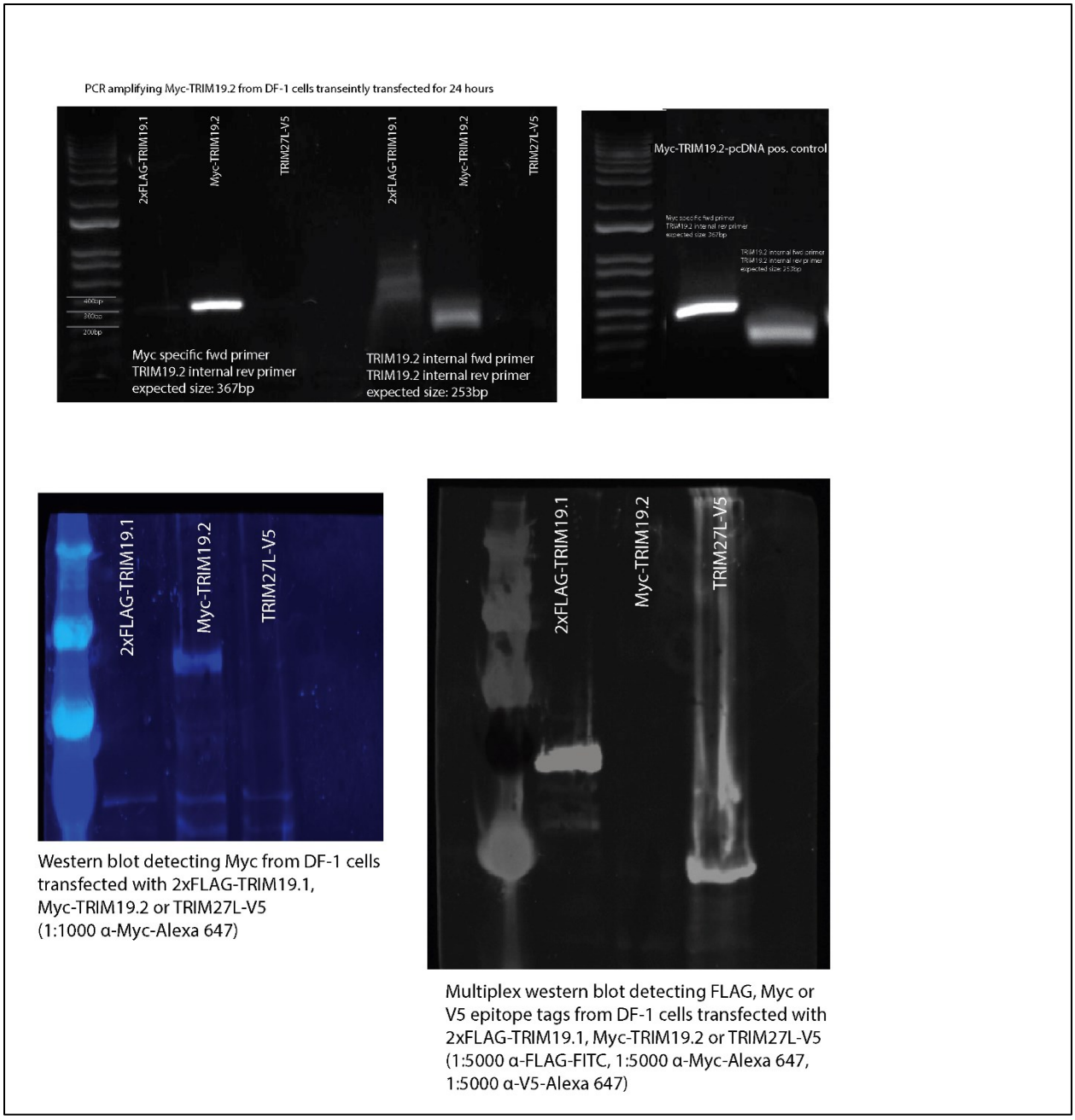


Figure 5.6. Duck TRIM27L, TRIM32 and diaTRIM58 restrict LPAI IAV in DEF cells. DF-1 (green) or DEF (blue) cells transiently overexpressing duck TRIM genes (19.1, 19.2, 27.1, 27L, 32, dia58) were challenged with an avian H6N2 (CA431) (A) or a mammalian adapted H1N1(PR8) (B) at an MOI of 1. 24 hours after infection cells were fixed and stained with anti-NP to assess for infection and analyzed using the Attune NX flow cytometer. The ratio of

infected cells is expressed relative to vector control. Statistical significance was calculated using the unpaired two-tailed student's t-test by comparing treatments to vector control (*, P<0.05, **,P <0.01). Each experiment was repeated 3 times independently except for PR8 infection in DEF cells which was repeated 2 times. Data is composite of three independent experiments.



Supplementary figure S5.1. PCR primer location and nested primer design for TRIM19.1 and TRIM19.2. GC content of sequence is indicted by blue to red vertical bars. High GC content (> 77%) indicated by red while low GC content (> 53%) indicated by blue. Purple and light blue horizontal bars indicate location and expected size of PCR and nested PCR amplification products.



Supplementary figure S5.2. PCR amplification and western blot of Myc-TRIM19.1 from transfected DF-1 cells.

CHAPTER 6

TRIM27L is both a positive and negative regulator of the MAVS signaling pathway

6.1 Introduction

Tripartite motif (TRIM) proteins are named for their conserved tripartite motif; that is a RING, B-box and coiled-coil domain. TRIM proteins are also a large and diverse family of genes that have many functions in the cell such as aiding in development, cell cycle regulation and in cellular immune processes (Ozato et al., 2008; Hatakeyama, 2011; Watanabe et al., 2017; Yang et al., 2020). The RING domain is most often associated with E3 ubiquitin ligase activity (Ardley et al., 2005; Meroni, 2012; Metzger et al., 2014; Koliopoulos et al., 2016; Esposito et al., 2017). This E3 ubiquitin ligase activity can lead to the degradation of target cellular or viral components by catalyzing the linkage of lysine 48 linked polyubiquitin (K48). Additionally, E3 ubiquitin ligase activity can lead to the stabilization or activation of cellular or viral proteins through K63-linked polyubiquitin chains. The B-box domain has a less defined function and can have E3 ubiquitin ligase activity or can aid in protein-protein interactions (Massiah et al., 2006; Bell et al., 2012; Napolitano et al., 2012). The coiled-coil domain is associated with higher-order protein multimerization (Meroni, 2012; Sanchez et al., 2014).

TRIM proteins are important modulators of innate signaling pathways. TRIM proteins can increase antiviral signaling through stabilization of important signaling proteins or their effectors. For example, TRIM25 and RNF135 stabilize RIG-I so it can interact with MAVS and increase antiviral signaling downstream (Gack et al., 2007; Oshiumi et al., 2010), while TRIM40 causes degradation of RIG-I and subsequently decreases downstream antiviral signaling (Zhao et al., 2017). Some TRIM proteins modulate innate signaling pathways by modifying multiple proteins in antiviral signaling pathways, such as TRIM38, which can increase antiviral responses

through its interactions with the RNA virus PRRs MDA5 and RIG-I (Hu et al., 2017), as well as cGAS (Hu et al., 2016), a DNA sensing PRR.

Ducks are the natural host and reservoir of influenza A virus (Webster et al., 1992). As ducks have shared a long co-evolutionary history with the virus, it is likely that the duck has evolved unique mechanisms to restrict viral replication. Chickens, however, are highly susceptible to influenza A virus (IAV) infection. We previously hypothesized that chickens are more permissive to IAV replication, and the detrimental effects of this replication is due to the chicken missing the key influenza A virus pattern recognition receptor (PRR) retinoic acid-inducible gene I (RIG-I). Chickens and quail appear to be also missing RIPLET/RNF135 (Magor et al., 2013; Morris et al., 2020) a protein that stabilizes RIG-I signaling through ubiquitination, which in turn causes an increase in MAVS signaling downstream of virus detection (Oshiumi et al., 2010; Hayman et al., 2019; Kouwaki et al., 2021). We also documented a novel TRIM protein, which we named TRIM27L (Blaine et al., 2015). TRIM27L is present in ducks but appears to be missing in Galliformes (Kaufman et al., 1999; Chaves et al., 2009; Blaine et al., 2015). When cotransfected with the constitutively active form of RIG-I duck 2CARD (d2CARD) TRIM27L increases IFN-β promoter activity in chicken cells but does not increase IFN-β activation on its own (Blaine et al., 2015). It is not known where in the pathway TRIM27L is interacting to augment this signaling. Additionally, TRIM27L is upregulated in both lungs and spleen of ducks infected with the highly pathogenic avian influenza (HPAI) virus VN1203 (Blaine et al, 2015) (Chapter 5). TRIM27L is unable to restrict influenza A replication in immortalized chicken DF-1 cells, however, it successfully restricts IAV replication when transfected into primary duck embryonic fibroblast (DEF) cells. Why it can restrict IAV replication in DEF but not DF-1 cells is not known.

In this study, we co-transfect TRIM27L with different components of the RIG-I signaling pathway to pinpoint where in the pathway TRIM27L is interacting. We also determine which domain of TRIM27L is responsible for the modulation of the RIG-I signaling pathway by creating mutant and domain constructs of TRIM27L.

6.2 Materials and methods

6.2.1 PCR amplification of genes and creation of expression constructs

TRIM27L and TRIM27.1 with C-terminal V5 epitope tags were cloned into pcDNA3.1+(hyg) vectors, as described previously (Blaine et al., 2015). TRIM27L domains were predicted through the NCBI protein database (NCBI) and the SMART domain server (http://smart.embl-heidelberg.de/). Domain constructs of TRIM27L were made according to these predictions and the RING, RING and B-box together, and PRYSPRY were deleted. A domain construct of the TRIM27L PRYSPRY domain on its own was also created. A RING mutant construct was made using primers with point mutations changing the TRIM27L cysteine 16 to alanine and cysteine 31 to alanine (C16AC31A). All domain mutants were cloned into a pcDNA3.1+(hyg) vector using NheI/NotI restriction sites at the 5′ and 3′ ends of the coding sequences (respectively). V5 epitope tags were added to all mutant constructs at the 3′ end of the coding sequence. All PCR reactions were performed using Phusion polymerase (NEB). 2xFLAG-TRIM19.1 was inserted into a pcDNA3.1 vector as previously described (Chapter 5). TRIM25-V5 was inserted into a pcDNA3.1 vector, as previously described (Miranzo-Navarro et al., 2014).

Duck V5-MAVS was cloned into a pcDNA3.1+(hyg) expression vector, as previously described (Xiao et al., 2020). Duck 2xFLAG-MAVS (2xFLAG-duMAVS) was created using V5-MAVS in pcDNA3.1+(hyg) as the template. Primers were designed to add NheI and BamHI as 5′ and 3′ restriction sites and a 2xFLAG (DYKDDDDKDYKDDDDK) 5′-epitope tag to the MAVS gene. The resulting contig was inserted into a pcDNA3.1+(hyg) expression vector. dIRF7-mCherry was amplified and cloned into expression vectors as previously described (Xiao et al., 2018). A 2xFLAG-dIRF7 expression construct was made using dIRF7-mCherry as a template. Primers were designed to add a 5′- and a 3′- restriction site to the gene, as well as a 5′-2xFLAG epitope tag. The resulting contig was inserted into a pcDNA3.1+(hyg) expression vector. All PCR amplifications were performed using Phusion polymerase (NEB).

Primers were designed to amplify the autophagosome marker, duck microtubule-associated proteins 1A/1B light chain 3B (MAPLC3B or LC3B) based on sequence annotation on NCBI (GeneID: 101798675). The gene was amplified from cDNA made from the RNA from the lungs of a healthy 6-week-old Pekin duck. cDNA was made using Superscript III (Invitrogen) and oligo dT primers. Resulting amplification fragments were placed into a pCR2.1 TOPO cloning vector (Invitrogen). Using pCR2.1-LC3B as a template, primers were designed to add a 5′-SalI restriction site and a 3′-NotI restriction site. A 3′-2xFLAG epitope tag was also

added to the template using a reverse primer. LC3B-2xFLAG was cloned into the pcDNA3.1+(hyg) cloning vector. All PCR reactions were performed using Phusion (NEB).

All primers used for creation and amplification of TRIM27L domain and mutant constructs, 2xFLAG-MAVS, 2xFLAG-dIRF7 and LC3B-2xFLAG can be found in Table 6.1.

6.2.2 DEF extraction and cell culture conditions

Duck embryonic fibroblasts (DEF) cells were harvested from 12-day old Pekin duck embryos provided by Duckscetera (Bonnyville, Alberta) and grown as previously described (Chapter 5). Both DF-1 and DEF cells were grown and maintained at 39 °C + 5 % CO_2 in DMEM + 10 % FBS (Gibco).

6.2.3 Transfection and infection of DF-1 cells with H6N2 IAV

Twenty-four hours prior to transfection DF-1 cells were seeded at $1x10^6$ cells per well, and DEF cells were seeded at $1.5x10^6$ cells per well in 6 well plates. Cells were transfected using lipofectamine 2000 (Invitrogen) according to the manufacturer's protocol with a total of 2 µg plasmid DNA per well as previously described (Chapter 5). Cells were transfected with either 2 µg of TRIM27L or TRIM27.1, or 2 µg of TRIM27L or TRIM27L plus 300 ng d2CARD. Eighteen hours post transfection, cells were infected with either an avian H6N2 strain (A/Ck/California/431/2000) at a multiplicity of infection (MOI) of 1 in DMEM + 0.3% BSA (Gibco and Sigma) infection media with 0.1 µg/mL of TPCK trypsin added. The infection was allowed to progress for 24 hours, at which point cells were detached using 0.25% trypsin in EDTA (Gibco). Cells were washed and fixed in 2% PFA before being permeabilized in 0.25% Tween-20 (Sigma). To confirm successful transfection, cells were stained with anti-V5-Alexa Fluor 647 (ThermoFisher) and anti-FLAG iFluor 647 (ThermoFisher). To detect influenza, cells were stained with anti-nucleoprotein (NP) conjugated to FITC MA-7322 (Invitrogen). Nuclei were stained using Hoechst 33324 (Sigma). Compensation controls consisted of anti-NP FITC only, anti-V5 Alexa Fluor 647, Hoechst only and unstained cells. Flow cytometry analysis was performed on stained cells in the University of Alberta Faculty of Medicine and Dentistry Flow Cytometry Facility, operated with grant support from Canadian Institutes of Health Research (CIHR) and financial support from the Faculty of Medicine and Dentistry.

6.2.4 Dual luciferase-reporter assays

DF-1 cells were plated at a density of $0.25x10^6$ cells per well and DEF cells were plated at a density of $0.5x10^6$ cells per well. Both cell types were allowed to grow for 24 hours before

transfection. Cells were transfected with chicken IFN-β promoter (150 ng/well) in the PGL3 luciferase expression vector and *Renilla* control expression vector (10 ng/well). 250 ng of TRIM27L and all TRIM27L mutant and wildtype constructs were transfected in each well. RIG-I signaling pathway components GST-d2CARD, V5-dMAVS and 2xFLAG-dIRF7 were added to each well at 15 ng/well, 100 ng/well and 100 ng/well (respectively). Total DNA was kept equal between wells by adding empty pcDNA3.1+(hyg) vector. Luciferase assays investigating TRIM27L wildtype, domain and mutant constructs cotransfected with TRIM19.1 and dIRF7 used 250 ng/well of TRIM27L and TRIM19.1 and 100 ng/well of dIRF7, and DNA was equalized using empty vector control. IFN-β promoter reporter activity was measured 24 h post-transfection using the Dual Luciferase Reporter Assay System (Promega). Cells were lysed using 1x passive lysis buffer (100 µL/well). DF-1 cells were incubated in the lysis buffer for 30 minutes at room temperature while DEF cells were incubated in the lysis buffer for 1 hour at room temperature. After the incubation period, the lysate was added to 100 µL of luciferase assay reagent II (LAR II buffer) (Promega). All luminescence measurements were completed using a the GloMax 20/20 Luminometer (Promega). The ratio of firefly to *Renilla* luciferase luminescence was calculated for each well.

For proteasome and lysosome inhibition assays cell culture grade MG132 (Sigma) and ammonium chloride (NH_4Cl) (Sigma) or DMSO control were added to cells 24-hours post transfection. Cells were incubated in MG132, NH_4Cl or DMSO for 5 hours before being lysed (as described above).

6.2.5 Confocal microscopy and analysis

To sterilize coverslips, they were washed in 75 % EtOH and dried under UV light for 30 minutes. Coverslips were then placed in the bottom of 24-well plates and washed 3x in PBS. DF-1 cells were seeded at a density of $0.25x10^6$ cells/well on these coverslips and allowed to grow for 24-hours. Cells were transfected with a maximum of 500 ng/well with their respective expression plasmid vectors. After 24 hours, wells were fixed in 0.5 % PFA for 30 minutes and then permeabilized in 0.25 % Triton-X for 30 minutes at room temperature. Nuclei were stained with Hoechst 33324 (2 µg/mL) for 15 minutes at room temperature. Coverslips were mounted onto microscope slides (1.0 mm thick) using in house made Mowiol mounting media (Appendix A3). Mounted coverslips were allowed to dry in dark for 24 hours at room temperature and stored in the dark at 4 °C. Slides were visualized using the WaveFX spinning disk confocal

microscope (Quorum Technologies) located at the Cell Imaging Centre located operated by the faculty of medicine and dentistry at the university of Alberta.

6.3 Results

6.3.1 TRIM27L increases IFNβ signaling when cotransfected with d2CARD and dMAVS

We previously demonstrated that TRIM27L restricted H6N2 in DEF but not DF1 cells (Chapter 5). To investigate whether addition of RIG-I rescued the anti-IAV activity of TRIM27L in DF-1 cells, we transfected DF-1 cells with the constitutively active 2CARD domains of RIG-I and TRIM27.1, TRIM27L or TRIM25 (Figure 6.1A). As expected, activation of the RIG-I signaling pathway with d2CARD decreased the ratio of IAV infected cells compared to vector. TRIM27.1, which slightly decreases IFN-β promoter activity downstream of RIG-I in DF-1 cells (Blaine et al., 2015) did not significantly increase the relative infection of DF-1 cells. TRIM25, which stabilizes RIG-I signaling in human and increases IFN signaling downstream of MDA5 in chicken (Zhou et al., 2020), did not significantly decrease relative infection in DF-1 cells. When TRIM27L was cotransfected with d2CARD there was a significant decrease in relative viral infection compared to both pcDNA3.1+(hyg) vector control, d2CARD alone or TRIM27L alone. Cotransfection with d2CARD and TRIM27.1 or TRIM25 did not significantly alter IAV replication compared to d2CARD alone. Our results suggest TRIM27L needs either RIG-I or RIG-I pathway components to restrict IAV in DF-1 cells.

To determine what component of the RIG-I signaling pathway TRIM27L was interacting with to exert its antiviral effect, we cotransfected TRIM27L with either d2CARD, dMAVS or dIRF7 in both DF-1 cells or primary DEF cells and measured the relative chIFNβ promoter activation using dual-luciferase assays (Figure 6.1B). TRIM27L significantly increased the IFN-β promoter activity when cotransfected with both d2CARD and dMAVS in both DF-1 and DEF cells. Unexpectedly, when TRIM27L was transfected with IRF7, it strongly inhibited the IFN-β promoter activity in both DF-1 and DEF cells. As TRIM27L inhibits IAV replication in DEF cells, it is likely that this inhibitory effect on dIRF7 is context dependent.

To determine if TRIM27L colocalized with any of the RIG-I signaling pathway components we cotransfected DF-1 cells with TRIM27L and either d2CARD, dMAVS or dIRF7 (Figure 6.1C). TRIM27L colocalized with d2CARD, although previous coimmunoprecipitation experiments in our lab previously demonstrated that TRIM27L did not interact with RIG-I (data

not shown). As TRIM27L can activate the IFN-β promoter when cotransfected with dMAVS in DF-1 cells (which do not have endogenous RIG-I), it is likely TRIM27L is interacting with a cofactor that is also in close proximity to RIG-I. TRIM27L did not colocalize with dMAVS, indeed there is an area separating aggregated TRIM27L and dMAVS. TRIM27L appears to moderately colocalize with dIRF7. Nuclear TRIM27L did not coincide with nuclear dIRF7, indeed much of the colocalization between TRIM27L and dIRF7 appears to be cytoplasmic, suggesting TRIM27L could be preventing dIRF7 nuclear translocation. To investigate the possibility that TRIM27L targeted IRF7 for destruction, we examined whether TRIM27L localized with lysosomes, mitochondria or autophagosomes (Figure 6.1D). There was no significant colocalization between TRIM27L and lysosomes. TRIM27L had moderate colocalization with the autophagosome marker LCB3 in the cytoplasm of DF-1 cells. Autophagosomes fuse with lysosomes to degrade cellular components (Mizushima, 2007; Glick et al., 2010), so if autophagic degradation was the mechanism used by TRIM27L to inhibit IRF7, a lysosomal inhibitor should restore IFN-β promoter activity. To determine if lysosomal degradation was responsible for IFN-β promoter inhibition downstream of dIRF7, we treated DF-1 cells cotransfected with dIRF7 and TRIM27L with the lysosomal inhibitor NH_4Cl to see if IFN-β promoter activity could be restored. We did not see a significant increase in IFN-β promoter activity in DF-1 cells after 5 hours of NH_4Cl treatment (Supplementary figure S6.1). We also tested to see if this inhibition was caused by proteasomal degradation, however treatment of DF-1 cells cotransfected with dIRF7 and TRIM27L with the proteasome inhibitor MG132 also did not restore IFN-β promoter activity in these cells.

6.3.2 Deletion of RING domain localizes TRIM27L to the nucleus

To determine which domain of TRIM27L was responsible for both the activation of the RIG-I signaling pathway upstream of dIRF7, and the inhibition of the RIG-I signaling pathway when TRIM27L was cotransfected with dIRF7, we made domain and mutant constructs of TRIM27L (Figure 6.2A). We made a RING deletion construct (ΔRING) and a construct where the cross-bracing architecture of the RING domain was disrupted by mutating cysteines 16 and 31 to alanine (C16AC31A). We also made a RING and BBOX deletion (ΔRBB) and a PRYSPRY domain deletion expression construct (ΔPS). Additionally, we made a construct of just the C-terminal PRYSPRY domain.

To verify that all domain and mutant constructs were expressed and to inspect the subcellular localization of all proteins, we used confocal microscopy. All domain and mutant TRIM27L proteins were expressed in DF-1 cells (Figure 6.2). When compared to the expression of wildtype TRIM27L (Figure 6.2B) deletion or mutation of the RING domain of TRIM27L caused the overexpressed proteins to locate diffusely in the cytoplasm and form punctate nuclear spots (Figure 6.2C and D). Further deletion of the RING and B-box domains resulted in the overexpressed protein localizing diffusely in both the cytoplasm and nucleus (Figure 6.2E). The PRYSPRY domain expressed on its own localized to the cytoplasm and nucleus, suggesting that the PRYSPRY domain has a nuclear localization signal (Figure 6.2F). Deletion of the C-terminal PRYSPRY domain did not change the overall phenotype of the protein, with large dense cytoplasmic aggregates evident as well as smaller nuclear spots (Figure 6.2G). The results of our mutant and domain construct expression assays suggests that the RING domain is necessary for the large cytoplasmic aggregates. Small nuclear dots were present during over expression of every construct except ΔRBB-V5 and PRYSPRY-V5 suggesting that the B-box domain localizes the protein to distinct punctate structures in the nucleus, potentially through higher order multimerization with other proteins. The PRYSPRY domain appears to aid in nuclear localization. As no nuclear localization signal (NLS) was detected on TRIM27L when it was analyzed using prediction software (data not shown) it is possible that the PRYSPRY domain of TRIM27L is binding to another protein that has an NLS.

6.3.3 TRIM27L PRYSPRY domain activates the RIG-I signaling pathway, while the RING domain inhibits downstream of IRF7.

We used dual-luciferase reporter assays to determine the TRIM27L domains used in activation and inhibition of the RIG-I signaling pathway in DEF and DF-1 cells (Figure 6.3). As previously reported, when TRIM27L is co-transfected with the constitutively active 2CARD domains, it greatly increases IFN-β promoter activity in both chicken and duck cells. When RING domain mutant constructs are co-transfected with d2CARD we saw that deletion or mutation of the RING domain does not abrogate IFN-β promoter activity in DF-1 or DEF cells (Figure 6.3A). In DEF cells, mutation of the RING domain increases IFN-β promoter activity above that of TRIM27L wildtype, whereas the RING deletion does not have this effect. Interestingly, in DF-1 cells when both the RING and B-box domain are deleted (ΔRBB) the IFN-β promoter activity is reduced to that of d2CARD alone, while in DEF cells this mutant activates

IFN-β promoter activity as much as wildtype TRIM27L. When the TRIM27L with PRYSPRY domain deleted is cotransfected with d2CARD the IFN-β promoter activity is decreased compared to wildtype, suggesting that in both DEF and DF-1 cells the PRYSPRY domain is necessary for the increase in IFN-β promoter activity. A similar pattern is seen when the TRIM27L domain and mutant constructs are cotransfected with dMAVS (Figure 6.3B). The PRYSPRY domain is responsible for the increase in IFN-β promoter activity, with deletion of this domain abrogating IFN-β promoter activity compared to the wildtype TRIM27L. Deletion of both the RING and B-box domains abrogates IFN-β promoter activity in DF-1 cells only. When TRIM27L is cotransfected with dIRF7 it significantly decreases IFN-β promoter activity. Transfecting domain and mutant constructs in both DEF and DF-1, we find that deletion or mutation of the RING domain restores IFN-β promoter activity (Figure 6.3C), whereas there is still significant inhibition when ΔPRYSPRY is cotransfected with dIRF7. Our results show that the PRYSPRY domain of TRIM27L is necessary for the activation of IFN-β signaling downstream of RIG-I and MAVS, while the RING domain is necessary for the inhibition of IFN-β promoter activity when TRIM27L is cotransfected with dIRF7.

6.3.4 TRIM27L PRYSPRY domain colocalizes with TRIM19.1

As deletion and mutation of the RING domain of TRIM27L causes the protein to localize to distinct punctate nuclear locations, we hypothesized that TRIM27L may be interacting with PML-nuclear bodies. We investigated if TRIM27L was interacting with duck TRIM19.1, one of the paralogous PML isoforms found in ducks that forms PML-like nuclear bodies in DF-1 cells (described in Chapter 5**).** We cotransfected DEF cells with TRIM27L-V5 expression constructs or 2xFLAG-TRIM19.1 and performed dual-luciferase assays as well as colocalization analysis (Figure 6.4). Full-length TRIM27L did not colocalize with TRIM19.1, nor did the nuclear localized ΔRING construct (Figure 6.4A), or TRIM27LC16AC31A (data not shown). The ΔPRYSPRY TRIM27L construct also did not colocalize with TRIM19.1. However, when the RING and B-box domains were both deleted from the constructs, TRIM27L deletion mutants began to colocalize with TRIM19.1. The TRIM27LΔRBB construct had moderate colocalization when cotransfected with TRIM19.1, while the PRYSPRY domain on its own had significant colocalization with TRIM19.1. These results suggest that the interactions of the B-box domain of TRIM27L prevent the PRYSPRY domain of TRIM27L from interacting with TRIM19.1. As the PRYSPRY domain is known to bind substrate proteins (Woo et al., 2006; James et al., 2007),

this suggests that TRIM27L may need post translational modifications, or specific isoform expression to interact with TRIM19.1.

To determine if the PRYSPRY domain interactions with TRIM19.1 influences the effects of TRIM27L overexpression on IFNβ promoter activity, we cotransfected TRIM27L constructs with TRIM19.1 and dIRF7 (Figure 6.4B). When cotransfected with dIRF7, TRIM19.1 causes a decrease in IFNβ promoter activity**.** When dIRF7 is cotransfected with TRIM19.1 and any of the TRIM27L constructs, IFN-β promoter activity is decreased below activity seen in treatments without TRIM19.1 added. If TRIM27L and TRIM19.1 were working synergistically to decrease IFN-β signaling, we would expect that deletion of one of the TRIM27L domains would cause the decrease in IFN-β promoter activity to be restored when it was cotransfected with TRIM19.1 We did not see this restoration, however, suggesting that TRIM27L and TRIM19.1 are not working together to regulate IFN-β promoter activity.

6.4 Discussion

Here we determine the modulatory effects of TRIM27L on the RIG-I signaling pathway in duck and chicken cells. TRIM27L was not able to restrict IAV in chicken DF-1 cells unless constitutively active RIG-I CARD domains were cotransfected with TRIM27L, however TRIM27L can restrict IAV in DEF cells. These results suggest that the resistance seen is due to IFN being produced by cells and not TRIM27L directly targeting the virus. We cotransfected TRIM27L with the constitutively active 2CARD domains of duck RIG-I, dMAVS and dIRF7 and demonstrate that TRIM27L increases MAVS signaling leading to IFN-β promoter activity when cotransfected with both d2CARD and dMAVS (Figure 6.5A), however this signaling is significantly inhibited when TRIM27L is cotransfected with dIRF7 (Figure 6.5B). TRIM27L did not colocalize with dMAVS but did show moderate colocalization when cotransfected with d2CARD and dIRF7 (Figure 6.5C). Because wildtype TRIM27L forms large dense insoluble cytoplasmic aggregates when overexpressed, we were unable to perform co-immunoprecipitation experiments to determine if TRIM27L was physically interacting with any of the RIG-I signaling pathway components. We have also tried using coimmunoprecipitation and mass spectrometry to identify what TRIM27L was interacting with in DF-1 cells. Our mass spectrometry results only found TRIM27L, suggesting it was oligomerizing with other TRIM27L proteins (Appendix A4). Additionally, TRIM27L can augment MAVS signaling in chicken cells from overexpressed duck

MAVS. As chickens appear to be lacking RIG-I, this suggests that TRIM27L is interacting with an adaptor or regulatory protein in the RIG-I signaling pathway that is present in both duck and chicken.

TRIM27L moderately colocalizes with the autophagic protein LC3B. LC3B proteins exhibit cytoplasmic subcellular localization until autophagic pathways are activated by environmental factors such as starvation or viral infection (Sharma et al., 2018). Activated LC3B form membrane associated puncta in the cytoplasm which decorate autophagosomes (Levine et al., 2008). Autophagy also selectively targets aggregated proteins (Lamark et al., 2012). It is possible that the colocalization of LC3B and TRIM27L is due to autophagic pathways being activated from overexpressed protein aggregates. As the LC3B and TRIM27L colocalization is cytoplasmic and diffuse and not punctate, it suggests that LC3B is not interacting with activated autophagosomes. This may suggest that TRIM27L is preventing autophagosome formation by interacting with LC3B. To test this, experiments activating LC3B autophagosome formation and inspecting the effect of TRIM27L overexpression on autophagosome formation need to be performed. It is also noteworthy that adding the lysosomal inhibitor NH_4Cl did not restore IFN-β signaling when added to cotransfections with dIRF7 and TRIM27L, suggesting autophagy or lysosomal degradation is not responsible for the TRIM27L inhibition of the IFN-β promoter. However, these experiments were lacking significant controls, as we are unaware of any avian proteins that have been documented as responding to either MG132 or NH_4Cl inhibition of cellular antiviral signaling pathways that are responsive in dual-luciferase assays. DF-1 cells do respond to MG132 treatment, in that MG132 inhibited infectious bursal disease virus-induced apoptosis in infected cells (Liu et al., 2007) suggesting that the chemical treatment works.

TRIM27L moderately colocalized with mitochondria but did not colocalize with MAVS. In fact, in colocalization analyses of TRIM27L and dMAVS, TRIM27L appears to surround MAVS, but there is distinct and visible space between TRIM27L aggregates and MAVS.

The domain and mutant constructs of TRIM27L allowed us to better understand the subcellular location of TRIM27L protein. When the RING domain was mutated or deleted, the dense cytoplasmic aggregates diminished, and the protein was either diffusely cytoplasmic or localized to punctate spots on the nucleus of the cell. These results suggest that the RING domain allowed TRIM27L to oligomerize in the cytoplasm. RING domains have been implicated in dimerization of other TRIM proteins such as TRIM32 (Koliopoulos et al., 2016)

and TRIM69 (Keown et al., 2020), while TRIM19 RING domains tetramerize to promote higher order self-assembly of TRIM19 proteins (Wang et al., 2018). Deletion of the RING domain also abolished the inhibitory effect of TRIM27L on dIRF7. Together these results suggest that the inhibition of IRF7 takes place in the cytoplasm and may be dependent on TRIM27L self-assembly. As inhibition of the proteasome using MG132 did not restore IFN-β promoter activity downstream of dIRF7, it is unlikely that TRIM27L is ubiquitinating dIRF7 for proteasomal degradation. Deletion of the PRYSPRY domain caused the protein to remain cytoplasmic, but the PRYSPRY domain expressed on its own, or further deletion of the N-terminal RING and B-box domains, caused the protein to become more localized to the nucleus. It is noteworthy that both the PRYSPRY and ΔRINGBBOX protein products are under 60 kDa, so part of the nuclear localization is potentially due to passive diffusion (Shimonozo et al., 2009). However, as the ΔRING mutant also shows nuclear localization, it is likely that the PRYSPRY and B-box domains contribute to the nuclear subcellular localization. TRIM22, a eutherian specific TRIM (Sawyer et al., 2007; Hattlmann et al., 2012), also uses its PRYSPRY domain to localize to the nucleus (Sivaramakrishnan et al., 2009). Like the wildtype TRIM27L, TRIM22 does not colocalize with PML bodies, and instead colocalizes with or is in adjacent location to Cajal bodies. Cajal bodies are punctate nuclear structures that often contain components involved in post translational modification of nuclear RNAs (Gall, 2000).

While our results demonstrating both negative and positive regulation of the RIG-I signaling pathway may seem counterintuitive, other TRIM proteins have demonstrated both positive and negative regulation on the same signaling pathway. TRIM13 is characterized by its C-terminal transmembrane domain (Hatakeyama, 2011; Narayan et al., 2014) and is documented to both activate and restrict immune signaling in cells depending on the pathway it is interacting with (Narayan et al., 2014; Huang et al., 2017; Li et al., 2022b). In mouse bone marrow derived macrophages, TRIM13 positively regulates RIG-I signaling and negatively regulates MDA5 signaling upstream of IRF3 (Narayan et al., 2014). TRIM13 also helps degrade the DNA virus sensing PRR STING (Li et al., 2022b) and increases TLR2-meidated immune responses through K26-linked polyubiquitination of TRAF6 (Huang et al., 2017). TRIM35 potentiates RIG-I signaling through K63-linked ubiquitination of TRAF3 in both mouse (Sun et al., 2020) and pig (Li et al., 2022a). Mouse TRIM35 also negatively regulates TLR7 and TLR9 signaling through K48-linked ubiquitination of IRF7 (Wang et al., 2015b). TRIM26 helps bridge the association

with TBK1 and NEMO at the TBK1-MAVS signaling complex, increasing IRF3 phosphorylation and cellular antiviral responses (Ran et al., 2016). TRIM26 also inhibits IRF3 by translocating with phosphorylated IRF3 to the nucleus and causing its degradation (Wang et al., 2015a). Like TRIM27L, TRIM26 is an MHC-linked TRIM gene (Jia et al., 2021). Also, similar to TRIM27L, it appears that the subcellular location of TRIM26 helps dictate its function, with nuclear TRIM26 causing inhibition of antiviral signaling, while cytoplasmic TRIM26 increases it. TRIM27L however, appears to decrease signaling when its location is primarily cytoplasmic and increases IFN-β signaling when its subcellular location is primarily nuclear. It is possible that TRIM27L acts in a temporal manner on the RIG-I signaling pathway in the duck, and that during initial infection with IAV TRIM27L increases IFN-β signaling, however as IRF7 accumulates in the cytoplasm during the course of infection, TRIM27L then targets IRF7 for degradation or physically blocks nuclear translocation.

We did not find any definitive substrate or interacting proteins for TRIM27L when it activates the IFN signaling pathway. This is largely due to the incredibly vast number of proteins that TRIM27L could be binding with. Several reviews have outlined TRIM proteins that can augment antiviral signaling pathways in the cell, and as well as ubiquitinating main effectors of antiviral signaling pathways, many TRIM proteins also augment lesser-known effector proteins to modify these pathways (Rajsbaum et al., 2014; van Gent et al., 2018; Yang et al., 2020). Previous attempts at coimmunoprecipitation and mass spectrometry to determine binding partners could have failed due to interactions of TRIM27L with substrate being too transient to be detected. Additionally, multiple TRIM proteins have the ability to bind RNA-targets rather than protein targets (Liu et al., 2016; Williams et al., 2019; Connacher et al., 2021). If TRIM27L is interacting with RNA, mass spectrometry would not detect this. Finally, while the current version of the duck genome is very well assembled, there are still missing genes. Protein databases are similarly incomplete, especially for non-human or non-mammalian species, such as ducks. It is possible that there was a plentiful protein present in our mass spectrometric analysis that was not identified as a binding partner of TRIM due to this protein sequence not being annotated.

Human TRIM27 (also known as ret finger protein or RFP) associates with PML nuclear bodies in human cells using its B-box domain (Cao et al., 1998). Our TRIM27L did not primarily associate in the nucleus nor did it colocalize with PML until the B-box domain was deleted,

RING deletion alone did not cause colocalization with the nucleus or TRIM19.1 (Figure 6.5C). However, the TRIM27L PRYSPRY domain did colocalize with TRIM19.1. The PRYSPRY domain is generally involved in substrate recognition (James et al., 2007; D'Cruz et al., 2013), suggesting that TRIM19.1 could potentially be a substrate of TRIM27L. It remains to be determined why the full-length TRIM27L does not appear to colocalize with TRIM19.1. Potentially, there are specific isoforms of TRIM27L that interact with TRIM19.1. Our data would suggest that if this interaction is isoform specific, it would be an isoform missing the B-box domain of TRIM27L. B-box domains can be involved in higher order multimerization or oligomerization, or even E3 ubiquitin ligase activity (Massiah et al., 2006; Han et al., 2011; Wagner et al., 2016). If this interaction is not isoform specific, it may be that TRIM27L needs additional post translational modification to exhibit the conformation needed to allow for the PRYSPRY domain to interact with PML. Additionally, we have documented two paralogs of TRIM19 in birds, in this study we used the shorter TRIM19.1, as the longer isoform TRIM19.2 is difficult to express and visualize in avian cells (Chapter 5). Both paralogs of TRIM19.1 are incredibly GC rich, especially in the exon coding their B-box type II domain. It is also worth noting that duck TRIM27L is likely related to human TRIM27 in name only, as phylogenetic analysis suggests that human TRIM27 and the three duck TRIM27 paralogs arose independently of each other, and from a different ancestral TRIM protein (Chapter 4), suggesting that TRIM27L is not functionally analogous to human TRIM27.

This study highlights the complex relationship between duck TRIM27L and the MAVS signaling pathway in both duck and chicken cells. While we did not identify substrate proteins of TRIM27L, we did gain further insight into the complex functional plasticity of TRIM proteins in the cell. As ducks are both permissive to influenza A replication and resistant to damaging effects of this viral replication, it is likely they have gained species specific mechanisms for this resistance through a long evolutionary relationship with IAV. Currently, there is no publicly available mallard duck cell line, however the development of such a line could help answer questions on TRIM27L function. By investigating subcellular location and pathway augmentation of TRIM27L in ducks, we gain insight into the complex evolution of TRIM proteins and pathway augmentation in vertebrate species.

6.5 References

Ardley, H.C., and Robinson, P.A. (2005). E3 ubiquitin ligases. *Essays Biochem* 41(1)**,** 15-30. doi: 10.1042/EB0410015.

Bell, J.L., Malyukova, A., Holien, J.K., Koach, J., Parker, M.W., Kavallaris, M., et al. (2012). TRIM16 acts as an E3 ubiquitin ligase and can heterodimerize with other TRIM family members. *PLoS One* 7(5)**,** e37470. doi: 10.1371/journal.pone.0037470.

Blaine, A.H., Miranzo-Navarro, D., Campbell, L.K., Aldridge, J.R., Jr., Webster, R.G., and Magor, K.E. (2015). Duck TRIM27-L enhances MAVS signaling and is absent in chickens and turkeys. *Mol Immunol* 67(2 Pt B)**,** 607-615. doi: 10.1016/j.molimm.2015.07.011.

Cao, T.Y., Duprez, E., Borden, K.L.B., Freemont, P.S., and Etkin, L.D. (1998). Ret finger protein is a normal component of PML nuclear bodies and interacts directly with PML. *Journal of Cell Science* 111(10)**,** 1319-1329.

Chaves, L.D., Krueth, S.B., and Reed, K.M. (2009). Defining the turkey MHC: sequence and genes of the B locus. *J Immunol* 183(10)**,** 6530-6537. doi: 10.4049/jimmunol.0901310.

Connacher, R.P., and Goldstrohm, A.C. (2021). Molecular and biological functions of TRIM-NHL RNA-binding proteins. *Wiley Interdiscip Rev RNA* 12(2)**,** e1620. doi: 10.1002/wrna.1620.

D'Cruz, A.A., Babon, J.J., Norton, R.S., Nicola, N.A., and Nicholson, S.E. (2013). Structure and function of the SPRY/B30.2 domain proteins involved in innate immunity. *Protein Sci* 22(1)**,** 1-10. doi: 10.1002/pro.2185.

Esposito, D., Koliopoulos, M.G., and Rittinger, K. (2017). Structural determinants of TRIM protein function. *Biochem Soc Trans* 45(1)**,** 183-191. doi: 10.1042/BST20160325.

Gall, J.G. (2000). Cajal bodies: the first 100 years. *Annu Rev Cell Dev Biol* 16**,** 273-300. doi: 10.1146/annurev.cellbio.16.1.273.

Glick, D., Barth, S., and Macleod, K.F. (2010). Autophagy: cellular and molecular mechanisms. *J Pathol* 221(1)**,** 3-12. doi: 10.1002/path.2697.

Han, X., Du, H., and Massiah, M.A. (2011). Detection and characterization of the in vitro e3 ligase activity of the human MID1 protein. *J Mol Biol* 407(4)**,** 505-520. doi: 10.1016/j.jmb.2011.01.048.

Hatakeyama, S. (2011). TRIM proteins and cancer. *Nat Rev Cancer* 11(11)**,** 792-804. doi: 10.1038/nrc3139.

Hattlmann, C.J., Kelly, J.N., and Barr, S.D. (2012). TRIM22: A Diverse and Dynamic Antiviral Protein. *Mol Biol Int* 2012**,** 153415. doi: 10.1155/2012/153415.

Hayman, T.J., Hsu, A.C., Kolesnik, T.B., Dagley, L.F., Willemsen, J., Tate, M.D., et al. (2019). RIPLET, and not TRIM25, is required for endogenous RIG-I-dependent antiviral responses. *Immunology & Cell Biology* 97(9)**,** 840-852. doi: https://doi.org/10.1111/imcb.12284.

Hu, M.M., Xie, X.Q., Yang, Q., Liao, C.Y., Ye, W., Lin, H., et al. (2015). TRIM38 Negatively Regulates TLR3/4-Mediated Innate Immune and Inflammatory Responses by Two Sequential and Distinct Mechanisms. J Immunol 195(9), 4415-4425. doi: 10.4049/jimmunol.1500859.

Hu, M.M., Yang, Q., Xie, X.Q., Liao, C.Y., Lin, H., Liu, T.T., et al. (2016). Sumoylation Promotes the Stability of the DNA Sensor cGAS and the Adaptor STING to Regulate the Kinetics of Response to DNA Virus. Immunity 45(3), 555-569. doi: 10.1016/j.immuni.2016.08.014.

Huang, B., and Baek, S.H. (2017). TRIM13 potentiates Toll-like receptor 2-mediated nuclear factor κB activation via K29-linked polyubiquitination of tumor necrosis factor receptor-associated factor 6. *Mol Pharmacol* 91(4)**,** 307-316. doi: 10.1124/mol.116.106716.

James, L.C., Keeble, A.H., Khan, Z., Rhodes, D.A., and Trowsdale, J. (2007). Structural basis for PRYSPRY-mediated tripartite motif (TRIM) protein function. *Proceedings of the National Academy of Sciences* 104(15)**,** 6200. doi: 10.1073/pnas.0609174104.

Jia, X., Zhao, C., and Zhao, W. (2021). Emerging Roles of MHC Class I Region-Encoded E3 Ubiquitin Ligases in Innate Immunity. *Frontiers in Immunology* 12. doi: 10.3389/fimmu.2021.687102.

Kaufman, J., Milne, S., Göbel, T.W.F., Walker, B.A., Jacob, J.P., Auffray, C., et al. (1999). The chicken B locus is a minimal essential major histocompatibility complex. *Nature* 401(6756)**,** 923-925. doi: 10.1038/44856.

Keown, J.R., Yang, J., Black, M.M., and Goldstone, D.C. (2020). The RING domain of TRIM69 promotes higher-order assembly. *Acta Crystallogr D Struct Biol* 76(Pt 10)**,** 954-961. doi: 10.1107/s2059798320010499.

Koliopoulos, M.G., Esposito, D., Christodoulou, E., Taylor, I.A., and Rittinger, K. (2016). Functional role of TRIM E3 ligase oligomerization and regulation of catalytic activity. *The EMBO Journal* 35(11)**,** 1204-1218. doi: https://doi.org/10.15252/embj.201593741.

Kouwaki, T., Nishimura, T., Wang, G., Nakagawa, R., and Oshiumi, H. (2021). Ubiquitin ligase RIPLET mediates polyubiquitination of RIG-I and LGP2 and regulates the innate immune responses to SARS-CoV-2 infection. *bioRxiv***,** 2021.2001.2025.428042. doi: 10.1101/2021.01.25.428042.

Lamark, T., and Johansen, T. (2012). Aggrephagy: selective disposal of protein aggregates by macroautophagy. *International Journal of Cell Biology* 2012**,** 736905. doi: 10.1155/2012/736905.

Levine, B., and Kroemer, G. (2008). Autophagy in the pathogenesis of disease. *Cell* 132(1)**,** 27-42. doi: https://doi.org/10.1016/j.cell.2007.12.018.

Li, C., Zhou, Y., Chen, X., Zhang, Y., Hu, J., Ren, C., et al. (2022a). Porcine TRIM35 positively regulate TRAF3-mediated IFN-β production and inhibit Japanese encephalitis virus replication. *Dev Comp Immunol* 127**,** 104290. doi: 10.1016/j.dci.2021.104290.

Li, X., Yu, Z., Fang, Q., Yang, M., Huang, J., Li, Z., et al. (2022b). The transmembrane endoplasmic reticulum-associated E3 ubiquitin ligase TRIM13 restrains the pathogenic-DNA-triggered inflammatory response. *Sci Adv* 8(4)**,** eabh0496. doi: 10.1126/sciadv.abh0496.

Liu, B., Li, N.L., Shen, Y., Bao, X., Fabrizio, T., Elbahesh, H., et al. (2016). The C-terminal tail of TRIM56 dictates antiviral restriction of influenza A and B viruses by impeding viral RNA synthesis. *J Virol* 90(9)**,** 4369-4382. doi: 10.1128/JVI.03172-15.

Liu, J., Wei, L., Jiang, T., Shi, L., and Wang, J. (2007). Reduction of infectious bursal disease virus replication in cultured cells by proteasome inhibitors. *Virus Genes* 35(3)**,** 719-727. doi: 10.1007/s11262-007-0147-3.

Magor, K.E., Miranzo Navarro, D., Barber, M.R., Petkau, K., Fleming-Canepa, X., Blyth, G.A., et al. (2013). Defense genes missing from the flight division. *Dev Comp Immunol* 41(3)**,** 377-388. doi: 10.1016/j.dci.2013.04.010.

Massiah, M.A., Simmons, B.N., Short, K.M., and Cox, T.C. (2006). Solution structure of the RBCC/TRIM B-box1 domain of human MID1: B-box with a RING. *J Mol Biol* 358(2)**,** 532-545. doi: 10.1016/j.jmb.2006.02.009.

Meroni, G. (2012). Genomics and evolution of the TRIM gene family. *Adv Exp Med Biol* 770**,** 1-9. doi: 10.1007/978-1-4614-5398-7_1.

Metzger, M.B., Pruneda, J.N., Klevit, R.E., and Weissman, A.M. (2014). RING-type E3 ligases: master manipulators of E2 ubiquitin-conjugating enzymes and ubiquitination. *Biochim Biophys Acta* 1843(1)**,** 47-60. doi: 10.1016/j.bbamcr.2013.05.026.

Miranzo-Navarro, D., and Magor, K.E. (2014). Activation of duck RIG-I by TRIM25 is independent of anchored ubiquitin. *PLoS One* 9(1)**,** e86968. doi: 10.1371/journal.pone.0086968.

Mizushima, N. (2007). Autophagy: process and function. *Genes Dev* 21(22)**,** 2861-2873. doi: 10.1101/gad.1599207.

Morris, K.M., Hindle, M.M., Boitard, S., Burt, D.W., Danner, A.F., Eory, L., et al. (2020). The quail genome: insights into social behaviour, seasonal biology and infectious disease response. *BMC Biol* 18(1)**,** 14. doi: 10.1186/s12915-020-0743-4.

Napolitano, L.M., and Meroni, G. (2012). TRIM family: Pleiotropy and diversification through homomultimer and heteromultimer formation. *IUBMB Life* 64(1)**,** 64-71. doi: 10.1002/iub.580.

Narayan, K., Waggoner, L., Pham, S.T., Hendricks, G.L., Waggoner, S.N., Conlon, J., et al. (2014). TRIM13 is a negative regulator of MDA5-mediated type I interferon production. *Journal of Virology* 88(18)**,** 10748. doi: 10.1128/JVI.02593-13.

Oshiumi, H., Miyashita, M., Inoue, N., Okabe, M., Matsumoto, M., and Seya, T. (2010). The ubiquitin ligase Riplet is essential for RIG-I-dependent innate immune responses to RNA virus infection. *Cell Host Microbe* 8(6)**,** 496-509. doi: 10.1016/j.chom.2010.11.008.

Ozato, K., Shin, D.M., Chang, T.H., and Morse, H.C., 3rd (2008). TRIM family proteins and their emerging roles in innate immunity. *Nat Rev Immunol* 8(11)**,** 849-860. doi: 10.1038/nri2413.

Rajsbaum, R., Garcia-Sastre, A., and Versteeg, G.A. (2014). TRIMmunity: the roles of the TRIM E3-ubiquitin ligase family in innate antiviral immunity. *J Mol Biol* 426(6)**,** 1265-1284. doi: 10.1016/j.jmb.2013.12.005.

Ran, Y., Zhang, J., Liu, L.L., Pan, Z.Y., Nie, Y., Zhang, H.Y., et al. (2016). Autoubiquitination of TRIM26 links TBK1 to NEMO in RLR-mediated innate antiviral immune response. *J Mol Cell Biol* 8(1)**,** 31-43. doi: 10.1093/jmcb/mjv068.

Rose, K.M., Spada, S.J., Broeckel, R., McNally, K.L., Hirsch, V.M., Best, S.M., et al. (2021). From capsids to complexes: Expanding the role of TRIM5α in the restriction of divergent RNA viruses and elements. *Viruses* 13(3). doi: 10.3390/v13030446.

Sanchez, J.G., Okreglicka, K., Chandrasekaran, V., Welker, J.M., Sundquist, W.I., and Pornillos, O. (2014). The tripartite motif coiled-coil is an elongated antiparallel hairpin dimer. *Proceedings of the National Academy of Sciences of the United States of America* 111(7)**,** 2494-2499. doi: 10.1073/pnas.1318962111.

Sawyer, S.L., Emerman, M., and Malik, H.S. (2007). Discordant evolution of the adjacent antiretroviral genes TRIM22 and TRIM5 in mammals. *PLOS Pathogens* 3(12)**,** e197. doi: 10.1371/journal.ppat.0030197.

Sawyer, S.L., Wu, L.I., Emerman, M., and Malik, H.S. (2005). Positive selection of primate TRIM5alpha identifies a critical species-specific retroviral restriction domain. *Proc Natl Acad Sci U S A* 102(8)**,** 2832-2837. doi: 10.1073/pnas.0409853102.

Sharma, V., Verma, S., Seranova, E., Sarkar, S., and Kumar, D. (2018). Selective autophagy and xenophagy in infection and disease. *Frontiers in Cell and Developmental Biology* 6.

Sivaramakrishnan, G., Sun, Y., Rajmohan, R., and Lin, V.C. (2009). B30.2/SPRY domain in tripartite motif-containing 22 is essential for the formation of distinct nuclear bodies. *FEBS Lett* 583(12)**,** 2093-2099. doi: 10.1016/j.febslet.2009.05.036.

Song, B., Javanbakht, H., Perron, M., Park, D.H., Stremlau, M., and Sodroski, J. (2005). Retrovirus restriction by TRIM5alpha variants from Old World and New World primates. *Journal of virology* 79(7)**,** 3930-3937. doi: 10.1128/JVI.79.7.3930-3937.2005.

Stremlau, M., Owens, C.M., Perron, M.J., Kiessling, M., Autissier, P., and Sodroski, J. (2004). The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. *Nature* 427(6977)**,** 848-853. doi: 10.1038/nature02343.

Sun, N., Jiang, L., Ye, M., Wang, Y., Wang, G., Wan, X., et al. (2020). TRIM35 mediates protection against influenza infection by activating TRAF3 and degrading viral PB2. *Protein Cell* 11(12)**,** 894-914. doi: 10.1007/s13238-020-00734-6.

van Gent, M., Sparrer, K.M.J., and Gack, M.U. (2018). TRIM proteins and their roles in antiviral host defenses. *Annu Rev Virol* 5(1)**,** 385-405. doi: 10.1146/annurev-virology-092917-043323.

Wagner, J.M., Roganowicz, M.D., Skorupka, K., Alam, S.L., Christensen, D., Doss, G., et al. (2016). Mechanism of B-box 2 domain-mediated higher-order assembly of the retroviral restriction factor TRIM5α. *Elife* 5. doi: 10.7554/eLife.16309.

Wang, P., Benhenda, S., Wu, H., Lallemand-Breitenbach, V., Zhen, T., Jollivet, F., et al. (2018). RING tetramerization is required for nuclear body biogenesis and PML sumoylation. *Nat Commun* 9(1)**,** 1277. doi: 10.1038/s41467-018-03498-0.

Wang, P., Zhao, W., Zhao, K., Zhang, L., and Gao, C. (2015a). TRIM26 negatively regulates interferon-β production and antiviral response through polyubiquitination and degradation of nuclear IRF3. *PLoS Pathog* 11(3)**,** e1004726. doi: 10.1371/journal.ppat.1004726.

Wang, Y., Yan, S., Yang, B., Wang, Y., Zhou, H., Lian, Q., et al. (2015b). TRIM35 negatively regulates TLR7- and TLR9-mediated type I interferon production by targeting IRF7. *FEBS Lett* 589(12)**,** 1322-1330. doi: 10.1016/j.febslet.2015.04.019.

Watanabe, M., and Hatakeyama, S. (2017). TRIM proteins and diseases. *J Biochem* 161(2)**,** 135-144. doi: 10.1093/jb/mvw087.

Webster, R.G., Bean, W.J., Gorman, O.T., Chambers, T.M., and Kawaoka, Y. (1992). Evolution and ecology of influenza A viruses. *Microbiol Rev* 56(1)**,** 152-179.

Williams, F.P., Haubrich, K., Perez-Borrajero, C., and Hennig, J. (2019). Emerging RNA-binding roles in the TRIM family of ubiquitin ligases. *Biological Chemistry* 400(11)**,** 1443-1464. doi: doi:10.1515/hsz-2019-0158.

Woo, J.S., Suh, H.Y., Park, S.Y., and Oh, B.H. (2006). Structural basis for protein recognition by B30.2/SPRY domains. *Mol Cell* 24(6)**,** 967-976. doi: 10.1016/j.molcel.2006.11.009.

Xiao, Y., Evseev, D., Stevens, C.A., Moghrabi, A., Miranzo-Navarro, D., Fleming-Canepa, X., et al. (2020). Influenza PB1-F2 Inhibits Avian MAVS Signaling. *Viruses* 12(4). doi: 10.3390/v12040409.

Xiao, Y., Reeves, M.B., Caulfield, A.F., Evseev, D., and Magor, K.E. (2018). The core promoter controls basal and inducible expression of duck retinoic acid inducible gene-I (RIG-I). *Mol Immunol* 103(September)**,** 156-165. doi: 10.1016/j.molimm.2018.09.002.

Yang, W., Gu, Z., Zhang, H., and Hu, H. (2020). To TRIM the immunity: From innate to adaptive immunity. *Frontiers in Immunology* 11.

Zheng, W., and Satta, Y. (2018). Functional evolution of avian RIG-I-like receptors. *Genes* 9(9). doi: 10.3390/genes9090456.

Zhou, J.-r., Liu, J.-h., Li, H.-m., Zhao, Y., Cheng, Z., Hou, Y.-m., et al. (2020). Regulatory effects of chicken TRIM25 on the replication of ALV-A and the MDA5-mediated type I interferon response. *Veterinary Research* 51(1)**,** 145. doi: 10.1186/s13567-020-00870-1.

Table 6.1. PCR primers used to create domain and mutant constructs and to amplify duck MAPLC3B.

Gene	Primer Name	Primer Sequence (5'-3')
MAPLC3B	LC3B Fwd	GCCGCCATGCCCTCGGAGAA
MAPLC3B	LC3B Rev	CTAAACGGAAGGTTGCGCTCCGAAAGTC
TRIM27L C16A	C16A fwd	GAAGCATCCGCCTCCATCTGC
TRIM27L C16A	C16A Rev	ATGGAGGCGGATGCTTCTTTCTG
TRIM27L C31A	C31A fwd	ATCAACGGCGGGCACAGCTTCTTCTGC
TRIM27L C31A	C31A Rev	GAAGCTGTGCCCGGCGTTGATGGAGAC
TRIM27LPRYSPRY	PRYSPRY fwd + NheI	GGCGCTAGCATGCTGGATGTGCAATGGGCAA
TRIM27LΔPRYSPRY	ΔPRYSPRY rev + NotI	CCCTCCCTGCCGTGAAGCCTTCG
TRIM27LΔRBB	ΔRBB fwd + NheI	GCTAGCATGGAAGAAGCCGCCCAGGA
TRIM27LΔRING	ΔRING Fwd + NheI	GGCGCTAGCATGCAGAAGAGAAAATTTCGGCCAAAC

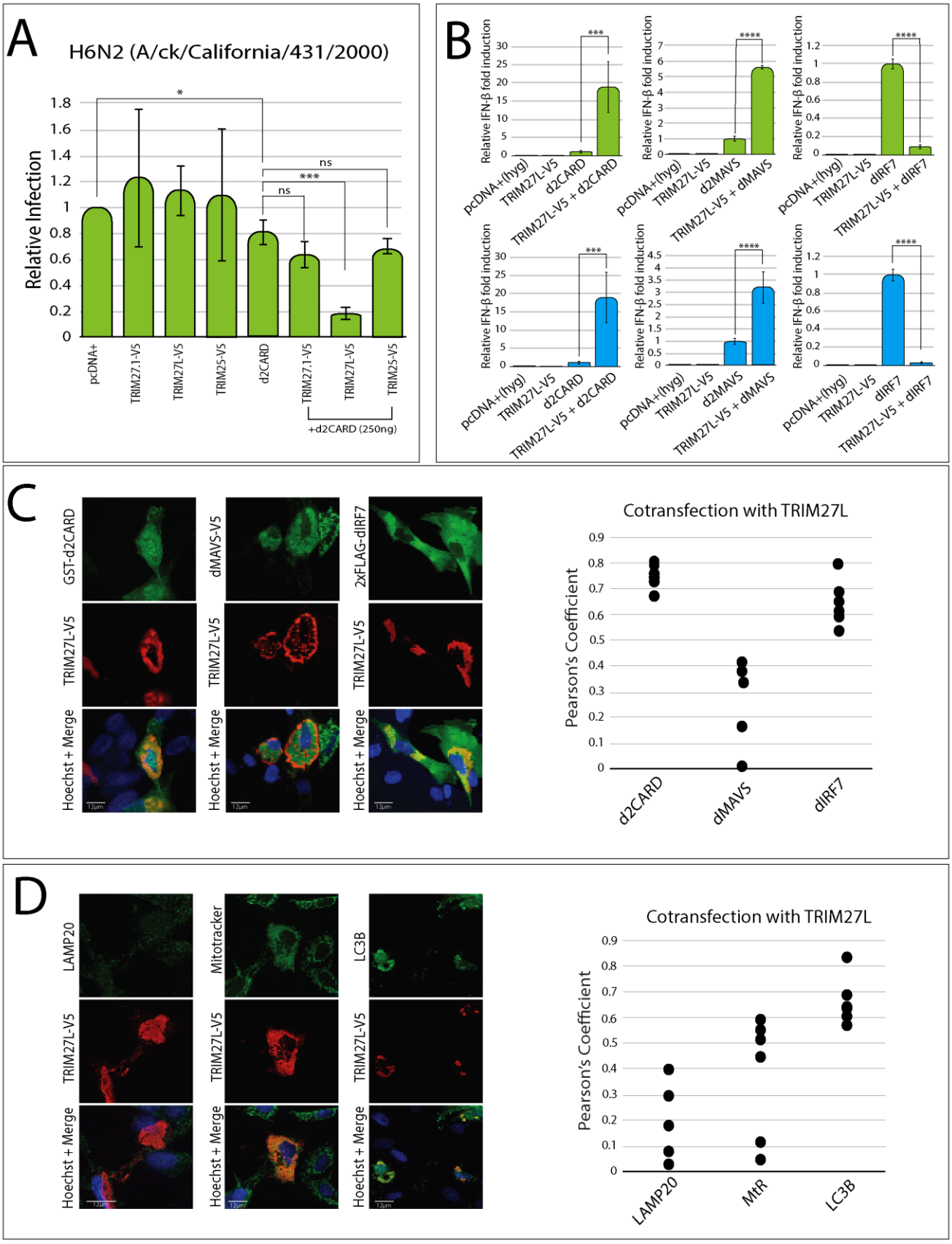

A
H6N2 (A/ck/California/431/2000)
Relative Infection
pcDNA+
TRIM27.1-V5
TRIM27L-V5
TRIM25-V5
d2CARD
TRIM27.1-V5
TRIM27L-V5
TRIM25-V5
+d2CARD (250ng)
B
Relative IFN-β fold induction
pcDNA+(hyg)
TRIM27L-V5
d2CARD
TRIM27L-V5 + d2CARD
d2MAVS
TRIM27L-V5 + dMAVS
dIRF7
TRIM27L-V5 + dIRF7
C
GST-d2CARD
dMAVS-V5
2xFLAG-dIRF7
TRIM27L-V5
Hoechst + Merge
Cotransfection with TRIM27L
Pearson's Coefficient
d2CARD
dMAVS
dIRF7
D
LAMP20
Mitotracker
LC3B
TRIM27L-V5
Hoechst + Merge
Cotransfection with TRIM27L
Pearson's Coefficient
LAMP20
MtR
LC3B

Figure 6.1. TRIM27L interacts with different components of the MAVS signaling pathway to both increase and decrease IFNβ promoter activity in chicken and duck cells. DF-1 cells transiently overexpressing duck TRIM27.1, 27L, 25 or empty vector or the TRIM genes plus 250ng of d2CARD were challenged with H6N2 (A) at an MOI of 1. Twenty-four hours post infection cells were fixed and stained for viral protein with α-NP-FITC. The percentage of infected cells was determined using flow cytometry and is expressed relative to the vector control. Statistical significance compared to the vector control cells or d2CARD was analyzed using an unpaired two-tailed Student's t test (*, $P \leq 0.05$; **, $P \leq 0.01$). The maximum percentage of cells infected was 33%, and each experiment was performed independently 3 times. DF-1 or DEF cells were transiently cotransfected with 250 ng of vector control, TRIM27L and either d2CARD, dMAVs or dIRF7 (15 ng, 100 ng or 100 ng, respectively) and the effect on IFN-β promoter activity was assessed using dual-luciferase reporter promoter assays using DF-1 (green) or DEF (blue) cells (B). Statistical significance was performed using a one-way ANOVA and comparing the means of d2CARD, dMAVS or dIRF7 to that of TRIM27L cotransfected with those components of the RIG-I signaling pathway (*, $P \leq 0.05$; **, $P \leq 0.01$, ***, $P \leq 0.005$, ****, $P \leq 0.0001$). Experiments were performed independently at least 3 times. DF-1 cells transiently coexpressing TRIM27L and d2CARD, dMAVS or dIRF7 were fixed, stained for epitope (V5 for TRIM27L, GST for d2CARD and 2xFLAG for dMAVS and dIRF7) and visualized using confocal microscopy (C). Pearson's coefficient was calculated using ImageJ. DF-1 cells transiently overexpressing TRIM27L and LC3B-2XFLAG, or overexpression TRIM27L on its own were stained for the lysosomal marker (LAMP2), the mitochondrial dye mitotracker red or 2xFLAG to visualize the autophagosomal protein LC3B, fixed, stained, and visualized using confocal microscopy (D). Pearson's coefficient was calculated using ImageJ

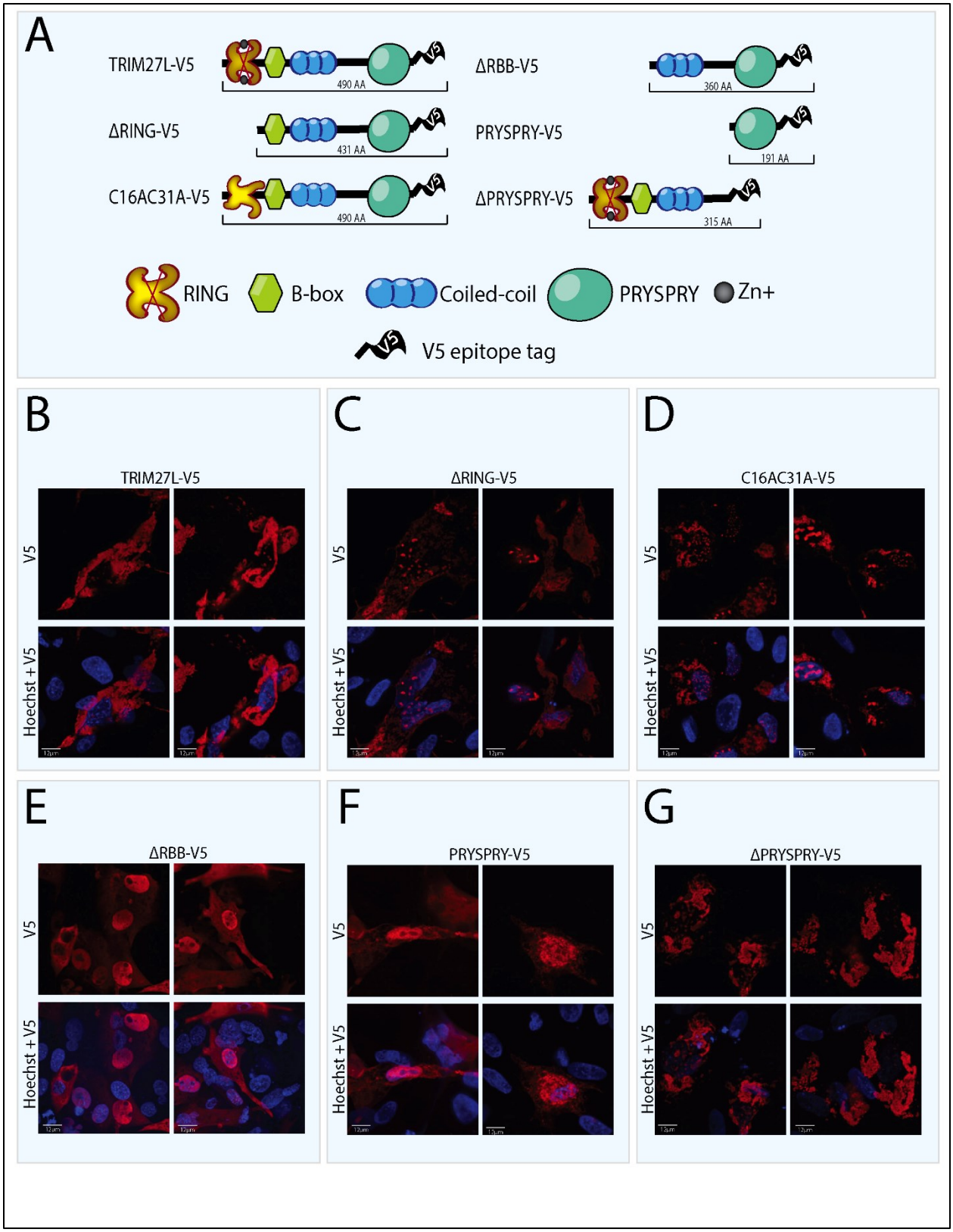


Figure 6.2. TRIM27L RING domain localized protein in distinct cytoplasmic aggregates,

while B-box and PRYSPRY domain localize protein to the nucleus. Domain and mutant expression constructs were made and compared to the full length TRIM27L (A). DF-1 cells transiently overexpressing TRIM27L (B), the RING deletion mutant (ΔRING) (C), the RING structure mutation (C16AC31A) (D), the deletion of both the RING and B-Box (ΔRBB) (E), the PRYSPRY domain on its own (F) or the deletion of the C-terminal PRYSPRY domain (ΔPRYSPRY) (G) were fixed and stained and visualized using confocal microscopy. Panels show the V5-epitope tag (red) and the V5 epitope tag with Hoechst 3324 (Blue).

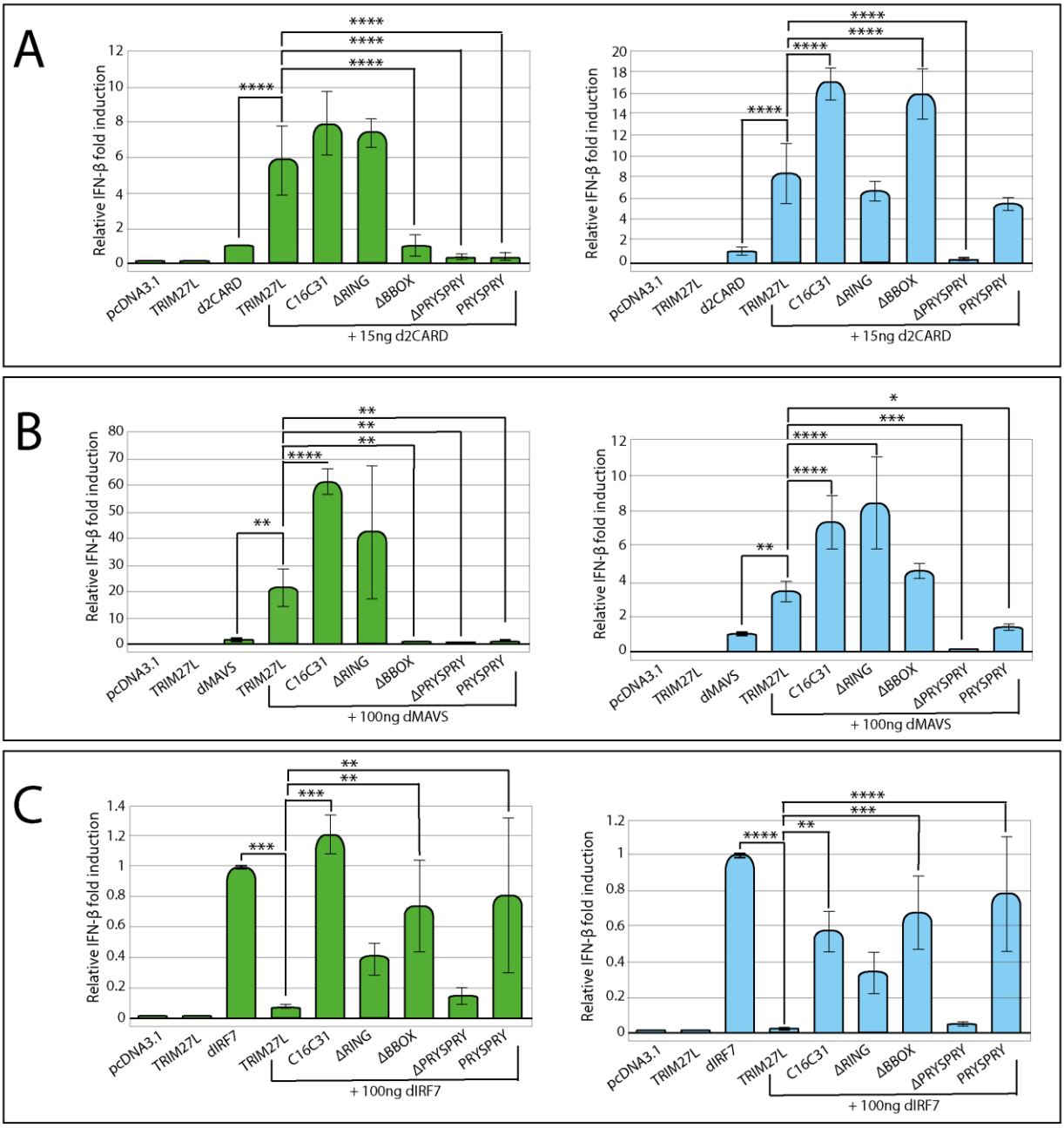


Figure 6.3. TRIM27L PRYSPRY domain is needed for enhancement of MAVS signaling activating the IFNβ promoter, while the RING domain is needed for inhibition. To determine domain functions during augmentation of signaling to activate the IFNβ promoter, DF-1 (green) or DEF cells (blue) were cotransfected for 24 hours with TRIM27L or domain constructs and d2CARD (A), dMAVS (B) or dIRF7 (C) and analyzed using dual-luciferase reporter assays. Statistics were analyzed using One-way ANOVA and comparing means of domain mutants to TRIM27L when cotransfected with various components of the RIG-I

signaling pathway (*, P ≤ 0.05; **, P ≤ 0.01, ***, P ≤ 0.005, ****, P ≤ 0.0001). Each experiment was performed 3 times independently.

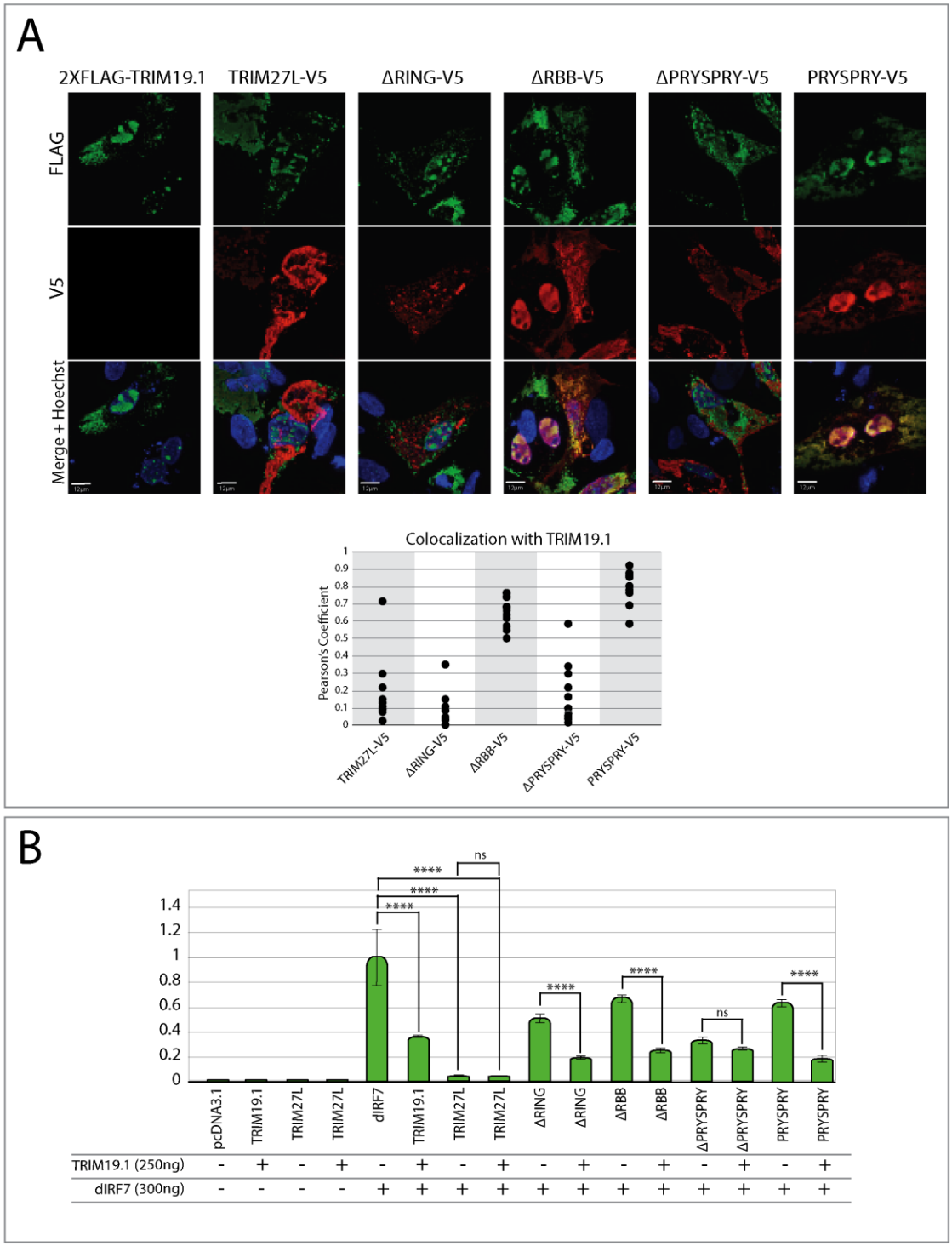


Figure 6.4. The PRYSPRY domain of TRIM27L colocalizes with TRIM19.1. The

PRYSPRY domain of TRIM27L colocalizes with TRIM19.1. DF-1 cells transiently expressing TRIM19.1 were cotransfected with TRIM27L and domain and mutant constructs, fixed, stained and visualized using confocal microscopy (A). Panels show the 2xFLAG-epitope tag (green) on TRIM19.1, the V5-epitope tag (Red) on TRIM27L and a merged image of the epitope tags plus Hoechst 3324 (Blue). Pearson's coefficient was calculated using ImageJ on ten random images of cells. To determine if TRIM27L and TRIM19.1 influenced IFNβ promoter activity in DF-1 cells, TRIM27L domain and mutant constructs were cotransfected with dIRF7 and TRIM19.1 and analyzed using dual-luciferase reporter assays (B). Statistical significance was performed using a one-way ANOVA and comparing the means of d2CARD, dMAVS or dIRF7 to that of TRIM27L cotransfected with those components of the RIG-I signaling pathway (*, $P \leq 0.05$; **, $P \leq 0.01$, ***, $P \leq 0.005$, ****, $P \leq 0.0001$). Luciferase experiment was performed independently two times.

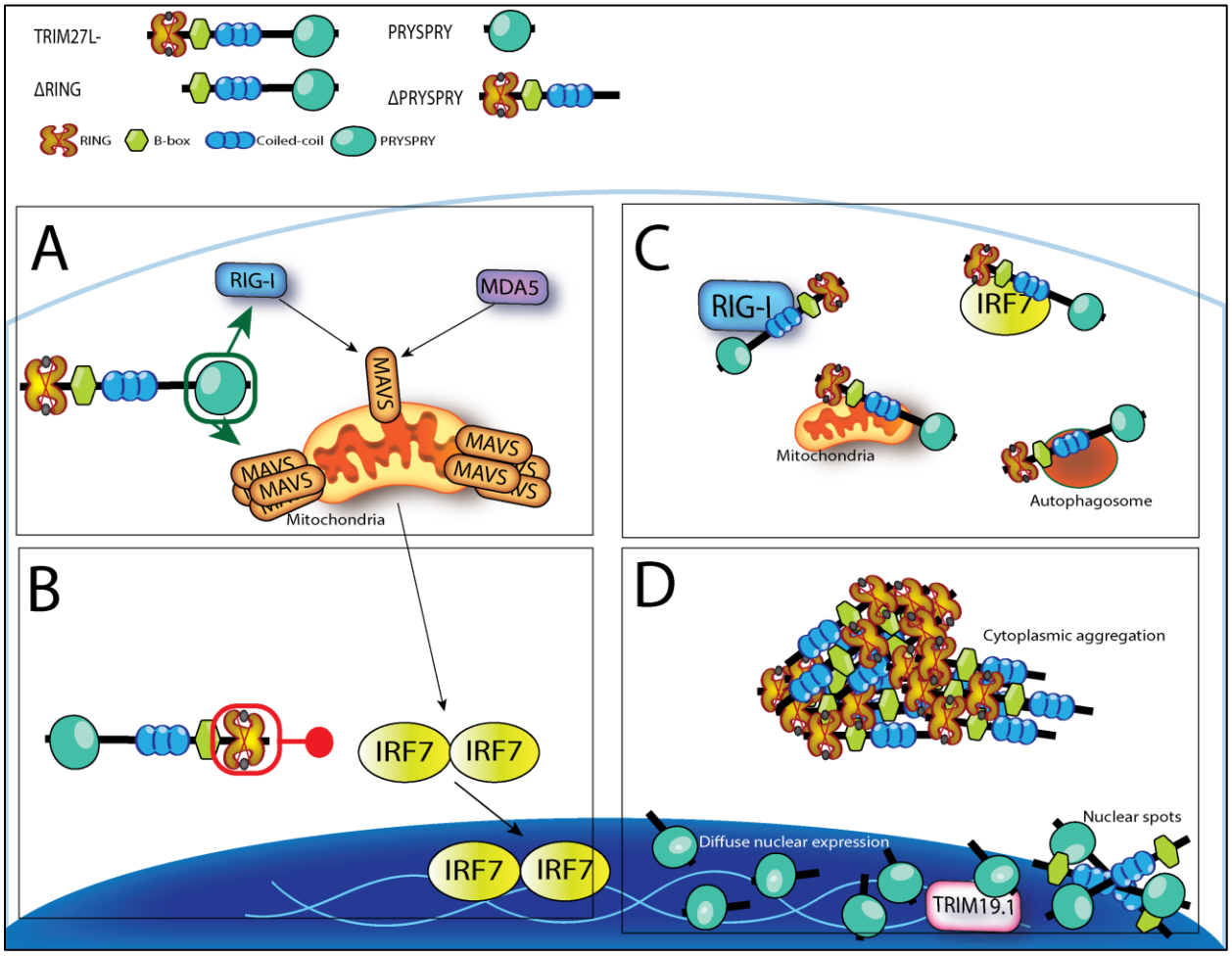
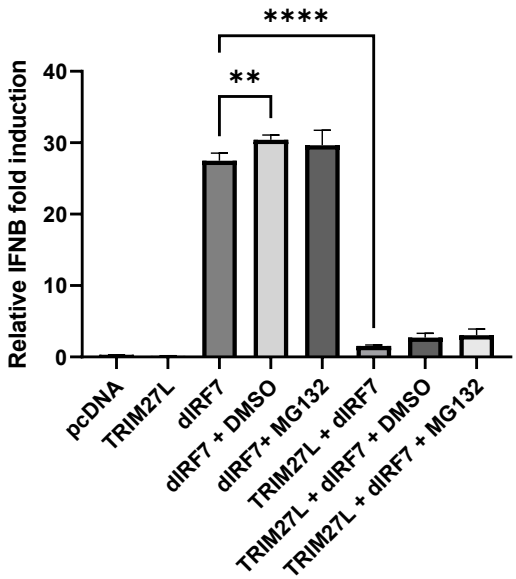


Figure 6.5. Summary of TRIM27L protein interactions with the RIG-I signaling pathway in chicken and duck cells. Overexpression of TRIM27L in both chicken and duck cells increases IFN-β promoter activity when cotransfected with the constitutively active CARD domains of duck RIG-I (d2CARD) and duck MAVS (A). The PRYSPRY domain of TRIM27L is necessary for this activation. Overexpression of TRIM27L in both chicken and duck cells inhibits IFN-β promoter activity when cotransfected duck IRF7 (B). The RING domains is necessary for this inhibition. TRIM27L colocalized with d2CARD, dIRF7, the autophagosomal marker LC3B and mitochondria in the cytoplasm of chicken cells (C). TRIM27L did not colocalize with duck MAVS. Overexpression of TRIM27L deletion and mutant constructs demonstrated that the RING domain of TRIM27L primarily localizes to the cytoplasm, deletion of the RING domain caused the protein to localize to distinct nuclear spots, while overexpressed PRYSPRY domain alone caused nuclear localization of the protein (D). The PRYPRY domain of TRIM27L also colocalized with overexpressed duck TRIM19.1 in the nucleus.

A



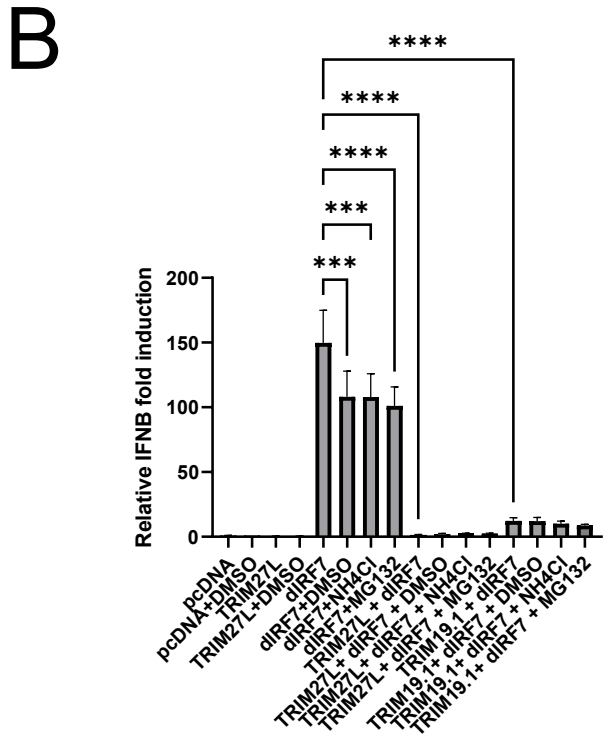


Figure S6.1. TRIM27L is not inhibiting dIRF7 IFNB promoter activity through lysosomal or proteasomal mechanisms. DF-1 cells were transfected for 24 hours then exposed for 5 hours to MG132 or DMSO control (A), or MG132, NH_4Cl or DMSO control (B). Relative IFN-β promoter activity was assessed using a dial luciferase reporter assay. Statistical significance was performed using a one-way ANOVA and comparing the means of dIRF7 against dIRF7 with the addition of DMSO, NH_4Cl or MG132. Cotransfections of TRIM27L with dIRF7 and TRIM19.1 with dIRF7 were compared against these cotransfections with the addition of DMSO, NH_4Cl or MG132. Only statistically significant pairwise comparisons are shown (*, $P \leq 0.05$; **, $P \leq 0.01$, ***, $P \leq 0.005$, ****, $P \leq 0.0001$).

Conclusions

7.1 Research summary

The primary aim of this thesis was to investigate how TRIM genes added to the defense to influenza A virus in the duck (Figure 7.1). Reservoir hosts to zoonotic viruses, such as the duck to IAV, may possess unique mechanisms of both viral restriction and tolerance that are not seen in incidental hosts, such as humans, and can lead to new pharmaceutical therapeutics.

The first aim of this thesis was to determine how these tissues were regulating gene

expression during IAV infection in the duck. I found that in ducks infected with either a low or highly pathogenic strain of influenza A virus, many of the genes that were upregulated in all the tissues tested in response to both viruses were already documented to be both antiviral or target IAV specifically in the host defense (Campbell et al, 2021). Many of the genes that were upregulated by both viruses were RLRs or genes which augment the RIG-I signaling pathway. The RLR pathway is likely upregulated to restrict IAV replication in both lung and intestine to control actively replicating virus. However, there were many genes that had tissue specific regulation that may influence tolerance or resistance specific to where the virus is actively replicating. Many more genes were flagged as involved in tissue specific responses that have not been implicated in antiviral defenses or simply did not have obvious orthologous genes in mammals for reference. The responses of annotated genes suggest that ducks control viral replication through upregulation of RIG-I pathway signaling components and selectively decrease proinflammatory genes. Some of the proinflammatory cytokines which were selectively downregulated in lungs and intestines in response to viral infection should be functionally characterized in ducks. There is limited capacity to do this in a duck model. There are no publicly available immortalized mallard duck cell lines, making knock-downs and knock-ins of target genes difficult. Additionally, there are minimal reagents available which are specific to ducks, such as antibodies or cytokines.

Many TRIM genes are involved in antiviral defenses (Nisole et al,, 2005; Ozato et al, 2008; Rajsbaum et al, 2014), including against IAV, and have demonstrated both species-specific (Sawyer et al, 2005; Song et al., 2005) and virus-specific antiviral defenses (Taylor et al., 2011; van Gent et al, 2018). The second Aim (Chapter 4) of this thesis was to categorize the duck TRIM repertoire and to identify candidate TRIM genes in the duck which could potentially have antiviral function (Figure 7.1A). This is the first instance of categorizing the TRIM gene repertoire in the duck, and only the second attempt at documenting TRIM genes in birds since a 2008 study by Sardiello and colleagues which documented 37 TRIM genes in chickens (Sardiello et al, 2008). I documented 57-TRIM or TRIM-like genes in the ducks. While many of these genes had direct orthologs to mammalian TRIM genes, some interesting exceptions were noted. For example, humans have a singular TRIM19/PML (promyelocytic leukemia) gene which is essential for PML nuclear body formation (Condemine et al., 2006; Lallemand-Breitenbach et al., 2010). I documented birds and reptiles have two paralogs of TRIM19, which I

named TRIM19.1 and TRIM19.2. It is unknown when TRIM19 arose in evolution, as there appear to be no TRIM19 genes in fish (Boudinot et al., 2011; Sardiello et al., 2008; van der Aa et al., 2009). It is unknown whether this duplication is specific to diapsids and the shorter paralog (TRIM19.1) was lost in mammals or if this duplication happened in the diapsids specifically. Additionally, two TRIM genes which augment the RIG-I signaling pathway, TRIM27L and RNF135, are in ducks but appear to be missing in chickens. Interestingly, chickens have two TRIM genes which appear missing in ducks that are phylogenetically related to RNF135/TRIM25, which may function to augment pathways compensating for the loss of RIG-I in chicken. Phylogenetic analysis from Chapter 4 suggests that some expansions of MHC-linked TRIM genes in diapsid and mammalian lineages happened independently of each other, and that the current accepted nomenclature of the TRIM27, TRIM39 and TRIM10 genes in birds may be wrong. This research only compared the duck TRIM repertoire to chicken TRIM genes, and in that only documented the annotated chicken TRIM repertoire. Some of the TRIM genes that appear to be present in duck but missing in chicken may be hidden in the chicken "dark DNA", that is GC-rich or repetitive DNA that is prone to amplification error or remain unplaced or discarded in genomic assemblies (Hron et al,, 2015; Weissensteiner et al, 2019). Indeed, many of these TRIM genes that appear in duck but not chicken, I was only able to identify from a *de novo* assembled transcriptome. When analyzing the relative tissue expression of TRIM genes in various duck tissues, many of the C-IV subfamily of TRIM proteins had higher relative expression in immune relevant tissues, such as lung, spleen, intestine and liver.

The third aim of my thesis (Chapter 5) was to determine which of the newly categorized duck TRIM genes changed expression in response to IAV, and if a select subset of these genes were antiviral (Figure 7.1B). While TRIM gene response to interferons and other cytokines has been documented in mammals (Carthagena et al., 2009; Rajsbaum et al., 2014), this is the first analysis of TRIM gene expression in response to infection in a non-mammalian vertebrate. My analyses demonstrated that over half the TRIM gene repertoire responded to HPAI IAV in ducks, with fewer of the TRIM genes being differentially expressing in response to BC500. Likely this is due to the higher cytokine response seen in tissues in ducks infected with VN1203. While some of the upregulated TRIM genes are known to modulate the RIG-I signaling pathway (TRIM25, 27L and 27.1), most of the other TRIM genes strongly upregulated by influenza infection have unknown in functions in the duck. I also tested the antiviral activities of several

duck TRIM proteins against an avian H6N2 IAV, and a mouse adapted H1N1 strain. The paralogs TRIM19.1 and TRIM19.2 did not show antiviral activity against either strain of IAV despite being consistently and most strongly upregulated in response to IAV, while diaTRIM41, specific to the Diapsid lineage and appears missing in mammals, restricted IAV in both duck and chicken cells.

I also continued the work by Alysson Blaine in her Master's thesis project on trying to functionally characterize TRIM27L and how it is modulating the MAVS signaling pathway (Blaine et al., 2015) in aim 4 (Chapter 6). TRIM27L activates the RIG-I signaling pathway predominantly through its PRYSPRY domain, and curiously restricts the same pathway when cotransfected with IRF7 primarily through its RING domain. TRIM27L appears to be missing in Galliformes (Blaine et al., 2015; Chaves et al, 2009; Kaufman et al., 1999; Shiina et al., 2007) suggesting that its function was not beneficial to Galliformes such as chicken. While it makes sense that TRIM27L would be lost in Galliformes if it only interacted with RIG-I, which also appears to be missing in Galliformes, my results suggest that TRIM27L is augmenting the MAVS signaling pathway in chicken independent of RIG-I. Indeed, it appears that either duck MAVS or duck RIG-I is needed for TRIM27L to increase IFN-β signaling in chicken cells, as viral infection alone does not induce TRIM27L activation of signaling. My results may not have found the binding partner of TRIM27L, but they demonstrate how complex not only TRIM augmentation of signaling pathways is but also the overall complexity of TRIM protein evolution and function. I may not have answered my initial question of "What is TRIM27L interacting with in order to augment the MAVS signaling pathway", however I have gained unique insight into the specific domains it uses to exert its functions and where the subcellular location of this function may take place. I have also recently improved the transfection efficiency of DEF cells through testing of newer transfection reagents and techniques, such as magnetofection (Appendix). With the increase in DEF transfection efficiency the immunoprecipitation and mass spectrometric experiments I previously attempted using chicken DF-1 cells can be repeated using DEF cells. It is possible it will be more informative to pull out the genes directly interacting with TRIM27L in its host system.

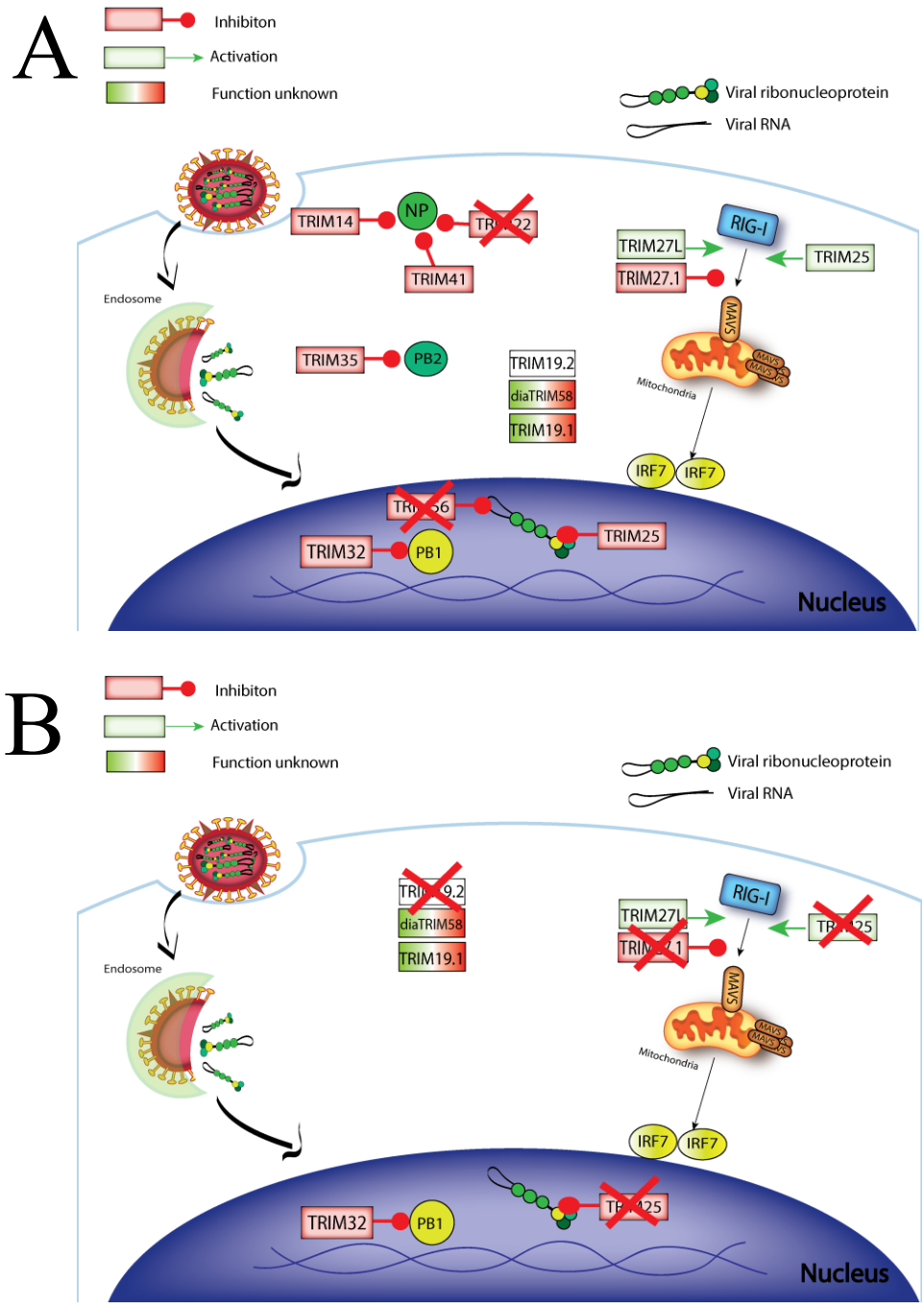


Figure 7.1. Summary of TRIM genes and their antiviral potential in ducks. Ducks have 57 TRIM or TRIM-like genes, some orthologous to TRIM proteins found in human which restrict IAV (A) such as TRIM25, TRIM32 and TRIM19. Ducks have lineage specific expansions of some TRIM genes such as the MHC-linked TRIM27,1 and TRIM27L, which appear absent in humans. Over half of the duck TRIM repertoire is differentially expressed in response to IAV in the duck. To determine if the duck TRIM proteins TRIM19.1, TRIM19.2, TRIM27.1, TRIM27L,

TRIM25, diaTRIM58 or TRIM32 were able to restrict IAV in cells, these TRIM proteins were overexpressed in both duck and chicken cells (B). TRIM25, TRIM19.2 and TRIM27.1 were not able to reduce viral replication in either cell type, while diaTRIM58, TRIM32 restrict IAV replication in both duck and chicken cells. TRIM27L and TRIM19.1 could only restrict IAV replication in duck cells. TRIM27L was further investigated to determine where in the RIG-I pathway it was augmenting in order to induce an antiviral state in duck cells.

7.2 Significance

The work in this thesis furthers our understanding of not only global transcriptional responses of the reservoir host to IAV, the duck, but also documents the TRIM gene repertoire in the duck. The findings in the first Aim of my thesis suggests that ducks tightly control regulation of gene expression during IAV infection to provide a rapid antiviral response, but also quickly limit inflammatory responses in key tissues utilized by IAV for replication (namely the lung and intestine). By analyzing the global transcriptional regulation of ducks to both HPAI and LPAI IAV, I found many potential candidate genes to study in both ducks and humans, for anti-inflammatory roles. These genes offer the potential to be utilized as therapeutics for humans, and even other agriculturally significant animals such as chickens, to control tissue damage resulting from IAV replication. I documented the TRIM gene repertoire in ducks and compare to the chicken gene TRIM repertoire available from NCBI. While TRIM proteins are known to be antiviral, and even offer species specific protection from viruses, this is the first instance of doing a thorough analysis of TRIM gene evolution in Anseriforme birds. I also documented TRIM gene specific regulation to HPAI and LPAI infection in duck. By analyzing the evolution and regulation of TRIM genes in ducks we can gain better understanding of TRIM gene evolution. Additionally, I was careful not to use only the NCBI automated prediction of TRIM gene names in birds, most of which are redundant or phylogenetically untrue. By determining the correct annotation of these TRIM genes, I will prevent further miss-annotation of TRIM genes in other species in the future. I also tested the anti-viral potential of several duck TRIM genes, some of which had never been analyzed as anti-IAV effectors before (Namely TRIM19.1, TRIM19.2, TRIM27.1, TRIM27L, TRIM25 and diaTRIM41). I described both diaTRIM41 and TRIM27L as antiviral effectors in duck cells. I also continued previous work done in our lab by demonstrating

that TRIM27L both increases and decreases the same antiviral signaling pathway, making this the first non-mammalian TRIM protein found to do so.

TRIM protein biology and function in antiviral immunity is a new and understudied area of immunological research. Many of these proteins are known to be upregulated or differentially expressed during viral infection, but most have not been characterized. By increasing our knowledge of the functions of TRIM proteins in influenza infection in ducks we can gain a better understanding of both TRIM protein biology and function in host-pathogen interactions in the reservoir host.

7.3 Future directions

This thesis work documented the differential regulation of many genes that were not previously known to be involved in viral restriction, especially in the intestine of ducks infected with LPAI. To continue the investigation on how the intestines of ducks are permitting IAV to replicate to high titres without causing tissue damage, we need to determine: A.) Where specifically in the intestine of infected ducks IAV is replicating B.) What cell types, specifically, is IAV using to replicate in in the intestine and C.) What transcriptional responses are these cells specifically exhibiting in response to infection. To carry out these future goals, we would need histological sections from intestines of infected ducks, stained with antibodies specific for viral proteins to find a more precise location of replication. Once the location of replication is found, single cell RNA-seq analysis on this tissue could help identify not only the type of cell permissive to IAV replication, but also the transcriptional responses of that cell type. By looking at cell-specific transcriptional response to actively replicating virus, and the transcriptional response of surrounding cells that are not permissive to the virus, we will gain more insight into how ducks are able to prevent widespread tissue damage from active replicating virus.

Much of the work of this thesis focused on documenting and characterizing TRIM genes in ducks. The 7 TRIM genes investigated for anti-IAV potential were chosen because of transcriptional regulation in response to infection or prior knowledge of anti-IAV activity in mammals or birds. This leaves about 50 TRIM or TRIM-like genes uncharacterized in the duck. While it would be beneficial to examine and characterize TRIM genes upregulated in response to HPAI in the first attempts to explore anti-IAV activity of TRIM proteins in the duck, the TRIM genes that were unresponsive to IAV infection should not be ignored. In humans, TRIM41

successfully targets and degrades IAV NP, however TRIM41 is not interferon inducible (Patil et al., 2018). It is possible that some TRIM proteins in the duck are acting as intrinsic antiviral effectors without changing regulation in response to infection. The entire TRIM repertoire in the duck should be examined for positive selection, to see which TRIM gene alleles are retained, expanded or deleted in different avian populations. As more genomic information becomes available for different species of birds, more TRIM genes can be compared across avian lineages to assess the rates of synonymous versus non-synonymous mutations in avian TRIM genes. By analyzing rates of positive selection among avian TRIM genes, more anti-viral TRIM protein candidates could be identified to functionally characterize.

Finally, transcriptional analysis on any subset of genes responding to infection or stimulation should always lead to *in vitro* experiments. This avenue of analysis presents challenges when working in the duck model. There is no publicly available *Anas platyrhynchos* derived immortalized cell line, either from domestic or wild mallard ducks. Immortalized duck cell lines would allow not only stable transfection of genes, but the ability to knock-in and knock-out genes of interest with techniques such as CRISPR (Roebroek et al., 2011).

7.4 References

Alexander, D.J., Parsons, G., and Manvell, R.J. (1986). Experimental assessment of the pathogenicity of eight avian influenza A viruses of H5 subtype for chickens, turkeys, ducks and quail. *Avian Pathol* 15(4)**,** 647-662. doi: 10.1080/03079458608436328.

Blaine, A.H., Miranzo-Navarro, D., Campbell, L.K., Aldridge, J.R., Jr., Webster, R.G., and Magor, K.E. (2015). Duck TRIM27-L enhances MAVS signaling and is absent in chickens and turkeys. *Mol Immunol* 67(2 Pt B)**,** 607-615. doi: 10.1016/j.molimm.2015.07.011.

Boudinot, P., van der Aa, L.M., Jouneau, L., Du Pasquier, L., Pontarotti, P., Briolat, V., et al. (2011). Origin and evolution of TRIM proteins: new insights from the complete TRIM repertoire of zebrafish and pufferfish. *PloS one* 6(7)**,** e22022-e22022. doi: 10.1371/journal.pone.0022022.

Campbell, L.K., Fleming-Canepa, X., Webster, R.G., and Magor, K.E. (2021). Tissue Specific Transcriptome Changes Upon Influenza A Virus Replication in the Duck. *Frontiers in Immunology* 12(4653). doi: 10.3389/fimmu.2021.786205.

Campbell, L.K., and Magor, K.E. (2020). Pattern Recognition Receptor Signaling and Innate Responses to Influenza A Viruses in the Mallard Duck, Compared to Humans and Chickens. *Frontiers in Cellular and Infection Microbiology* 10(209). doi: 10.3389/fcimb.2020.00209.

Carthagena, L., Bergamaschi, A., Luna, J.M., David, A., Uchil, P.D., Margottin-Goguet, F., et al. (2009). Human TRIM gene expression in response to interferons. *PLoS One* 4(3)**,** e4894. doi: 10.1371/journal.pone.0004894.

Chaves, L.D., Krueth, S.B., and Reed, K.M. (2009). Defining the turkey MHC: sequence and genes of the B locus. *J Immunol* 183(10)**,** 6530-6537. doi: 10.4049/jimmunol.0901310.

Condemine, W., Takahashi, Y., Zhu, J., Puvion-Dutilleul, F., Guegan, S., Janin, A., et al. (2006). Characterization of Endogenous Human Promyelocytic Leukemia Isoforms. *Cancer Research* 66(12)**,** 6192-6198. doi: 10.1158/0008-5472.CAN-05-3792.

Davison, T.F. (2003). The immunologists' debt to the chicken. *British Poultry Science* 44(1)**,** 6-21. doi: 10.1080/0007166031000085364.

Hron, T., Pajer, P., Paces, J., Bartunek, P., and Elleder, D. (2015). Hidden genes in birds. *Genome Biol* 16(1)**,** 164. doi: 10.1186/s13059-015-0724-z.

Jordan, I., Vos, A., Beilfuß, S., Neubert, A., Breul, S., and Sandig, V. (2009). An avian cell line designed for production of highly attenuated viruses. *Vaccine* 27(5)**,** 748-756. doi: https://doi.org/10.1016/j.vaccine.2008.11.066.

Kaufman, J., Milne, S., Göbel, T.W.F., Walker, B.A., Jacob, J.P., Auffray, C., et al. (1999). The chicken B locus is a minimal essential major histocompatibility complex. *Nature* 401(6756)**,** 923-925. doi: 10.1038/44856.

Kida, H., Yanagawa, R., and Matsuoka, Y. (1980). Duck Influenza Lacking Evidence of Disease Signs and Immune-Response. *Infection and Immunity* 30(2)**,** 547-553.

Lallemand-Breitenbach, V., and de Thé, H. (2010). PML nuclear bodies. *Cold Spring Harb Perspect Biol* 2(5)**,** a000661. doi: 10.1101/cshperspect.a000661.

Nisole, S., Stoye, J.P., and Saib, A. (2005). TRIM family proteins: retroviral restriction and antiviral defence. *Nat Rev Microbiol* 3(10)**,** 799-808. doi: 10.1038/nrmicro1248.

Ozato, K., Shin, D.M., Chang, T.H., and Morse, H.C., 3rd (2008). TRIM family proteins and their emerging roles in innate immunity. *Nat Rev Immunol* 8(11)**,** 849-860. doi: 10.1038/nri2413.

Pantin-Jackwood, M., Swayne, D.E., Smith, D., and Shepherd, E. (2013). Effect of species, breed and route of virus inoculation on the pathogenicity of H5N1 highly pathogenic influenza (HPAI) viruses in domestic ducks. *Vet Res* 44(1)**,** 62. doi: 10.1186/1297-9716-44-62.

Patil, G., Zhao, M., Song, K., Hao, W., Bouchereau, D., Wang, L., et al. (2018). TRIM41-Mediated Ubiquitination of Nucleoprotein Limits Influenza A Virus Infection. *J Virol* 92(16). doi: 10.1128/JVI.00905-18.

Rajsbaum, R., Garcia-Sastre, A., and Versteeg, G.A. (2014). TRIMmunity: the roles of the TRIM E3-ubiquitin ligase family in innate antiviral immunity. *J Mol Biol* 426(6)**,** 1265-1284. doi: 10.1016/j.jmb.2013.12.005.

Roebroek, A.J.M., Gordts, P.L.S.M., and Reekmans, S. (2011). "Knock-In Approaches," in *Transgenic Mouse Methods and Protocols,* eds. M.H. Hofker & J. van Deursen. (Totowa, NJ: Humana Press), 257-275.

Sardiello, M., Cairo, S., Fontanella, B., Ballabio, A., and Meroni, G. (2008). Genomic analysis of the TRIM family reveals two groups of genes with distinct evolutionary properties. *BMC Evol Biol* 8(1)**,** 225. doi: 10.1186/1471-2148-8-225.

Sawyer, S.L., Wu, L.I., Emerman, M., and Malik, H.S. (2005). Positive selection of primate TRIM5alpha identifies a critical species-specific retroviral restriction domain. *Proc Natl Acad Sci U S A* 102(8)**,** 2832-2837. doi: 10.1073/pnas.0409853102.

Shiina, T., Briles, W.E., Goto, R.M., Hosomichi, K., Yanagiya, K., Shimizu, S., et al. (2007). Extended gene map reveals tripartite motif, C-type lectin, and Ig superfamily type genes within a subregion of the chicken MHC-B affecting infectious disease. *J Immunol* 178(11)**,** 7162-7172. doi: 10.4049/jimmunol.178.11.7162.

Song, B., Javanbakht, H., Perron, M., Park, D.H., Stremlau, M., and Sodroski, J. (2005). Retrovirus restriction by TRIM5alpha variants from Old World and New World primates. *Journal of virology* 79(7)**,** 3930-3937. doi: 10.1128/JVI.79.7.3930-3937.2005.

Taylor, R.T., Lubick, K.J., Robertson, S.J., Broughton, J.P., Bloom, M.E., Bresnahan, W.A., et al. (2011). TRIM79alpha, an interferon-stimulated gene product, restricts tick-borne encephalitis virus replication by degrading the viral RNA polymerase. *Cell Host Microbe* 10(3)**,** 185-196. doi: 10.1016/j.chom.2011.08.004.

van der Aa, L.M., Levraud, J.-P., Yahmi, M., Lauret, E., Briolat, V., Herbomel, P., et al. (2009). A large new subset of TRIM genes highly diversified by duplication and positive selection in teleost fish. *BMC Biology* 7(1)**,** 7. doi: 10.1186/1741-7007-7-7.

van Gent, M., Sparrer, K.M.J., and Gack, M.U. (2018). TRIM Proteins and Their Roles in Antiviral Host Defenses. *Annu Rev Virol* 5(1)**,** 385-405. doi: 10.1146/annurev-virology-092917-043323.

Webster, R.G., Yakhno, M., Hinshaw, V.S., Bean, W.J., and Murti, K.G. (1978). Intestinal influenza: replication and characterization of influenza viruses in ducks. *Virology* 84(2)**,** 268-278. doi: 10.1016/0042-6822(78)90247-7.

Weissensteiner, M.H., and Suh, A. (2019). "Repetitive DNA: The Dark Matter of Avian Genomics," in *Avian Genomics in Ecology and Evolution: From the Lab into the Wild,* ed. R.H.S. Kraus. (Cham: Springer International Publishing), 93-150.

Williams, F.P., Haubrich, K., Perez-Borrajero, C., and Hennig, J. (2019). Emerging RNA-binding roles in the TRIM family of ubiquitin ligases. Biological Chemistry 400(11), 1443-1464. doi: doi:10.1515/hsz-2019-0158.

Woo, J.S., Suh, H.Y., Park, S.Y., and Oh, B.H. (2006). Structural basis for protein recognition by B30.2/SPRY domains. Mol Cell 24(6), 967-976. doi: 10.1016/j.molcel.2006.11.009.

Xiao, Y., Evseev, D., Stevens, C.A., Moghrabi, A., Miranzo-Navarro, D., Fleming-Canepa, X., et al. (2020). Influenza PB1-F2 Inhibits Avian MAVS Signaling. Viruses 12(4). doi: 10.3390/v12040409.

Xiao, Y., Reeves, M.B., Caulfield, A.F., Evseev, D., and Magor, K.E. (2018). The core promoter controls basal and inducible expression of duck retinoic acid inducible gene-I (RIG-I). Mol Immunol 103(September), 156-165. doi: 10.1016/j.molimm.2018.09.002.

Yang, W., Gu, Z., Zhang, H., and Hu, H. (2020). To TRIM the Immunity: From Innate to Adaptive Immunity. Frontiers in Immunology 11.

Zheng, W., and Satta, Y. (2018). Functional Evolution of Avian RIG-I-Like Receptors. Genes 9(9). doi: 10.3390/genes9090456.

Zhao, C., Jia, M., Song, H., Yu, Z., Wang, W., Li, Q., et al. (2017). The E3 Ubiquitin Ligase TRIM40 Attenuates Antiviral Immune Responses by Targeting MDA5 and RIG-I. Cell Rep 21(6), 1613-1623. doi: 10.1016/j.celrep.2017.10.020.

Zhou, J.-r., Liu, J.-h., Li, H.-m., Zhao, Y., Cheng, Z., Hou, Y.-m., et al. (2020). Regulatory effects of chicken TRIM25 on the replication of ALV-A and the MDA5-mediated type I interferon response. Veterinary Research 51(1), 145. doi: 10.1186/s13567-020-00870-1.

REFERENCES

Abbas, Y.M., Pichlmair, A., Gorna, M.W., Superti-Furga, G., and Nagar, B. (2013). Structural basis for viral 5'-PPP-RNA recognition by human IFIT proteins. *Nature* 494(7435)**,** 60-64. doi: 10.1038/nature11783.

Abdul-Cader, M.S., De Silva Senapathi, U., Nagy, E., Sharif, S., and Abdul-Careem, M.F. (2018). Antiviral response elicited against avian influenza virus infection following activation of toll-like receptor (TLR)7 signaling pathway is attributable to interleukin (IL)-1β production. *BMC Research Notes* 11(1)**,** 859. doi: 10.1186/s13104-018-3975-4.

Abi Rached, L., McDermott, M.F., and Pontarotti, P. (1999). The MHC big bang. *Immunol Rev* 167**,** 33-44. doi: 10.1111/j.1600-065x.1999.tb01380.x.

Abolnik, C. (2014). A current review of avian influenza in pigeons and doves (Columbidae). *Veterinary Microbiology* 170(3)**,** 181-196. doi: https://doi.org/10.1016/j.vetmic.2014.02.042.

Agarwal, N., Rinaldetti, S., Cheikh, B.B., Zhou, Q., Hass, E.P., Jones, R.T., et al. (2021). TRIM28 is a transcriptional activator of the mutant TERT promoter in human bladder cancer. *Proc Natl Acad Sci U S A* 118(38). doi: 10.1073/pnas.2102423118.

Alexander, D.J., Parsons, G., and Manvell, R.J. (1986). Experimental assessment of the pathogenicity of eight avian influenza A viruses of H5 subtype for chickens, turkeys, ducks and quail. *Avian Pathol* 15(4)**,** 647-662. doi: 10.1080/03079458608436328.

Alexopoulou, L., Holt, A.C., Medzhitov, R., and Flavell, R.A. (2001). Recognition of double-stranded RNA and activation of NF-kappaB by Toll-like receptor 3. *Nature* 413(6857)**,** 732-738. doi: 10.1038/35099560.

Allen, I.C., Scull, M.A., Moore, C.B., Holl, E.K., McElvania-TeKippe, E., Taxman, D.J., et al. (2009). The NLRP3 inflammasome mediates in vivo innate immunity to influenza A virus through recognition of viral RNA. *Immunity* 30(4)**,** 556-565. doi: 10.1016/j.immuni.2009.02.005.

Andrews, S. (2010). "FASTQC. A quality control tool for high throughput sequence data".

Ardley, H.C., and Robinson, P.A. (2005). E3 ubiquitin ligases. *Essays Biochem* 41(1)**,** 15-30. doi: 10.1042/EB0410015.

Arimori, Y., Nakamura, R., Yamada, H., Shibata, K., Maeda, N., Kase, T., et al. (2013). Type I interferon limits influenza virus-induced acute lung injury by regulation of excessive inflammation in mice. *Antiviral Res* 99(3)**,** 230-237. doi: 10.1016/j.antiviral.2013.05.007.

Atasheva, S., Akhrymuk, M., Frolova, E.I., and Frolov, I. (2012). New PARP gene with an anti-alphavirus function. *Journal of Virology* 86(15)**,** 8147. doi: 10.1128/JVI.00733-12.

Balachandran, S., Roberts, P.C., Brown, L.E., Truong, H., Pattnaik, A.K., Archer, D.R., et al. (2000). Essential role for the dsRNA-dependent protein kinase PKR in innate immunity to viral infection. *Immunity* 13(1)**,** 129-141. doi: 10.1016/s1074-7613(00)00014-5.

Banerjee, A., Baker, M.L., Kulcsar, K., Misra, V., Plowright, R., and Mossman, K. (2020). Novel Insights Into Immune Systems of Bats. *Frontiers in Immunology* 11.

Barber, M.R., Aldridge, J.R., Jr., Webster, R.G., and Magor, K.E. (2010). Association of RIG-I with innate immunity of ducks to influenza. *Proc Natl Acad Sci U S A* 107(13)**,** 5913-5918. doi: 10.1073/pnas.1001755107.

Barber, M.R.W., Aldridge, J.R., Jr., Fleming-Canepa, X., Wang, Y.-D., Webster, R.G., and Magor, K.E. (2013). Identification of avian RIG-I responsive genes during influenza infection. *Molecular immunology* 54(1)**,** 89-97. doi: 10.1016/j.molimm.2012.10.038.

Barjesteh, N., Behboudi, S., Brisbin, J.T., Villanueva, A.I., Nagy, E., and Sharif, S. (2014). TLR ligands induce antiviral responses in chicken macrophages. *PloS one* 9(8)**,** e105713-e105713. doi: 10.1371/journal.pone.0105713.

Barr, S.D., Smiley, J.R., and Bushman, F.D. (2008). The interferon response inhibits HIV particle production by induction of TRIM22. *PLOS Pathogens* 4(2)**,** e1000007. doi: 10.1371/journal.ppat.1000007.

Baum, L.G., and Paulson, J.C. (1990). Sialyloligosaccharides of the respiratory epithelium in the selection of human influenza virus receptor specificity. *Acta Histochem Suppl* 40**,** 35-38.

Bazzigher, L., Schwarz, A., and Staeheli, P. (1993). No enhanced influenza virus resistance of murine and avian cells expressing cloned duck Mx protein. *Virology* 195(1)**,** 100-112. doi: 10.1006/viro.1993.1350.

Bell, J.L., Malyukova, A., Holien, J.K., Koach, J., Parker, M.W., Kavallaris, M., et al. (2012). TRIM16 acts as an E3 ubiquitin ligase and can heterodimerize with other TRIM family members. *PLoS One* 7(5)**,** e37470. doi: 10.1371/journal.pone.0037470.

Ben, L., and Steven, L.S. (2011). Fast gapped-read alignment with Bowtie 2. 1-24. doi: 10.1002/0471250953.bi1107s32.Aligning.

Benitez, A.A., Panis, M., Xue, J., Varble, A., Shim, J.V., Frick, A.L., et al. (2015). In vivo RNAi screening identifies MDA5 as a significant contributor to the cellular defense against influenza A virus. *Cell Rep* 11(11)**,** 1714-1726. doi: 10.1016/j.celrep.2015.05.032.

Berti, C., Messali, S., Ballabio, A., Reymond, A., and Meroni, G. (2002b). TRIM9 is specifically expressed in the embryonic and adult nervous system. *Mechanisms of Development* 113(2)**,** 159-162. doi: https://doi.org/10.1016/S0925-4773(02)00013-8.

Billiau, A. (2006). Anti-inflammatory properties of Type I interferons. *Antiviral Res* 71(2-3)**,** 108-116. doi: 10.1016/j.antiviral.2006.03.006.

Bingham, A.M., Graham, S.P., Burkett-Cadena, N.D., White, G.S., Hassan, H.K., and Unnasch, T.R. (2012). Detection of eastern equine encephalomyelitis virus RNA in North American snakes. *Am J Trop Med Hyg* 87(6)**,** 1140-1144. doi: 10.4269/ajtmh.2012.12-0257.

Bingham, J., Green, D.J., Lowther, S., Klippel, J., Burggraaf, S., Anderson, D.E., et al. (2009). Infection studies with two highly pathogenic avian influenza strains (Vietnamese and Indonesian) in Pekin ducks (Anas platyrhynchos), with particular reference to clinical disease, tissue tropism and viral shedding. *Avian Pathol* 38(4)**,** 267-278. doi: 10.1080/03079450903055371.

Birol, I., Jackman, S.D., Nielsen, C.B., Qian, J.Q., Varhol, R., Stazyk, G., et al. (2009). De novo transcriptome assembly with ABySS. *Bioinformatics* 25(21)**,** 2872-2877. doi: 10.1093/bioinformatics/btp367.

Blaine, A.H., Miranzo-Navarro, D., Campbell, L.K., Aldridge, J.R., Jr., Webster, R.G., and Magor, K.E. (2015). Duck TRIM27-L enhances MAVS signaling and is absent in chickens and turkeys. *Mol Immunol* 67(2 Pt B)**,** 607-615. doi: 10.1016/j.molimm.2015.07.011.

Blondel, D., Kheddache, S., Lahaye, X., Dianoux, L., and Chelbi-Alix, M.K. (2010). Resistance to rabies virus infection conferred by the PMLIV isoform. *J Virol* 84(20)**,** 10719-10726. doi: 10.1128/jvi.01286-10.

Blyth, G.A., Chan, W.F., Webster, R.G., and Magor, K.E. (2016). Duck interferon-inducible transmembrane protein 3 mediates restriction of influenza viruses. *J Virol* 90(1)**,** 103-116. doi: 10.1128/JVI.01593-15.

Bodenhofer, U., Bonatesta, E., Horejš-Kainrath, C., and Hochreiter, S. (2015). msa: an R package for multiple sequence alignment. *Bioinformatics* 31(24)**,** 3997-3999. doi: 10.1093/bioinformatics/btv494.

Bolger, A.M., Lohse, M., and Usadel, B. (2014a). Trimmomatic: a flexible trimmer for Illumina sequence data. *Bioinformatics* 30(15)**,** 2114-2120. doi: 10.1093/bioinformatics/btu170.

Boon, A.C.M., Williams, R.W., Sinasac, D.S., and Webby, R.J. (2014). A novel genetic locus linked to pro-inflammatory cytokines after virulent H5N1 virus infection in mice. *BMC Genomics* 15(1)**,** 1017. doi: 10.1186/1471-2164-15-1017.

Borden, K.L., and Freemont, P.S. (1996). The RING finger domain: a recent example of a sequence-structure family. *Curr Opin Struct Biol* 6(3)**,** 395-401. doi: 10.1016/s0959-440x(96)80060-1.

Bosco-Lauth, A.M., Hartwig, A.E., and Bowen, R.A. (2018). Reptiles and amphibians as potential reservoir hosts of Chikungunya virus. *Am J Trop Med Hyg* 98(3)**,** 841-844. doi: 10.4269/ajtmh.17-0730.

Botos, I., Segal, D.M., and Davies, D.R. (2011). The structural biology of Toll-like receptors. *Structure* 19(4)**,** 447-459. doi: 10.1016/j.str.2011.02.004.

Boudinot, P., van der Aa, L.M., Jouneau, L., Du Pasquier, L., Pontarotti, P., Briolat, V., et al. (2011). Origin and evolution of TRIM proteins: new insights from the complete TRIM repertoire of zebrafish and pufferfish. *PloS one* 6(7)**,** e22022-e22022. doi: 10.1371/journal.pone.0022022.

Boyd, A., Philbin, V.J., and Smith, A.L. (2007). Conserved and distinct aspects of the avian Toll-like receptor (TLR) system: implications for transmission and control of bird-borne zoonoses. *Biochemical Society transactions* 35(Pt 6)**,** 1504-1507. doi: 10.1042/bst0351504.

Boyd, A.C., Peroval, M.Y., Hammond, J.A., Prickett, M.D., Young, J.R., and Smith, A.L. (2012). TLR15 Is Unique to avian and reptilian lineages and recognizes a yeast-derived agonist. *The Journal of Immunology* 189(10)**,** 4930. doi: 10.4049/jimmunol.1101790.

Boyer, N.P., Monkiewicz, C., Menon, S., Moy, S.S., and Gupton, S.L. (2018). Mammalian TRIM67 functions in brain development and behavior. *eNeuro* 5(3). doi: 10.1523/eneuro.0186-18.2018.

Brass, A.L., Huang, I.C., Benita, Y., John, S.P., Krishnan, M.N., Feeley, E.M., et al. (2009). The IFITM proteins mediate cellular resistance to influenza A H1N1 virus, West Nile virus, and dengue virus. *Cell* 139(7)**,** 1243-1254. doi: 10.1016/j.cell.2009.12.017.

Brooks A., Harkins R., Wang P., Qian H., Liu P. and Rubanyi G (2004). Transcriptional silencing is associated with extensive methylation of the CMV promoter following adenoviral gene delivery to muscle. *J Gene Med*. 6(4):395-404. doi: 10.1002/jgm.516. PMID: 15079814.

Brownlie, R., and Allan, B. (2011). Avian toll-like receptors. *Cell Tissue Res* 343(1)**,** 121-130. doi: 10.1007/s00441-010-1026-0.

Brownlie, R., Zhu, J., Allan, B., Mutwiri, G.K., Babiuk, L.A., Potter, A., et al. (2009). Chicken TLR21 acts as a functional homologue to mammalian TLR9 in the recognition of CpG oligodeoxynucleotides. *Molecular immunology* 46(15)**,** 3163-3170. doi: 10.1016/j.molimm.2009.06.002.

Burggraaf, S., Karpala, A.J., Bingham, J., Lowther, S., Selleck, P., Kimpton, W., et al. (2014). H5N1 infection causes rapid mortality and high cytokine levels in chickens compared to ducks. *Virus Res* 185**,** 23-31. doi: 10.1016/j.virusres.2014.03.012.

Burton Althea, N., McLintock, J., and Rempel, J.G. (1966). Western equine encephalitis virus in Saskatchewan garter snakes and leopard frogs. *Science* 154(3752)**,** 1029-1031. doi: 10.1126/science.154.3752.1029.

Bush, S.J., Freem, L., MacCallum, A.J., O'Dell, J., Wu, C., Afrasiabi, C., et al. (2018). Combination of novel and public RNA-seq datasets to generate an mRNA expression atlas for the domestic chicken. *BMC Genomics* 19(1)**,** 594. doi: 10.1186/s12864-018-4972-7.

Byrd-Leotis, L., Jia, N., Dutta, S., Trost, J.F., Gao, C., Cummings, S.F., et al. (2019). Influenza binds phosphorylated glycans from human lung. *Science Advances* 5(2)**,** eaav2554. doi: 10.1126/sciadv.aav2554.

Cadena, C., Ahmad, S., Xavier, A., Willemsen, J., Park, S., Park, J.W., et al. (2019). Ubiquitin-dependent and -independent roles of E3 ligase RIPLET in innate immunity. *Cell* 177(5)**,** 1187-1200.e1116. doi: 10.1016/j.cell.2019.03.017.

Cagle, C., To, T.L., Nguyen, T., Wasilenko, J., Adams, S.C., Cardona, C.J., et al. (2011). Pekin and Muscovy ducks respond differently to vaccination with a H5N1 highly pathogenic avian influenza (HPAI) commercial inactivated vaccine. *Vaccine* 29(38)**,** 6549-6557. doi: 10.1016/j.vaccine.2011.07.004.

Califano, D., Furuya, Y., Roberts, S., Avram, D., McKenzie, A.N.J., and Metzger, D.W. (2018). IFN-gamma increases susceptibility to influenza A infection through suppression of group II innate lymphoid cells. *Mucosal Immunol* 11(1)**,** 209-219. doi: 10.1038/mi.2017.41.

Camacho, C., Coulouris, G., Avagyan, V., Ma, N., Papadopoulos, J., Bealer, K., et al. (2009). BLAST+: architecture and applications. *BMC Bioinformatics* 10**,** 421. doi: 10.1186/1471-2105-10-421.

Campbell, L.K., Fleming-Canepa, X., Webster, R.G., and Magor, K.E. (2021). Tissue specific transcriptome changes upon influenza A virus replication in the duck. *Frontiers in Immunology* 12(4653). doi: 10.3389/fimmu.2021.786205.

Campbell, L.K., and Magor, K.E. (2020). Pattern recognition receptor signaling and innate responses to influenza A viruses in the mallard duck, compared to humans and chickens. *Frontiers in Cellular and Infection Microbiology* 10(209). doi: 10.3389/fcimb.2020.00209.

Cao, T.Y., Duprez, E., Borden, K.L.B., Freemont, P.S., and Etkin, L.D. (1998). Ret finger protein is a normal component of PML nuclear bodies and interacts directly with PML. *Journal of Cell Science* 111(10)**,** 1319-1329.

Caprara, G., Prosperini, E., Piccolo, V., Sigismondo, G., Melacarne, A., Cuomo, A., et al. (2018). PARP14 Controls the Nuclear Accumulation of a Subset of Type I IFN–Inducible Proteins. *The Journal of Immunology* 200(7)**,** 2439. doi: 10.4049/jimmunol.1701117.

Carthagena, L., Bergamaschi, A., Luna, J.M., David, A., Uchil, P.D., Margottin-Goguet, F., et al. (2009). Human TRIM Gene Expression in Response to Interferons. *PLOS ONE* 4(3)**,** e4894. doi: 10.1371/journal.pone.0004894.

Castanier, C., Garcin, D., Vazquez, A., and Arnoult, D. (2010). Mitochondrial dynamics regulate the RIG-I-like receptor antiviral pathway. *EMBO Rep* 11(2)**,** 133-138. doi: 10.1038/embor.2009.258.

Castanier, C., Zemirli, N., Portier, A., Garcin, D., Bidère, N., Vazquez, A., et al. (2012). MAVS ubiquitination by the E3 ligase TRIM25 and degradation by the proteasome is involved in type I interferon production after activation of the antiviral RIG-I-like receptors. *BMC Biol* 10**,** 44. doi: 10.1186/1741-7007-10-44.

Castello, A., Fischer B Fau - Eichelbaum, K., Eichelbaum K Fau - Horos, R., Horos R Fau - Beckmann, B.M., Beckmann Bm Fau - Strein, C., Strein C Fau - Davey, N.E., et al. Insights into RNA biology from an atlas of mammalian mRNA-binding proteins. (1097-4172 (Electronic)).

Cella, M., Salio, M., Sakakibara, Y., Langen, H., Julkunen, I., and Lanzavecchia, A. (1999). Maturation, activation, and protection of dendritic cells induced by double-stranded RNA. *J Exp Med* 189(5)**,** 821-829. doi: 10.1084/jem.189.5.821.

Chang, H.-M., Martinez, N.J., Thornton, J.E., Hagan, J.P., Nguyen, K.D., and Gregory, R.I. (2012). Trim71 cooperates with microRNAs to repress Cdkn1a expression and promote embryonic stem cell proliferation. *Nature Communications* 3(1)**,** 923. doi: 10.1038/ncomms1909.

Charif, D., and Lobry, J.R. (2007). "SeqinR 1.0-2: A contributed package to the R project for statistical computing devoted to biological sequences retrieval and analysis," in *Structural Approaches to Sequence Evolution: Molecules, Networks, Populations,* eds. U. Bastolla, M. Porto, H.E. Roman & M. Vendruscolo. (Berlin, Heidelberg: Springer Berlin Heidelberg), 207-232.

Chaves, L.D., Krueth, S.B., and Reed, K.M. (2009). Defining the turkey MHC: sequence and genes of the B locus. *J Immunol* 183(10)**,** 6530-6537. doi: 10.4049/jimmunol.0901310.

Chelbi-Alix, M.K., Quignon, F., Pelicano, L., Koken, M.H., and de Thé, H. (1998). Resistance to virus infection conferred by the interferon-induced promyelocytic leukemia protein. *J Virol* 72(2)**,** 1043-1051. doi: 10.1128/jvi.72.2.1043-1051.1998.

Chen, J., Wang, Z., Guo, X., Li, F., Wei, Q., Chen, X., et al. (2019). TRIM66 reads unmodified H3R2K4 and H3K56ac to respond to DNA damage in embryonic stem cells. *Nat Commun* 10(1)**,** 4273. doi: 10.1038/s41467-019-12126-4.

Chen, M., Meng, Q., Qin, Y., Liang, P., Tan, P., He, L., et al. (2016). TRIM14 Inhibits cGAS degradation mediated by selective autophagy receptor p62 to promote innate immune responses. *Mol Cell* 64(1)**,** 105-119. doi: 10.1016/j.molcel.2016.08.025.

Chen, S., Cheng, A., and Wang, M. (2013). Innate sensing of viruses by pattern recognition receptors in birds. *Vet Res* 44(1)**,** 82. doi: 10.1186/1297-9716-44-82.

Chen, S., Wang, L., Chen, J., Zhang, L., Wang, S., Goraya, M.U., et al. (2017). Avian interferon-inducible transmembrane protein family effectively restricts avian Tembusu virus infection. *Front Microbiol* 8(APR)**,** 672. doi: 10.3389/fmicb.2017.00672.

Chen, S., Wang, T., Liu, P., Yang, C., Wang, M., Jia, R., et al. (2019). Duck interferon regulatory factor 7 (IRF7) can control duck Tembusu virus (DTMUV) infection by triggering type I interferon production and its signal transduction pathway. *Cytokine* 113(January 2018)**,** 31-38. doi: 10.1016/j.cyto.2018.06.001.

Chen, S.N., Zhang, X.W., Li, L., Ruan, B.Y., Huang, B., Huang, W.S., et al. (2016). Evolution of IFN-λ in tetrapod vertebrates and its functional characterization in green anole lizard (Anolis carolinensis). *Developmental & Comparative Immunology* 61**,** 208-224. doi: https://doi.org/10.1016/j.dci.2016.04.004.

Chen, Z.J., and Sun, L.J. (2009). Nonproteolytic functions of ubiquitin in cell signaling. *Mol Cell* 33(3)**,** 275-286. doi: 10.1016/j.molcel.2009.01.014.

Cheng, D., Wu, X., Jia, R., Wang, M., Chen, S., Liu, M., et al. (2019). CpG oligodeoxynucleotide-specific duck TLR21 mediates activation of NF-κB signaling pathway and plays an important role in the host defence of DPV infection. *Molecular immunology* 106**,** 87-98. doi: 10.1016/j.molimm.2018.12.019.

Cheng, Y., Huang, Q., Ji, W., Du, B., Fu, Q., An, H., et al. (2015). Muscovy duck retinoic acid-induced gene I (MdRIG-I) functions in innate immunity against H9N2 avian influenza viruses (AIV) infections. *Vet Immunol Immunopathol* 163(3-4)**,** 183-193. doi: 10.1016/j.vetimm.2014.12.009.

Cheng, Y., Liu, Y., Shi, S., Niu, Q., Zhu, W., Wang, Z., et al. (2019). Functional characterization of duck STING in IFN-beta induction and anti-H9N2 avian influenza viruses infections. *Front Immunol* 10(September)**,** 2224. doi: 10.3389/fimmu.2019.02224.

Cheng, Y., Wang, H., Yan, Y., Ding, C., and Sun, J. (2015). Two myeloid differentiation factor 88 (MyD88) isoforms identified in ducks. *Dev Comp Immunol* 52(2)**,** 144-154. doi: 10.1016/j.dci.2015.03.015.

Cheng, Y., Zhu, W., Ding, C., Niu, Q., Wang, H., Yan, Y., et al. (2019). IRF7 Is involved in both STING and MAVS mediating IFN-beta signaling in IRF3-lacking chickens. *J Immunol* 203(7)**,** 1930-1942. doi: 10.4049/jimmunol.1900293.

Choudhury, N.R., Heikel, G., Trubitsyna, M., Kubik, P., Nowak, J.S., Webb, S., et al. (2017). RNA-binding activity of TRIM25 is mediated by its PRY/SPRY domain and is required for ubiquitination. *BMC Biol* 15(1)**,** 105. doi: 10.1186/s12915-017-0444-9.

Ciancanelli, M.J., Huang, S.X., Luthra, P., Garner, H., Itan, Y., Volpi, S., et al. (2015). Infectious disease. Life-threatening influenza and impaired interferon amplification in human IRF7 deficiency. *Science* 348(6233)**,** 448-453. doi: 10.1126/science.aaa1578.

Cluff, C.W., Baldridge, J.R., Stöver, A.G., Evans, J.T., Johnson, D.A., Lacy, M.J., et al. (2005). Synthetic toll-Like receptor 4 agonists stimulate innate resistance to infectious challenge. *Infection and Immunity* 73(5)**,** 3044. doi: 10.1128/IAI.73.5.3044-3052.2005.

Coch, C., Stümpel, J.P., Lilien-Waldau, V., Wohlleber, D., Kümmerer, B.M., Bekeredjian-Ding, I., et al. (2017). RIG-I activation protects and rescues from lethal influenza virus infection and bacterial superinfection. *Mol Ther* 25(9)**,** 2093-2103. doi: 10.1016/j.ymthe.2017.07.003.

Condemine, W., Takahashi, Y., Le Bras, M., and de Thé, H. (2007). A nucleolar targeting signal in PML-I addresses PML to nucleolar caps in stressed or senescent cells. *J Cell Sci* 120(Pt 18)**,** 3219-3227. doi: 10.1242/jcs.007492.

Condemine, W., Takahashi, Y., Zhu, J., Puvion-Dutilleul, F., Guegan, S., Janin, A., et al. (2006). Characterization of endogenous human promyelocytic leukemia isoforms. *Cancer Research* 66(12)**,** 6192-6198. doi: 10.1158/0008-5472.CAN-05-3792.

Connacher, R.P., and Goldstrohm, A.C. (2021). Molecular and biological functions of TRIM-NHL RNA-binding proteins. *Wiley Interdiscip Rev RNA* 12(2)**,** e1620. doi: 10.1002/wrna.1620.

Cooley, A.J., Van Campen, H., Philpott, M.S., Easterday, B.C., and Hinshaw, V.S. (1989). Pathological lesions in the lungs of ducks infected with influenza A viruses. *Vet Pathol* 26(1)**,** 1-5. doi: 10.1177/030098588902600101.

Cormican, P., Lloyd, A.T., Downing, T., Connell, S.J., Bradley, D., and O'Farrelly, C. (2009). The avian Toll-Like receptor pathway—Subtle differences amidst general conformity. *Developmental & Comparative Immunology* 33(9)**,** 967-973. doi: https://doi.org/10.1016/j.dci.2009.04.001.

Cornelissen, J.B., Vervelde, L., Post, J., and Rebel, J.M. (2013). Differences in highly pathogenic avian influenza viral pathogenesis and associated early inflammatory response in chickens and ducks. *Avian Pathol* 42(4)**,** 347-364. doi: 10.1080/03079457.2013.807325.

Cornelissen, J.B.W.J., Post, J., Peeters, B., Vervelde, L., and Rebel, J.M.J. (2012). Differential innate responses of chickens and ducks to low-pathogenic avian influenza. *Avian Pathology* 41(6)**,** 519-529. doi: 10.1080/03079457.2012.732691.

Costa, T., Chaves, A.J., Valle, R., Darji, A., van Riel, D., Kuiken, T., et al. (2012). Distribution patterns of influenza virus receptors and viral attachment patterns in the respiratory and intestinal tracts of seven avian species. *Vet Res* 43**,** 28. doi: 10.1186/1297-9716-43-28.

Couceiro, J.N., Paulson, J.C., and Baum, L.G. (1993). Influenza virus strains selectively recognize sialyloligosaccharides on human respiratory epithelium; the role of the host cell in selection of hemagglutinin receptor specificity. *Virus Res* 29(2)**,** 155-165. doi: 10.1016/0168-1702(93)90056-s.

Crotta, S., Davidson, S., Mahlakoiv, T., Desmet, C.J., Buckwalter, M.R., Albert, M.L., et al. (2013). Type I and type III interferons drive redundant amplification loops to induce a transcriptional signature in influenza-infected airway epithelia. *PLoS Pathog* 9(11)**,** e1003773. doi: 10.1371/journal.ppat.1003773.

Cui, H., Liu, Y., and Huang, Y. (2017). Roles of TRIM32 in corneal epithelial cells after infection with herpes simplex virus. *Cell Physiol Biochem* 43(2)**,** 801-811. doi: 10.1159/000481563.

Cupp, E.W., Zhang, D., Yue, X., Cupp, M.S., Guyer, C., Sprenger, T.R., et al. (2004). Identification of reptilian and amphibian blood meals from mosquitoes in an eastern equine encephalomyelitis virus focus in central Alabama. *Am J Trop Med Hyg* 71(3)**,** 272-276.

D'Cruz, A.A., Babon, J.J., Norton, R.S., Nicola, N.A., and Nicholson, S.E. (2013). Structure and function of the SPRY/B30.2 domain proteins involved in innate immunity. *Protein Sci* 22(1)**,** 1-10. doi: 10.1002/pro.2185.

Daoust, P.Y., Kibenge, F.S., Fouchier, R.A., van de Bildt, M.W., van Riel, D., and Kuiken, T. (2011). Replication of low pathogenic avian influenza virus in naturally infected Mallard ducks (Anas platyrhynchos) causes no morphologic lesions. *J Wildl Dis* 47(2)**,** 401-409. doi: 10.7589/0090-3558-47.2.401.

Daoust, P.Y., van de Bildt, M., van Riel, D., van Amerongen, G., Bestebroer, T., Vanderstichel, R., et al. (2013). Replication of 2 subtypes of low-pathogenicity avian influenza virus of duck and gull origins in experimentally infected Mallard ducks. *Vet Pathol* 50(3)**,** 548-559. doi: 10.1177/0300985812469633.

Daviet, S., Van Borm, S., Habyarimana, A., Ahanda, M.L., Morin, V., Oudin, A., et al. (2009). Induction of Mx and PKR failed to protect chickens from H5N1 infection. *Viral Immunol* 22(6)**,** 467-472. doi: 10.1089/vim.2009.0053.

Davison, T.F. (2003). The immunologists' debt to the chicken. *British Poultry Science* 44(1)**,** 6-21. doi: 10.1080/0007166031000085364.

Di Pietro, A., Kajaste-Rudnitski, A., Oteiza, A., Nicora, L., Towers, G.J., Mechti, N., et al. (2013). TRIM22 inhibits influenza A virus infection by targeting the viral nucleoprotein for degradation. *J Virol* 87(8)**,** 4523-4533. doi: 10.1128/JVI.02548-12.

Diamond, M.S., and Farzan, M. (2013). The broad-spectrum antiviral functions of IFIT and IFITM proteins. *Nat Rev Immunol* 13(1)**,** 46-57. doi: 10.1038/nri3344.

Dickson, C., Fletcher, A.J., Vaysburd, M., Yang, J.-C., Mallery, D.L., Zeng, J., et al. (2018). Intracellular antibody signalling is regulated by phosphorylation of the Fc receptor TRIM21. *eLife* 7**,** e32660. doi: 10.7554/eLife.32660.

Diebold, S.S., Kaisho, T., Hemmi, H., Akira, S., and Reis e Sousa, C. (2004). Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. *Science* 303(5663)**,** 1529-1531. doi: 10.1126/science.1093616.

Dillon, D., and Runstadler, J. (2010). Mx gene diversity and influenza association among five wild dabbling duck species (*Anas spp.)* in Alaska. *Infection, Genetics and Evolution* 10(7)**,** 1085-1093. doi: https://doi.org/10.1016/j.meegid.2010.07.004.

Doncheva, N.T., Morris, J.H., Gorodkin, J., and Jensen, L.J. (2019). Cytoscape StringApp: network analysis and visualization of proteomics data. *J Proteome Res* 18(2)**,** 623-632. doi: 10.1021/acs.jproteome.8b00702.

Doyle, T., Moncorgé, O., Bonaventure, B., Pollpeter, D., Lussignol, M., Tauziet, M., et al. (2018). The interferon-inducible isoform of NCOA7 inhibits endosome-mediated viral entry. *Nat Microbiol* 3(12)**,** 1369-1376. doi: 10.1038/s41564-018-0273-9.

Ducatez, M., Sonnberg, S., Crumpton, J.C., Rubrum, A., Phommachanh, P., Douangngeun, B., et al. (2017). Highly pathogenic avian influenza H5N1 clade 2.3.2.1 and clade 2.3.4 viruses do not induce a clade-specific phenotype in mallard ducks. *Journal of General Virology* 98(6)**,** 1232-1244. doi: https://doi.org/10.1099/jgv.0.000806.

Dutrieux, J., Maarifi, G., Portilho, D.M., Arhel, N.J., Chelbi-Alix, M.K., and Nisole, S. (2015). PML/TRIM19-dependent inhibition of retroviral reverse-transcription by Daxx. *PLOS Pathogens* 11(11)**,** e1005280. doi: 10.1371/journal.ppat.1005280.

Eisen, M.B., Spellman, P.T., Brown, P.O., and Botstein, D. (1998). Cluster analysis and display of genome-wide expression patterns. *Proceedings of the National Academy of Sciences* 95(25)**,** 14863.

El Bougrini, J., Dianoux, L., and Chelbi-Alix, M.K. (2011). PML positively regulates interferon gamma signaling. *Biochimie* 93(3)**,** 389-398. doi: 10.1016/j.biochi.2010.11.005.

El McHichi, B., Regad, T., Maroui, M.-A., Rodriguez, M.S., Aminev, A., Gerbaud, S., et al. (2010). SUMOylation promotes PML degradation during encephalomyocarditis virus infection. *Journal of virology* 84(22)**,** 11634-11645. doi: 10.1128/JVI.01321-10.

Esposito, D., Koliopoulos, M.G., and Rittinger, K. (2017). Structural determinants of TRIM protein function. *Biochem Soc Trans* 45(1)**,** 183-191. doi: 10.1042/BST20160325.

Evseev, D., and Magor, K.E. (2019). Innate immune responses to avian influenza viruses in Ducks and Chickens. *Vet Sci* 6(1)**,** 5-5. doi: 10.3390/vetsci6010005.

Evseev, D., Miranzo-Navarro, D., Fleming-Canepa, X., Webster, R. and Magor, K. (2022) Avian influenza NS1 proteins inhibit human, bt not duck, RIG-I ubiquitination and interferon signaling. Journal of virology. doi: 10.1128/jvi.00776-22. Epub ahead of print. PMID: 36069546

Fan, W., Liu, X., Zhang, J., Qin, L., Du, J., Li, X., et al. (2022). TRIM67 Suppresses TNFalpha-Triggered NF-kB Activation by Competitively Binding Beta-TrCP to IkBa. *Frontiers in Immunology* 13.

Fang, R., Jiang, Q., Zhou, X., Wang, C., Guan, Y., Tao, J., et al. (2017). MAVS activates TBK1 and IKKε through TRAFs in NEMO dependent and independent manner. *PLOS Pathogens* 13(11)**,** e1006720. doi: 10.1371/journal.ppat.1006720.

Fang, R., Wang, C., Jiang, Q., Lv, M., Gao, P., Yu, X., et al. (2017). NEMO-IKKβ are essential for IRF3 and NF-κB activation in the cGAS-STING pathway. *J Immunol* 199(9)**,** 3222-3233. doi: 10.4049/jimmunol.1700699.

Farnsworth, M.L., Miller, R.S., Pedersen, K., Lutman, M.W., Swafford, S.R., Riggs, P.D., et al. (2012). Environmental and demographic determinants of avian influenza viruses in waterfowl across the contiguous United States. *PLoS One* 7(3)**,** e32729. doi: 10.1371/journal.pone.0032729.

Feng, P., Zeng, T., Yang, H., Chen, G., Du, J., Chen, L., et al. (2021). Whole-genome resequencing provides insights into the population structure and domestication signatures of ducks in eastern China. *BMC Genomics* 22(1)**,** 401. doi: 10.1186/s12864-021-07710-2.

Feng, Z.Q., Cheng, Y., Yang, H.L., Zhu, Q., Yu, D., and Liu, Y.P. (2015). Molecular characterization, tissue distribution and expression analysis of TRIM25 in Gallus gallus domesticus. *Gene* 561(1)**,** 138-147. doi: 10.1016/j.gene.2015.02.025.

Fensterl, V., and Sen, G.C. (2015). Interferon-induced IFIT proteins: their role in viral pathogenesis. *J Virol* 89(5)**,** 2462-2468. doi: 10.1128/JVI.02744-14.

Fernandes, A.P., Águeda-Pinto, A., Pinheiro, A., Rebelo, H., and Esteves, P.J. (2022). Evolution of TRIM5 and TRIM22 in bats reveals a complex duplication process. *Viruses* 14(2). doi: 10.3390/v14020345.

Fernandez, M.V., Miller, E., Krammer, F., Gopal, R., Greenbaum, B.D., and Bhardwaj, N. (2016). Ion efflux and influenza infection trigger NLRP3 inflammasome signaling in human dendritic cells. *J Leukoc Biol* 99(5)**,** 723-734. doi: 10.1189/jlb.3A0614-313RRR.

Ferri, F., Parcelier, A., Petit, V., Gallouet, A.-S., Lewandowski, D., Dalloz, M., et al. (2015). TRIM33 switches off Ifnb1 gene transcription during the late phase of macrophage activation. *Nature Communications* 6(1)**,** 8900. doi: 10.1038/ncomms9900.

Finley, D. (2009). Recognition and processing of ubiquitin-protein conjugates by the proteasome. *Annu Rev Biochem* 78**,** 477-513. doi: 10.1146/annurev.biochem.78.081507.101607.

Finn, R.D., Clements, J., and Eddy, S.R. (2011). HMMER web server: interactive sequence similarity searching. *Nucleic Acids Res* 39(Web Server issue)**,** W29-37. doi: 10.1093/nar/gkr367.

Fitzgerald, K.A., McWhirter, S.M., Faia, K.L., Rowe, D.C., Latz, E., Golenbock, D.T., et al. (2003). IKKepsilon and TBK1 are essential components of the IRF3 signaling pathway. *Nat Immunol* 4(5)**,** 491-496. doi: 10.1038/ni921.

Flajnik, M.F., and Kasahara, M. (2001). Comparative genomics of the MHC: glimpses into the evolution of the adaptive immune system. *Immunity* 15(3)**,** 351-362. doi: 10.1016/s1074-7613(01)00198-4.

Fleming-Canepa, X., Aldridge, J.R., Jr., Canniff, L., Kobewka, M., Jax, E., Webster, R.G., et al. (2019). Duck innate immune responses to high and low pathogenicity H5 avian influenza viruses. *Vet Microbiol* 228**,** 101-111. doi: 10.1016/j.vetmic.2018.11.018.

Fleming-Canepa, X., Brusnyk, C., Aldridge, J.R., Ross, K.L., Moon, D., Wang, D., et al. (2011). Expression of duck CCL19 and CCL21 and CCR7 receptor in lymphoid and influenza-infected tissues. *Mol Immunol* 48(15-16)**,** 1950-1957. doi: 10.1016/j.molimm.2011.05.025.

Foletta, V.C., White, L.J., Larsen, A.E., Léger, B., and Russell, A.P. (2011). The role and regulation of MAFbx/atrogin-1 and MuRF1 in skeletal muscle atrophy. *Pflügers Archiv - European Journal of Physiology* 461(3)**,** 325-335. doi: 10.1007/s00424-010-0919-9.

Förster, R., Davalos-Misslitz, A.C., and Rot, A. (2008). CCR7 and its ligands: balancing immunity and tolerance. *Nat Rev Immunol* 8(5)**,** 362-371. doi: 10.1038/nri2297.

Francisco, E., Suthar, M., Gale, M., Jr., Rosenfeld, A.B., and Racaniello, V.R. (2019). Cell-type specificity and functional redundancy of RIG-I-like receptors in innate immune sensing of Coxsackievirus B3 and encephalomyocarditis virus. *Virology* 528**,** 7-18. doi: 10.1016/j.virol.2018.12.003.

Fridell, R.A., Harding, L.S., Bogerd, H.P., and Cullen, B.R. (1995). Identification of a novel human zinc finger protein that specifically interacts with the activation domain of lentiviral Tat proteins. *Virology* 209(2)**,** 347-357. doi: 10.1006/viro.1995.1266.

Fu, B., Wang, L., Ding, H., Schwamborn, J.C., Li, S., and Dorf, M.E. (2015). TRIM32 senses and restricts influenza A virus by ubiquitination of PB1 polymerase. *PLoS Pathog* 11(6)**,** e1004960. doi: 10.1371/journal.ppat.1004960.

Full, F., van Gent, M., Sparrer, K.M.J., Chiang, C., Zurenski, M.A., Scherer, M., et al. (2019). Centrosomal protein TRIM43 restricts herpesvirus infection by regulating nuclear lamina integrity. *Nat Microbiol* 4(1)**,** 164-176. doi: 10.1038/s41564-018-0285-5.

Fulton, J.E., Arango, J., Ali, R.A., Bohorquez, E.B., Lund, A.R., Ashwell, C.M., et al. (2014). Genetic variation within the Mx gene of commercially selected chicken lines reveals multiple haplotypes, recombination and a protein under selection pressure. *PLoS One* 9(9)**,** e108054. doi: 10.1371/journal.pone.0108054.

Gack, M.U., Albrecht, R.A., Urano, T., Inn, K.S., Huang, I.C., Carnero, E., et al. (2009). Influenza A virus NS1 targets the ubiquitin ligase TRIM25 to evade recognition by the host viral RNA sensor RIG-I. *Cell Host Microbe* 5(5)**,** 439-449. doi: 10.1016/j.chom.2009.04.006.

Gack, M.U., Shin, Y.C., Joo, C.H., Urano, T., Liang, C., Sun, L., et al. (2007). TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity. *Nature* 446(7138)**,** 916-920. doi: 10.1038/nature05732.

Gaidet, N., Cappelle, J., Takekawa, J.Y., Prosser, D.J., Iverson, S.A., Douglas, D.C., et al. (2010). Potential spread of highly pathogenic avian influenza H5N1 by wildfowl: dispersal ranges and rates determined from large-scale satellite telemetry. *Journal of Applied Ecology* 47(5)**,** 1147-1157. doi: 10.1111/j.1365-2664.2010.01845.x.

Galabru, J., and Hovanessian, A. (1987). Autophosphorylation of the protein kinase dependent on double-stranded RNA. *J Biol Chem* 262(32)**,** 15538-15544.

Gall, J.G. (2000). Cajal bodies: the first 100 years. *Annu Rev Cell Dev Biol* 16**,** 273-300. doi: 10.1146/annurev.cellbio.16.1.273.

Gao, B., Duan, Z., Xu, W., and Xiong, S. (2009). Tripartite motif-containing 22 inhibits the activity of hepatitis B virus core promoter, which is dependent on nuclear-located RING domain. *Hepatology* 50(2)**,** 424-433. doi: https://doi.org/10.1002/hep.23011.

Gao, P., Fan, L., Du, H., Xiang, B., Li, Y., Sun, M., et al. (2018). Recombinant duck interferon gamma inhibits H5N1 influenza virus replication in vitro and in vivo. *J Interferon Cytokine Res* 38(7)**,** 290-297. doi: 10.1089/jir.2018.0034.

Gao, P., Xiang, B., Li, Y., Li, Y., Sun, M., Kang, Y., et al. (2018). Therapeutic effect of duck interferon-alpha against H5N1 highly pathogenic avian influenza virus infection in Peking ducks. *J Interferon Cytokine Res* 38(4)**,** 145-152. doi: 10.1089/jir.2017.0116.

García-Posadas, L., Arranz-Valsero, I., López-García, A., Soriano-Romaní, L., and Diebold, Y. (2013). A new human primary epithelial cell culture model to study conjunctival inflammation. *Investigative Ophthalmology & Visual Science* 54(10)**,** 7143-7152. doi: 10.1167/iovs.13-12866.

Garcia, C.C., Weston-Davies, W., Russo, R.C., Tavares, L.P., Rachid, M.A., Alves-Filho, J.C., et al. (2013). Complement C5 activation during influenza A infection in mice contributes to neutrophil recruitment and lung injury. *PLOS ONE* 8(5)**,** e64443. doi: 10.1371/journal.pone.0064443.

Garcia, M.A., Gil, J., Ventoso, I., Guerra, S., Domingo, E., Rivas, C., et al. (2006). Impact of protein kinase PKR in cell biology: from antiviral to antiproliferative action. *Microbiol Mol Biol Rev* 70(4)**,** 1032-1060. doi: 10.1128/MMBR.00027-06.

Gel, B., and Serra, E. (2017). karyoploteR: an R/Bioconductor package to plot customizable genomes displaying arbitrary data. *Bioinformatics* 33(19)**,** 3088-3090. doi: 10.1093/bioinformatics/btx346.

Glasauer, S.M., and Neuhauss, S.C. (2014). Whole-genome duplication in teleost fishes and its evolutionary consequences. *Mol Genet Genomics* 289(6)**,** 1045-1060. doi: 10.1007/s00438-014-0889-2.

Glick, D., Barth, S., and Macleod, K.F. (2010). Autophagy: cellular and molecular mechanisms. *J Pathol* 221(1)**,** 3-12. doi: 10.1002/path.2697.

Gonzalez, S.F., Jayasekera, J.P., and Carroll, M.C. (2008). Complement and natural antibody are required in the long-term memory response to influenza virus. *Vaccine* 26**,** I86-I93. doi: https://doi.org/10.1016/j.vaccine.2008.11.057.

Goossens, K.E., Karpala, A.J., Rohringer, A., Ward, A., and Bean, A.G. (2015). Characterisation of chicken viperin. *Mol Immunol* 63(2)**,** 373-380. doi: 10.1016/j.molimm.2014.09.011.

Goyani, S., Roy, M., and Singh, R. (2021). TRIM-NHL as RNA Binding Ubiquitin E3 Ligase (RBUL): Implication in development and disease pathogenesis. *Biochim Biophys Acta Mol Basis Dis* 1867(7)**,** 166066. doi: 10.1016/j.bbadis.2020.166066.

Gregory R. Warnes, B.B., Lodewijk Bonebakker, Robert Gentleman, Wolfgang Huber, Andy Liaw, Thomas Lumley, Martin Maechler, Arni Magnusson, Steffen Moeller, Marc Schwartz and Bill Venables (2022). "gplots: various R programming tools for plotting data."

Guarda, G., Braun, M., Staehli, F., Tardivel, A., Mattmann, C., Forster, I., et al. (2011). Type I interferon inhibits interleukin-1 production and inflammasome activation. *Immunity* 34(2)**,** 213-223. doi: 10.1016/j.immuni.2011.02.006.

Guillot, L., Le Goffic, R., Bloch, S., Escriou, N., Akira, S., Chignard, M., et al. (2005). Involvement of toll-like receptor 3 in the immune response of lung epithelial cells to double-stranded RNA and influenza A virus. *J Biol Chem* 280(7)**,** 5571-5580. doi: 10.1074/jbc.M410592200.

Guion, L., Bienkowska-Haba, M., DiGiuseppe, S., Florin, L., and Sapp, M. (2019). PML nuclear body-residing proteins sequentially associate with HPV genome after infectious nuclear delivery. *PLoS Pathog* 15(2)**,** e1007590. doi: 10.1371/journal.ppat.1007590.

Guo, B., and Cheng, G. (2007). Modulation of the interferon antiviral response by the TBK1/IKKi adaptor protein TANK. *J Biol Chem* 282(16)**,** 11817-11826. doi: 10.1074/jbc.M700017200.

Guo, X., He, X.X., Chen, H., Wang, Z.C., Li, H.F., Wang, J.X., et al. (2021). Revisiting the evolutionary history of domestic and wild ducks based on genomic analyses. *Zool Res* 42(1)**,** 43-50. doi: 10.24272/j.issn.2095-8137.2020.133.

Habjan, M., Hubel, P., Lacerda, L., Benda, C., Holze, C., Eberl, C.H., et al. (2013). Sequestration by IFIT1 impairs translation of 2'O-unmethylated capped RNA. *PLoS Pathog* 9(10)**,** e1003663. doi: 10.1371/journal.ppat.1003663.

Hacker, H., Redecke, V., Blagoev, B., Kratchmarova, I., Hsu, L.C., Wang, G.G., et al. (2006). Specificity in Toll-like receptor signalling through distinct effector functions of TRAF3 and TRAF6. *Nature* 439(7073)**,** 204-207. doi: 10.1038/nature04369.

Hagag, I.T., Mansour, S.M., Zhang, Z., Ali, A.A., Ismaiel el, B.M., Salama, A.A., et al. (2015). Pathogenicity of highly pathogenic avian influenza virus H5N1 in naturally infected poultry in Egypt. *PLoS One* 10(5)**,** e0120061. doi: 10.1371/journal.pone.0120061.

Haider, N., Sturm-Ramirez, K., Khan, S.U., Rahman, M.Z., Sarkar, S., Poh, M.K., et al. (2017). Unusually high mortality in waterfowl caused by highly pathogenic avian influenza

A(H5N1) in Bangladesh. *Transbound Emerg Dis* 64(1)**,** 144-156. doi: 10.1111/tbed.12354.

Haider, N., Sturm-Ramirez, K., Khan, S.U., Rahman, M.Z., Sarkar, S., Poh, M.K., et al. (2017). Unusually high mortality in waterfowl caused by highly pathogenic avian influenza A(H5N1) in Bangladesh. *Transboundary and Emerging Diseases* 64(1)**,** 144-156. doi: 10.1111/tbed.12354.

Haller, O., Staeheli, P., Schwemmle, M., and Kochs, G. (2015). Mx GTPases: dynamin-like antiviral machines of innate immunity. *Trends Microbiol* 23(3)**,** 154-163. doi: 10.1016/j.tim.2014.12.003.

Hammell, C.M., Lubin, I., Boag, P.R., Blackwell, T.K., and Ambros, V. (2009). nhl-2 modulates microRNA activity in *Caenorhabditis elegans*. *Cell* 136(5)**,** 926-938. doi: 10.1016/j.cell.2009.01.053.

Han, X., Du, H., and Massiah, M.A. (2011). Detection and characterization of the in vitro e3 ligase activity of the human MID1 protein. *J Mol Biol* 407(4)**,** 505-520. doi: 10.1016/j.jmb.2011.01.048.

Hatakeyama, S. (2011). TRIM proteins and cancer. *Nat Rev Cancer* 11(11)**,** 792-804. doi: 10.1038/nrc3139.

Hatakeyama, S. (2017). TRIM family proteins: Roles in autophagy, immunity, and carcinogenesis. *Trends Biochem Sci* 42(4)**,** 297-311. doi: 10.1016/j.tibs.2017.01.002.

Hatesuer, B., Hoang, H.T., Riese, P., Trittel, S., Gerhauser, I., Elbahesh, H., et al. (2017). Deletion of Irf3 and Irf7 genes in mice results in altered interferon pathway activation and granulocyte-dominated inflammatory responses to influenza A infection. *J Innate Immun* 9(2)**,** 145-161. doi: 10.1159/000450705.

Hattlmann, C.J., Kelly, J.N., and Barr, S.D. (2012). TRIM22: A diverse and dynamic antiviral protein. *Mol Biol Int* 2012**,** 153415. doi: 10.1155/2012/153415.

Hayashi, T., Watanabe, C., Suzuki, Y., Tanikawa, T., Uchida, Y., and Saito, T. (2014). Chicken MDA5 senses short double-stranded RNA with implications for antiviral response against avian influenza viruses in chicken. *J Innate Immun* 6(1)**,** 58-71. doi: 10.1159/000351583.

Hayman, D.T., Yu, M., Crameri, G., Wang, L.F., Suu-Ire, R., Wood, J.L., et al. (2012). Ebola virus antibodies in fruit bats, Ghana, West Africa. *Emerg Infect Dis* 18(7)**,** 1207-1209. doi: 10.3201/eid1807.111654.

Hayman, T.J., Hsu, A.C., Kolesnik, T.B., Dagley, L.F., Willemsen, J., Tate, M.D., et al. (2019). RIPLET, and not TRIM25, is required for endogenous RIG-I-dependent antiviral responses. *Immunology & Cell Biology* 97(9)**,** 840-852. doi: https://doi.org/10.1111/imcb.12284.

He, H., Genovese, K.J., Swaggerty, C.L., MacKinnon, K.M., and Kogut, M.H. (2012). Co-stimulation with TLR3 and TLR21 ligands synergistically up-regulates Th1-cytokine IFN-γ and regulatory cytokine IL-10 expression in chicken monocytes. *Developmental & Comparative Immunology* 36(4)**,** 756-760. doi: https://doi.org/10.1016/j.dci.2011.11.006.

He, L., Martins, P., Huguenin, J., Van, T.N., Manso, T., Galindo, T., et al. (2019). Simple, sensitive and robust chicken specific sexing assays, compliant with large scale analysis. *PLoS One* 14(3)**,** e0213033. doi: 10.1371/journal.pone.0213033.

Hemmi, H., Kaisho, T., Takeuchi, O., Sato, S., Sanjo, H., Hoshino, K., et al. (2002). Small anti-viral compounds activate immune cells via the TLR7 MyD88–dependent signaling pathway. *Nature Immunology* 3(2)**,** 196-200. doi: 10.1038/ni758.

Henkels, K.M., Frondorf, K., Gonzalez-Mejia, M.E., Doseff, A.L., and Gomez-Cambronero, J. (2011). IL-8-induced neutrophil chemotaxis is mediated by Janus kinase 3 (JAK3). *FEBS letters* 585(1)**,** 159-166. doi: 10.1016/j.febslet.2010.11.031.

Herman, C.M., Barrow, J.H., Jr., and Tarshis, I.B. (1975). Leucocytozoonosis in Canada geese at the Seney National Wildlife Refuge. *J Wildl Dis* 11(3)**,** 404-411. doi: 10.7589/0090-3558-11.3.404.

Hershko, A., and Ciechanover, A. (1998). The ubiquitin system. *Annu Rev Biochem* 67**,** 425-479. doi: 10.1146/annurev.biochem.67.1.425.

Herz, C., Stavnezer, E., Krug, R., and Gurney, T., Jr. (1981). Influenza virus, an RNA virus, synthesizes its messenger RNA in the nucleus of infected cells. *Cell* 26(3 Pt 1)**,** 391-400. doi: 10.1016/0092-8674(81)90208-7.

Higgs, R., Gabhann, J.N., Larbi, N.B., Breen, E.P., Fitzgerald, K.A., and Jefferies, C.A. (2008). The E3 Ubiquitin Ligase Ro52 Negatively Regulates IFN-β Production Post-Pathogen Recognition by Polyubiquitin-Mediated Degradation of IRF3. *The Journal of Immunology* 181(3)**,** 1780. doi: 10.4049/jimmunol.181.3.1780.

Hiscott, J. (2007). Triggering the innate antiviral response through IRF-3 activation. *J Biol Chem* 282(21)**,** 15325-15329. doi: 10.1074/jbc.R700002200.

Hisham, I., Ellakany, H.F., Selim, A.A., Abdalla, M.A.M., Zain El-Abideen, M.A., Kilany, W.H., et al. (2020). Comparative pathogenicity of duck hepatitis A virus-1 isolates in experimentally infected Pekin and muscovy ducklings. *Frontiers in Veterinary Science* 7.

Hoischen, C., Monajembashi, S., Weisshart, K., and Hemmerich, P. (2018). Multimodal light microscopy approaches to reveal structural and functional properties of promyelocytic leukemia nuclear bodies. *Frontiers in Oncology* 8.

Holm, C.K., Jensen, S.B., Jakobsen, M.R., Cheshenko, N., Horan, K.A., Moeller, H.B., et al. (2012). Virus-cell fusion as a trigger of innate immunity dependent on the adaptor STING. *Nat Immunol* 13(8)**,** 737-743. doi: 10.1038/ni.2350.

Holm, C.K., Rahbek, S.H., Gad, H.H., Bak, R.O., Jakobsen, M.R., Jiang, Z., et al. (2016). Influenza A virus targets a cGAS-independent STING pathway that controls enveloped RNA viruses. *Nat Commun* 7**,** 10680. doi: 10.1038/ncomms10680.

Honda, K., and Taniguchi, T. (2006). IRFs: master regulators of signalling by Toll-like receptors and cytosolic pattern-recognition receptors. *Nat Rev Immunol* 6(9)**,** 644-658. doi: 10.1038/nri1900.

Hopkins, P.A., and Sriskandan, S. (2005). Mammalian Toll-like receptors: to immunity and beyond. *Clin Exp Immunol* 140(3)**,** 395-407. doi: 10.1111/j.1365-2249.2005.02801.x.

Horisberger, M.A., Staeheli, P., and Haller, O. (1983). Interferon induces a unique protein in mouse cells bearing a gene for resistance to influenza virus. *Proc Natl Acad Sci U S A* 80(7)**,** 1910-1914. doi: 10.1073/pnas.80.7.1910.

Hornung, V., Ellegast, J., Kim, S., Brzozka, K., Jung, A., Kato, H., et al. (2006). 5'-Triphosphate RNA is the ligand for RIG-I. *Science* 314(5801)**,** 994-997. doi: 10.1126/science.1132505.

Hou, F., Sun, L., Zheng, H., Skaug, B., Jiang, Q.X., and Chen, Z.J. (2011). MAVS forms functional prion-like aggregates to activate and propagate antiviral innate immune response. *Cell* 146(3)**,** 448-461. doi: 10.1016/j.cell.2011.06.041.

Hovanessian, A.G. (1989). The double stranded RNA-activated protein kinase induced by interferon: dsRNA-PK. *J Interferon Res* 9(6)**,** 641-647. doi: 10.1089/jir.1989.9.641.

Hron, T., Pajer, P., Paces, J., Bartunek, P., and Elleder, D. (2015). Hidden genes in birds. *Genome Biol* 16(1)**,** 164. doi: 10.1186/s13059-015-0724-z.

Hsu, K.S., Guan, B.J., Cheng, X., Guan, D., Lam, M., Hatzoglou, M., et al. (2016). Translational control of PML contributes to TNFα-induced apoptosis of MCF7 breast cancer cells and

decreased angiogenesis in HUVECs. *Cell Death Differ* 23(3)**,** 469-483. doi: 10.1038/cdd.2015.114.

Hu, J., Zhao, K., Liu, X., Wang, X., Chen, Z., and Liu, X. (2013). Two highly pathogenic avian influenza H5N1 viruses of clade 2.3.2.1 with similar genetic background but with different pathogenicity in mice and ducks. *Transboundary and Emerging Diseases* 60(2)**,** 127-139. doi: 10.1111/j.1865-1682.2012.01325.x.

Hu, M.M., Liao, C.Y., Yang, Q., Xie, X.Q., and Shu, H.B. (2017). Innate immunity to RNA virus is regulated by temporal and reversible sumoylation of RIG-I and MDA5. *J Exp Med* 214(4)**,** 973-989. doi: 10.1084/jem.20161015.

Hu, M.M., Xie, X.Q., Yang, Q., Liao, C.Y., Ye, W., Lin, H., et al. (2015). TRIM38 negatively regulates TLR3/4-mediated innate immune and inflammatory responses by two sequential and distinct mechanisms. *J Immunol* 195(9)**,** 4415-4425. doi: 10.4049/jimmunol.1500859.

Hu, M.M., Yang, Q., Xie, X.Q., Liao, C.Y., Lin, H., Liu, T.T., et al. (2016). Sumoylation promotes the stability of the DNA sensor cGAS and the adaptor STING to regulate the kinetics of response to DNA virus. *Immunity* 45(3)**,** 555-569. doi: 10.1016/j.immuni.2016.08.014.

Hua, K., Li, Y., Chen, H., Ni, J., Bi, D., Luo, R., et al. (2018). Functional characterization of duck TBK1 in IFN-beta induction. *Cytokine* 111(1)**,** 325-333. doi: 10.1016/j.cyto.2018.09.007.

Huang, B., and Baek, S.H. (2017). TRIM13 Potentiates Toll-Like Receptor 2-Mediated Nuclear Factor κB Activation via K29-Linked Polyubiquitination of Tumor Necrosis Factor Receptor-Associated Factor 6. *Mol Pharmacol* 91(4)**,** 307-316. doi: 10.1124/mol.116.106716.

Huang, B., Qi, Z.T., Xu, Z., and Nie, P. (2010). Global characterization of interferon regulatory factor (IRF) genes in vertebrates: glimpse of the diversification in evolution. *BMC Immunol* 11(1)**,** 22. doi: 10.1186/1471-2172-11-22.

Huang, Y., Li, Y., Burt, D.W., Chen, H., Zhang, Y., Qian, W., et al. (2013). The duck genome and transcriptome provide insight into an avian influenza virus reservoir species. *Nat Genet* 45(7)**,** 776-783. doi: 10.1038/ng.2657.

Huo, H., Zhao, L., Wang, D., Chen, X., and Chen, H. (2019). LGP2 plays a critical role in MDA5-mediated antiviral activity against duck enteritis virus. *Mol Immunol* 116**,** 160-166. doi: 10.1016/j.molimm.2019.10.006.

Huth, S., Lausier, J., Rudolph, C., Planck, C., Gersting, S., Welsch, U., et al. (2003). "Characterisation of the Mechanism of Magnetofection", in: *MOLECULAR THERAPY*: ACADEMIC PRESS INC ELSEVIER SCIENCE 525 B ST, STE 1900, SAN DIEGO, CA 92101 …), S372-S372.

Huynh, J., Li, S., Yount, B., Smith, A., Sturges, L., Olsen, J.C., et al. (2012). Evidence supporting a zoonotic origin of human coronavirus strain NL63. *J Virol* 86(23)**,** 12816-12825. doi: 10.1128/jvi.00906-12.

Ibsen, M.S., Gad, H.H., Andersen, L.L., Hornung, V., Julkunen, I., Sarkar, S.N., et al. (2015). Structural and functional analysis reveals that human OASL binds dsRNA to enhance RIG-I signaling. *Nucleic Acids Res* 43(10)**,** 5236-5248. doi: 10.1093/nar/gkv389.

Iki, S., Yokota, S., Okabayashi, T., Yokosawa, N., Nagata, K., and Fujii, N. (2005). Serum-dependent expression of promyelocytic leukemia protein suppresses propagation of influenza virus. *Virology* 343(1)**,** 106-115. doi: 10.1016/j.virol.2005.08.010.

Ip, H.S., Flint, P.L., Franson, J.C., Dusek, R.J., Derksen, D.V., Gill, R.E., Jr., et al. (2008). Prevalence of Influenza A viruses in wild migratory birds in Alaska: patterns of variation in detection at a crossroads of intercontinental flyways. *Virol J* 5**,** 71. doi: 10.1186/1743-422x-5-71.

Iqbal, M., Philbin, V.J., and Smith, A.L. (2005). Expression patterns of chicken Toll-like receptor mRNA in tissues, immune cell subsets and cell lines. *Veterinary Immunology and Immunopathology* 104(1)**,** 117-127. doi: https://doi.org/10.1016/j.vetimm.2004.11.003.

Irving, A.T., Ahn, M., Goh, G., Anderson, D.E., and Wang, L.-F. (2021). Lessons from the host defences of bats, a unique viral reservoir. *Nature* 589(7842)**,** 363-370. doi: 10.1038/s41586-020-03128-0.

Ishiguro, K., Watanabe, O., Nakamura, M., Yamamura, T., Matsushita, M., Goto, H., et al. (2017). Combinational use of lipid-based reagents for efficient transfection of primary fibroblasts and hepatoblasts. *Biotechniques* 63(1)**,** 37-39. doi: 10.2144/000114569.

Ishikawa, H., Ma, Z., and Barber, G.N. (2009). STING regulates intracellular DNA-mediated, type I interferon-dependent innate immunity. *Nature* 461(7265)**,** 788-792. doi: 10.1038/nature08476.

Ito, T., Wang, Y.H., and Liu, Y.J. (2005). Plasmacytoid dendritic cell precursors/type I interferon-producing cells sense viral infection by Toll-like receptor (TLR) 7 and TLR9. *Springer Semin Immunopathol* 26(3)**,** 221-229. doi: 10.1007/s00281-004-0180-4.

Ito, Y., Correll, K., Zemans, R.L., Leslie, C.C., Murphy, R.C., and Mason, R.J. (2015). Influenza induces IL-8 and GM-CSF secretion by human alveolar epithelial cells through HGF/c-Met and TGF-α/EGFR signaling. *American journal of physiology. Lung cellular and molecular physiology* 308(11)**,** L1178-L1188. doi: 10.1152/ajplung.00290.2014.

Iwata, H., Goettsch, C., Sharma, A., Ricchiuto, P., Goh, W.W.B., Halu, A., et al. (2016). PARP9 and PARP14 cross-regulate macrophage activation via STAT1 ADP-ribosylation. *Nature Communications* 7(1)**,** 12849. doi: 10.1038/ncomms12849.

Jackson, D.A., Caton, A.J., McCready, S.J., and Cook, P.R. (1982). Influenza virus RNA is synthesized at fixed sites in the nucleus. *Nature* 296(5855)**,** 366-368. doi: 10.1038/296366a0.

Jacobs, J.L., and Coyne, C.B. (2013). Mechanisms of MAVS regulation at the mitochondrial membrane. *J Mol Biol* 425(24)**,** 5009-5019. doi: 10.1016/j.jmb.2013.10.007.

James, L.C., Keeble, A.H., Khan, Z., Rhodes, D.A., and Trowsdale, J. (2007). Structural basis for PRYSPRY-mediated tripartite motif (TRIM) protein function. *Proc Natl Acad Sci U S A* 104(15)**,** 6200-6205. doi: 10.1073/pnas.0609174104.

Jang, H.J., Lee, H.J., Kang, K.S., Song, K.D., Kim, T.H., Song, C.S., et al. (2015). Molecular responses to the influenza A virus in chicken trachea-derived cells. *Poult Sci* 94(6)**,** 1190-1201. doi: 10.3382/ps/pev033.

Jia, X., Zhao, C., and Zhao, W. (2021). Emerging roles of MHC class I region-encoded E3 ubiquitin ligases in innate immunity. *Frontiers in Immunology* 12. doi: 10.3389/fimmu.2021.687102.

Jiang, H., Yang, H., and Kapczynski, D.R. (2011). Chicken interferon alpha pretreatment reduces virus replication of pandemic H1N1 and H5N9 avian influenza viruses in lung cell cultures from different avian species. *Virol J* 8(1)**,** 447. doi: 10.1186/1743-422X-8-447.

Jiao, P.R., Wei, L.M., Cheng, Y.Q., Yuan, R.Y., Han, F., Liang, J., et al. (2012). Molecular cloning, characterization, and expression analysis of the Muscovy duck Toll-like receptor 3 (MdTLR3) gene. *Poult Sci* 91(10)**,** 2475-2481. doi: 10.3382/ps.2012-02394.

Jiao, P.R., Wei, L.M., Song, Y.F., Cui, J., Zhang, S., Han, F., et al. (2015). Molecular cloning and immune responsive expression of LGP2 gene, a pivotal member of the RLR gene family from Muscovy duck Cairina moschata. *Poult Sci* 94(6)**,** 1170-1176. doi: 10.3382/ps/pev082.

Joazeiro, C.A., and Weissman, A.M. (2000). RING finger proteins: mediators of ubiquitin ligase activity. *Cell* 102(5)**,** 549-552. doi: 10.1016/s0092-8674(00)00077-5.

Jordan, I., Vos, A., Beilfuß, S., Neubert, A., Breul, S., and Sandig, V. (2009). An avian cell line designed for production of highly attenuated viruses. *Vaccine* 27(5)**,** 748-756. doi: https://doi.org/10.1016/j.vaccine.2008.11.066.

Jourdain, E., Gunnarsson, G., Wahlgren, J., Latorre-Margalef, N., Brojer, C., Sahlin, S., et al. (2010). Influenza virus in a natural host, the mallard: experimental infection data. *PLoS One* 5(1)**,** e8935. doi: 10.1371/journal.pone.0008935.

Jul-Larsen, Å., Grudic, A., Bjerkvig, R., and Bøe, S.O. (2010). Subcellular distribution of nuclear import-defective isoforms of the promyelocytic leukemia protein. *BMC Molecular Biology* 11(1)**,** 89. doi: 10.1186/1471-2199-11-89.

Justesen, J., Hartmann, R., and Kjeldgaard, N.O. (2000). Gene structure and function of the 2'-5'-oligoadenylate synthetase family. *Cell Mol Life Sci* 57(11)**,** 1593-1612. doi: 10.1007/pl00000644.

Kahle, T., Volkmann, B., Eissmann, K., Herrmann, A., Schmitt, S., Wittmann, S., et al. (2015). TRIM19/PML restricts HIV infection in a cell type-dependent manner. *Viruses* 8(1). doi: 10.3390/v8010002.

Kaikai, H., Zhao, D., Liu, Y., Liu, Q., Huang, X., Yang, J., et al. (2021). The E3 ubiquitin ligase TRIM25 inhibits Tembusu virus replication in vitro. *Front Vet Sci* 8**,** 722113. doi: 10.3389/fvets.2021.722113.

Kaiser, A., Willer, T., Sid, H., Petersen, H., Baumgärtner, W., Steinberg, P., et al. (2016). Susceptibility of primary chicken intestinal epithelial cells for low pathogenic avian influenza virus and velogenic viscerotropic Newcastle disease virus. *Virus Research* 225**,** 50-63. doi: https://doi.org/10.1016/j.virusres.2016.09.001.

Kalyaanamoorthy, S., Minh, B.Q., Wong, T.K.F., von Haeseler, A., and Jermiin, L.S. (2017). ModelFinder: fast model selection for accurate phylogenetic estimates. *Nature Methods* 14(6)**,** 587-589. doi: 10.1038/nmeth.4285.

Kandasamy, M., Suryawanshi, A., Tundup, S., Perez, J.T., Schmolke, M., Manicassamy, S., et al. (2016). RIG-I signaling Is critical for efficient polyfunctional T Cell responses during influenza virus infection. *PLoS Pathogens* 12(1553-7374 (Electronic)).

Kang, H.M., Choi, J.G., Kim, K.I., Kim, B.S., Batchuluun, D., Erdene-Ochir, T.O., et al. (2013). Pathogenicity in domestic ducks and mice of highly pathogenic H5N1 clade 2.3.2.1 influenza viruses recently circulating in Eastern Asia. *Vet Microbiol* 167(3-4)**,** 327-333. doi: 10.1016/j.vetmic.2013.09.013.

Kannaki, T.R., Verma, P.C., Reddy, M.R., and Shanmugam, M. (2018). Molecular characterization of duck (Anas platyrhynchos) Toll-like receptors, mRNA expressions profile in day-old duckling's tissues and cytokine response to in vitro TLR agonsists stimulation. *Indian Journal of Animal Research* 52(6)**,** 851-857. doi: UNSP B-329010.18805/ijar.B-3290.

Kar, S., Rodriguez, S.E., Akyildiz, G., Cajimat, M.N.B., Bircan, R., Mears, M.C., et al. (2020). Crimean-Congo hemorrhagic fever virus in tortoises and Hyalomma aegyptium ticks in East Thrace, Turkey: potential of a cryptic transmission cycle. *Parasites & Vectors* 13(1)**,** 201. doi: 10.1186/s13071-020-04074-6.

Karpala, A.J., Morris, K.R., Broadway, M.M., McWaters, P.G.D., O'Neil, T.E., Goossens, K.E., et al. (2008). Molecular cloning, expression, and characterization of chicken IFN -λ. *Journal of Interferon & Cytokine Research* 28(6)**,** 341-350. doi: 10.1089/jir.2007.0117.

Karpala, A.J., Stewart, C., McKay, J., Lowenthal, J.W., and Bean, A.G. (2011). Characterization of chicken MDA5 activity: regulation of IFN-beta in the absence of RIG-I functionality. *J Immunol* 186(9)**,** 5397-5405. doi: 10.4049/jimmunol.1003712.

Kato, H., Sato, S., Yoneyama, M., Yamamoto, M., Uematsu, S., Matsui, K., et al. (2005). Cell type-specific involvement of RIG-I in antiviral response. *Immunity* 23(1)**,** 19-28. doi: 10.1016/j.immuni.2005.04.010.

Kato, H., Takeuchi, O., Mikamo-Satoh, E., Hirai, R., Kawai, T., Matsushita, K., et al. (2008). Length-dependent recognition of double-stranded ribonucleic acids by retinoic acid-

inducible gene-I and melanoma differentiation-associated gene 5. *J Exp Med* 205(7)**,** 1601-1610. doi: 10.1084/jem.20080091.

Kato, H., Takeuchi, O., Sato, S., Yoneyama, M., Yamamoto, M., Matsui, K., et al. (2006). Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. *Nature* 441(7089)**,** 101-105. doi: 10.1038/nature04734.

Kato, K., Ahmad, S., Zhu, Z., Young, J.M., Mu, X., Park, S., et al. (2021). Structural analysis of RIG-I-like receptors reveals ancient rules of engagement between diverse RNA helicases and TRIM ubiquitin ligases. *Mol Cell* 81(3)**,** 599-613.e598. doi: 10.1016/j.molcel.2020.11.047.

Katoh, K., Rozewicki, J., and Yamada, K.D. (2019). MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. *Briefings in Bioinformatics* 20(4)**,** 1160-1166. doi: 10.1093/bib/bbx108.

Kaufman, J. (2022). Innate immune genes of the chicken MHC and related regions. *Immunogenetics* 74(1)**,** 167-177. doi: 10.1007/s00251-021-01229-2.

Kaufman, J., Milne, S., Göbel, T.W.F., Walker, B.A., Jacob, J.P., Auffray, C., et al. (1999). The chicken B locus is a minimal essential major histocompatibility complex. *Nature* 401(6756)**,** 923-925. doi: 10.1038/44856.

Kaufman, J., Völk, H., and Wallny, H.J. (1995). A "minimal essential Mhc" and an "unrecognized Mhc": two extremes in selection for polymorphism. *Immunol Rev* 143**,** 63-88. doi: 10.1111/j.1600-065x.1995.tb00670.x.

Kawai, T., and Akira, S. (2010). The role of pattern-recognition receptors in innate immunity: update on Toll-like receptors. *Nature Immunology* 11(5)**,** 373-384. doi: 10.1038/ni.1863.

Kawai, T., Takahashi, K., Sato, S., Coban, C., Kumar, H., Kato, H., et al. (2005). IPS-1, an adaptor triggering RIG-I- and MDA5-mediated type I interferon induction. *Nat Immunol* 6(10)**,** 981-988. doi: 10.1038/ni1243.

Keestra, A.M., de Zoete, M.R., Bouwman, L.I., Vaezirad, M.M., and van Putten, J.P.M. (2013). Unique features of chicken Toll-like receptors. *Developmental & Comparative Immunology* 41(3)**,** 316-323. doi: https://doi.org/10.1016/j.dci.2013.04.009.

Keiser, M.J., Roth, B.L., Armbruster, B.N., Ernsberger, P., Irwin, J.J., and Shoichet, B.K. (2007). Relating protein pharmacology by ligand chemistry. *Nature Biotechnology* 25(2)**,** 197-206. doi: 10.1038/nbt1284.

Keown, J.R., Yang, J., Black, M.M., and Goldstone, D.C. (2020). The RING domain of TRIM69 promotes higher-order assembly. *Acta Crystallogr D Struct Biol* 76(Pt 10)**,** 954-961. doi: 10.1107/s2059798320010499.

Khaitov, M.R., Laza-Stanca, V., Edwards, M.R., Walton, R.P., Rohde, G., Contoli, M., et al. (2009). Respiratory virus induction of alpha-, beta- and lambda-interferons in bronchial epithelial cells and peripheral blood mononuclear cells. *Allergy* 64(3)**,** 375-386. doi: 10.1111/j.1398-9995.2008.01826.x.

Khan, R.A., and Fallis, A.M. (1968). Comparison of infections with *Leucocytozoon simondi* in black ducks (*Anas rubripes*), mallards (*Anas platyrhynchos* and white Pekins (*Anas bochas*). *Can J Zool* 46(4)**,** 773-780. doi: 10.1139/z68-107.

Kida, H., Yanagawa, R., and Matsuoka, Y. (1980). Duck influenza lacking evidence of disease signs and immune-response. *Infection and Immunity* 30(2)**,** 547-553.

Kim, D., Paggi, J.M., Park, C., Bennett, C., and Salzberg, S.L. (2019). Graph-based genome alignment and genotyping with HISAT2 and HISAT-genotype. *Nature Biotechnology* 37(8)**,** 907-915. doi: 10.1038/s41587-019-0201-4.

Kim, J.-K., Negovetich, N.J., Forrest, H.L., and Webster, R.G. (2009). Ducks: the "Trojan horses" of H5N1 influenza. *Influenza and other respiratory viruses* 3(4)**,** 121-128. doi: 10.1111/j.1750-2659.2009.00084.x.

Kim, T.H., and Zhou, H. (2015). Functional analysis of chicken IRF7 in response to dsRNA analog poly(I:C) by integrating overexpression and knockdown. *PLoS One* 10(7)**,** e0133450. doi: 10.1371/journal.pone.0133450.

Kim, T.H., and Zhou, H. (2018). Overexpression of chicken IRF7 increased viral replication and programmed cell death to the avian influenza virus infection through TGF-Beta/FoxO signaling axis in DF-1. *Front Genet* 9(September)**,** 415. doi: 10.3389/fgene.2018.00415.

Kitaura, M., Suzuki, N., Imai, T., Takagi, S., Suzuki, R., Nakajima, T., et al. (1999). Molecular cloning of a novel human CC chemokine (Eotaxin-3) that is a functional ligand of CC chemokine receptor 3. *J Biol Chem* 274(39)**,** 27975-27980. doi: 10.1074/jbc.274.39.27975.

Klein, S.L., and Flanagan, K.L. (2016). Sex differences in immune responses. *Nature Reviews Immunology* 16(10)**,** 626-638. doi: 10.1038/nri.2016.90.

Ko, J.H., Asano, A., Kon, Y., Watanabe, T., and Agui, T. (2004). Characterization of the chicken PKR: polymorphism of the gene and antiviral activity against vesicular stomatitis virus. *Jpn J Vet Res* 51(3-4)**,** 123-133.

Ko, J.H., Jin, H.K., Asano, A., Takada, A., Ninomiya, A., Kida, H., et al. (2002). Polymorphisms and the differential antiviral activity of the chicken Mx gene. *Genome Res* 12(4)**,** 595-601. doi: 10.1101/gr.210702.

Koch, L.K., Cunze, S., Kochmann, J., and Klimpel, S. (2020). Bats as putative Zaire ebolavirus reservoir hosts and their habitat suitability in Africa. *Scientific Reports* 10(1)**,** 14268. doi: 10.1038/s41598-020-71226-0.

Koerner, I., Kochs, G., Kalinke, U., Weiss, S., and Staeheli, P. (2007). Protective role of beta interferon in host defense against influenza A virus. *Journal of Virology* 81(4)**,** 2025. doi: 10.1128/JVI.01718-06.

Kogut, M.H., Iqbal, M., He, H., Philbin, V., Kaiser, P., and Smith, A. (2005). Expression and function of Toll-like receptors in chicken heterophils. *Developmental & Comparative Immunology* 29(9)**,** 791-807. doi: https://doi.org/10.1016/j.dci.2005.02.002.

Koliopoulos, M.G., Esposito, D., Christodoulou, E., Taylor, I.A., and Rittinger, K. (2016). Functional role of TRIM E3 ligase oligomerization and regulation of catalytic activity. *The EMBO Journal* 35(11)**,** 1204-1218. doi: https://doi.org/10.15252/embj.201593741.

Kondo, T., Watanabe, M., and Hatakeyama, S. (2012). TRIM59 interacts with ECSIT and negatively regulates NF-κB and IRF-3/7-mediated signal pathways. *Biochemical and Biophysical Research Communications* 422(3)**,** 501-507. doi: https://doi.org/10.1016/j.bbrc.2012.05.028.

Kotenko, S.V., Gallagher, G., Baurin, V.V., Lewis-Antes, A., Shen, M., Shah, N.K., et al. (2003). IFN-lambdas mediate antiviral protection through a distinct class II cytokine receptor complex. *Nat Immunol* 4(1)**,** 69-77. doi: 10.1038/ni875.

Kouwaki, T., Nishimura, T., Wang, G., Nakagawa, R., and Oshiumi, H. (2021). Ubiquitin ligase RIPLET mediates polyubiquitination of RIG-I and LGP2 and regulates the innate immune responses to SARS-CoV-2 infection. *bioRxiv***,** 2021.2001.2025.428042. doi: 10.1101/2021.01.25.428042.

Kuchipudi, S.V., Dunham, S.P., Nelli, R., White, G.A., Coward, V.J., Slomka, M.J., et al. (2012). Rapid death of duck cells infected with influenza: a potential mechanism for host resistance to H5N1. *Immunol Cell Biol* 90(1)**,** 116-123. doi: 10.1038/icb.2011.17.

Kuchipudi, S.V., Nelli, R., White, G.A., Bain, M., Chang, K.C., and Dunham, S. (2009). Differences in influenza virus receptors in chickens and ducks: Implications for interspecies transmission. *J Mol Genet Med* 3(1)**,** 143-151. doi: 10.4172/1747-0862.1000026.

Kumar, A., Vijayakumar, P., Gandhale, P.N., Ranaware, P.B., Kumar, H., Kulkarni, D.D., et al. (2017). Genome-wide gene expression pattern underlying differential host response to high or low pathogenic H5N1 avian influenza virus in ducks. *Acta Virol* 61(1)**,** 66-76. doi: 10.4149/av_2017_01_66.

Kuriakose, T., Zheng, M., Neale, G., and Kanneganti, T.D. (2018). IRF1 Is a transcriptional regulator of ZBP1 promoting NLRP3 inflammasome activation and cell death during influenza virus infection. *J Immunol* 200(4)**,** 1489-1495. doi: 10.4049/jimmunol.1701538.

Kurihara, M., Kato, K., Sanbo, C., Shigenobu, S., Ohkawa, Y., Fuchigami, T., et al. (2020). Genomic profiling by ALaP-Seq reveals transcriptional regulation by PML bodies through DNMT3A exclusion. *Mol Cell* 78(3)**,** 493-505.e498. doi: 10.1016/j.molcel.2020.04.004.

Kwon, Y.K., Thomas, C., and Swayne, D.E. (2010). Variability in pathobiology of South Korean H5N1 high-pathogenicity avian influenza virus infection for 5 species of migratory waterfowl. *Veterinary Pathology* 47(3)**,** 495-506. doi: 10.1177/0300985809359602.

Lakadamyali, M., Rust, M.J., Babcock, H.P., and Zhuang, X. (2003). Visualizing infection of individual influenza viruses. *Proc Natl Acad Sci U S A* 100(16)**,** 9280-9285. doi: 10.1073/pnas.0832269100.

Lallemand-Breitenbach, V., and de Thé, H. (2010). PML nuclear bodies. *Cold Spring Harb Perspect Biol* 2(5)**,** a000661. doi: 10.1101/cshperspect.a000661.

Lallemand-Breitenbach, V., and de Thé, H. (2018). PML nuclear bodies: from architecture to function. *Curr Opin Cell Biol* 52**,** 154-161. doi: 10.1016/j.ceb.2018.03.011.

Lamark, T., and Johansen, T. (2012). Aggrephagy: selective disposal of protein aggregates by macroautophagy. *International Journal of Cell Biology* 2012**,** 736905. doi: 10.1155/2012/736905.

Lang, X., Tang, T., Jin, T., Ding, C., Zhou, R., and Jiang, W. (2017). TRIM65-catalized ubiquitination is essential for MDA5-mediated antiviral innate immunity. *J Exp Med* 214(2)**,** 459-473. doi: 10.1084/jem.20160592.

Latinne, A., Hu, B., Olival, K.J., Zhu, G., Zhang, L., Li, H., et al. (2020). Origin and cross-species transmission of bat coronaviruses in China. *Nature Communications* 11(1)**,** 4235. doi: 10.1038/s41467-020-17687-3.

Le Goffic, R., Balloy, V., Lagranderie, M., Alexopoulou, L., Escriou, N., Flavell, R., et al. (2006). Detrimental contribution of the Toll-like receptor (TLR)3 to influenza A virus-induced acute pneumonia. *PLoS Pathog* 2(6)**,** e53. doi: 10.1371/journal.ppat.0020053.

Le Goffic, R., Bouguyon, E., Chevalier, C., Vidic, J., Da Costa, B., Leymarie, O., et al. (2010). Influenza A virus protein PB1-F2 exacerbates IFN-β expression of human respiratory epithelial cells. *The Journal of Immunology* 185(8)**,** 4812. doi: 10.4049/jimmunol.0903952.

Lee, N.R., Kim, H.I., Choi, M.S., Yi, C.M., and Inn, K.S. (2015). Regulation of MDA5-MAVS antiviral signaling axis by TRIM25 through TRAF6-mediated NF-κB activation. *Mol Cells* 38(9)**,** 759-764. doi: 10.14348/molcells.2015.0047.

Lee, Y., Song, B., Park, C., and Kwon, K.S. (2013). TRIM11 negatively regulates IFNβ production and antiviral activity by targeting TBK1. *PLoS One* 8(5)**,** e63255. doi: 10.1371/journal.pone.0063255.

Lei, C.Q., Zhang, Y., Li, M., Jiang, L.Q., Zhong, B., Kim, Y.H., et al. (2015). ECSIT bridges RIG-I-like receptors to VISA in signaling events of innate antiviral responses. *J Innate Immun* 7(2)**,** 153-164. doi: 10.1159/000365971.

Lei, C.Q., Zhong, B., Zhang, Y., Zhang, J., Wang, S., and Shu, H.B. (2010). Glycogen synthase kinase 3β regulates IRF3 transcription factor-mediated antiviral response via activation of the kinase TBK1. *Immunity* 33(6)**,** 878-889. doi: 10.1016/j.immuni.2010.11.021.

Lepetz, V., Massot, M., Chaine, A.S., and Clobert, J. (2009). Climate warming and the evolution of morphotypes in a reptile. *Global Change Biology* 15(2)**,** 454-466. doi: https://doi.org/10.1111/j.1365-2486.2008.01761.x.

Letko, M., Seifert, S.N., Olival, K.J., Plowright, R.K., and Munster, V.J. (2020). Bat-borne virus diversity, spillover and emergence. *Nature Reviews Microbiology* 18(8)**,** 461-471. doi: 10.1038/s41579-020-0394-z.

Letunic, I., Doerks, T., and Bork, P. (2015). SMART: recent updates, new developments and status in 2015. *Nucleic Acids Res* 43(Database issue)**,** D257-260. doi: 10.1093/nar/gku949.

Levine, B., and Kroemer, G. (2008). Autophagy in the pathogenesis of disease. *Cell* 132(1)**,** 27-42. doi: https://doi.org/10.1016/j.cell.2007.12.018.

Li, C., Yang, P., Sun, Y., Li, T., Wang, C., Wang, Z., et al. (2012). IL-17 response mediates acute lung injury induced by the 2009 pandemic influenza A (H1N1) virus. *Cell research* 22(3)**,** 528-538. doi: 10.1038/cr.2011.165.

Li, C., Zhou, Y., Chen, X., Zhang, Y., Hu, J., Ren, C., et al. (2022). Porcine TRIM35 positively regulate TRAF3-mediated IFN-β production and inhibit Japanese encephalitis virus replication. *Dev Comp Immunol* 127**,** 104290. doi: 10.1016/j.dci.2021.104290.

Li, H.-F., Zhu, W.-Q., Song, W.-T., Shu, J.-T., Han, W., and Chen, K.-W. (2010). Origin and genetic diversity of Chinese domestic ducks. *Molecular Phylogenetics and Evolution* 57(2)**,** 634-640. doi: https://doi.org/10.1016/j.ympev.2010.07.011.

Li, H., Zhai, Y., Fan, Y., Chen, H., Zhang, A., Jin, H., et al. (2016). Molecular cloning and functional characterization of duck mitochondrial antiviral-signaling protein (MAVS). *Dev Comp Immunol* 56**,** 1-6. doi: 10.1016/j.dci.2015.11.004.

Li, L., Feng, W., Cheng, Z., Yang, J., Bi, J., Wang, X., et al. (2019). TRIM62-mediated restriction of avian leukosis virus subgroup J replication is dependent on the SPRY domain. *Poult Sci* 98(11)**,** 6019-6025. doi: 10.3382/ps/pez408.

Li, L., Niu, D., Yang, J., Bi, J., Zhang, L., Cheng, Z., et al. (2020). TRIM62 From Chicken as a Negative Regulator of Reticuloendotheliosis Virus Replication. *Frontiers in Veterinary Science* 7.

Li, N., Hong, T., Li, R., Wang, Y., Guo, M., Cao, Z., et al. (2016). Cherry Valley ducks mitochondrial antiviral-aignaling protein-mediated signaling pathway and antiviral activity research. *Front Immunol* 7(SEP)**,** 377. doi: 10.3389/fimmu.2016.00377.

Li, R., Lin, J., Hou, X., Han, S., Weng, H., Xu, T., et al. (2018). Characterization and roles of Cherry Valley duck NLRP3 in innate immunity during avian pathogenic *Escherichia coli* infection. *Front Immunol* 9(OCT)**,** 2300. doi: 10.3389/fimmu.2018.02300.

Li, S., Min, J.-Y., Krug, R.M., and Sen, G.C. (2006). Binding of the influenza A virus NS1 protein to PKR mediates the inhibition of its activation by either PACT or double-stranded RNA. *Virology* 349(1)**,** 13-21. doi: https://doi.org/10.1016/j.virol.2006.01.005.

Li, T., Hu, X., Zhang, T., Song, X., Zhang, H., Dai, N., et al. (2021). Cloning, analysis, and anti-duck Tembusu virus innate immune response of Cherry Valley duck tripartite motif-containing 32. *Poult Sci* 100(5)**,** 101048. doi: 10.1016/j.psj.2021.101048.

Li, W., Wang, G., Zhang, H., Zhang, D., Zeng, J., Chen, X., et al. (2009). Differential suppressive effect of promyelocytic leukemia protein on the replication of different subtypes/strains of influenza A virus. *Biochem Biophys Res Commun* 389(1)**,** 84-89. doi: 10.1016/j.bbrc.2009.08.091.

Li, X., Yu, Z., Fang, Q., Yang, M., Huang, J., Li, Z., et al. (2022). The transmembrane endoplasmic reticulum-associated E3 ubiquitin ligase TRIM13 restrains the pathogenic-DNA-triggered inflammatory response. *Sci Adv* 8(4)**,** eabh0496. doi: 10.1126/sciadv.abh0496.

Li, Y., Ma, X., Chen, Z., Wu, H., Wang, P., Wu, W., et al. (2019). B1 oligomerization regulates PML nuclear body biogenesis and leukemogenesis. *Nature Communications* 10(1)**,** 3789. doi: 10.1038/s41467-019-11746-0.

Liang, Q., Deng, H., Li, X., Wu, X., Tang, Q., Chang, T.H., et al. (2011). Tripartite motif-containing protein 28 is a small ubiquitin-related modifier E3 ligase and negative regulator of IFN regulatory factor 7. *J Immunol* 187(9)**,** 4754-4763. doi: 10.4049/jimmunol.1101704.

Liao, Y., Smyth, G.K., and Shi, W. (2014). featureCounts: an efficient general purpose program for assigning sequence reads to genomic features. *Bioinformatics* 30(7)**,** 923-930. doi: 10.1093/bioinformatics/btt656.

Lin, H., Jiang, M., Liu, L., Yang, Z., Ma, Z., Liu, S., et al. (2019). The long noncoding RNA Lnczc3h7a promotes a TRIM25-mediated RIG-I antiviral innate immune response. *Nat Immunol* 20(7)**,** 812-823. doi: 10.1038/s41590-019-0379-0.

Lindenmann, J. (1962). Resistance of mice to mouse-adapted influenza A virus. *Virology* 16**,** 203-204. doi: 10.1016/0042-6822(62)90297-0.

Liniger, M., Summerfield, A., Zimmer, G., McCullough, K.C., and Ruggli, N. (2012). Chicken cells sense influenza A virus infection through MDA5 and CARDIF signaling involving LGP2. *J Virol* 86(2)**,** 705-717. doi: 10.1128/JVI.00742-11.

Liu, B., Li, N.L., Shen, Y., Bao, X., Fabrizio, T., Elbahesh, H., et al. (2016). The C-Terminal Tail of TRIM56 Dictates Antiviral Restriction of Influenza A and B Viruses by Impeding Viral RNA Synthesis. *J Virol* 90(9)**,** 4369-4382. doi: 10.1128/JVI.03172-15.

Liu, B., Li, N.L., Wang, J., Shi, P.Y., Wang, T., Miller, M.A., et al. (2014). Overlapping and distinct molecular determinants dictating the antiviral activities of TRIM56 against flaviviruses and coronavirus. *J Virol* 88(23)**,** 13821-13835. doi: 10.1128/jvi.02505-14.

Liu, B., Zhang, M., Chu, H., Zhang, H., Wu, H., Song, G., et al. (2017). The ubiquitin E3 ligase TRIM31 promotes aggregation and activation of the signaling adaptor MAVS through Lys63-linked polyubiquitination. *Nat Immunol* 18(2)**,** 214-224. doi: 10.1038/ni.3641.

Liu, G., Lu, Y., Thulasi Raman, S.N., Xu, F., Wu, Q., Li, Z., et al. (2018). Nuclear-resident RIG-I senses viral replication inducing antiviral immunity. *Nat Commun* 9(1)**,** 3199. doi: 10.1038/s41467-018-05745-w.

Liu, G., Park, H.-S., Pyo, H.-M., Liu, Q., and Zhou, Y. (2015). Influenza A virus panhandle structure is directly involved in RIG-I activation and interferon induction. *Journal of Virology* 89(11)**,** 6067. doi: 10.1128/JVI.00232-15.

Liu, G., and Zhou, Y. (2019). Cytoplasm and beyond: dynamic innate immune sensing of influenza A virus by RIG-I. *Journal of Virology* 93(8)**,** e02299-02218. doi: 10.1128/JVI.02299-18.

Liu, J., and McFadden, G. (2014). SAMD9 is an innate antiviral host factor with stress response properties that can be antagonized by poxviruses. *Journal of Virology***,** JVI.02262-02214. doi: 10.1128/JVI.02262-14.

Liu, J., Wei, L., Jiang, T., Shi, L., and Wang, J. (2007). Reduction of infectious bursal disease virus replication in cultured cells by proteasome inhibitors. *Virus Genes* 35(3)**,** 719-727. doi: 10.1007/s11262-007-0147-3.

Liu, R., Shi, S., Huang, Y., Chen, Z., Chen, C., Cheng, L., et al. (2019b. Comparative pathogenicity of different subtypes of duck hepatitis A virus in Pekin ducklings. *Vet Microbiol* 228**,** 181-187. doi: 10.1016/j.vetmic.2018.11.030.

Liu, S., Cai, X., Wu, J., Cong, Q., Chen, X., Li, T., et al. (2015). Phosphorylation of innate immune adaptor proteins MAVS, STING, and TRIF induces IRF3 activation. *Science* 347(6227)**,** aaa2630. doi: 10.1126/science.aaa2630.

Liu, W.J., Yang, Y.T., Huang, Y.M., Zhou, D.R., Xu, D.N., Cao, N., et al. (2018). Identification of goose PKR Gene: structure, expression profiling, and antiviral activity against Newcastle disease virus. *J Interferon Cytokine Res* 38(8)**,** 333-340. doi: 10.1089/jir.2018.0025.

Liu, Y., Cheng, Y., Shan, W., Ma, J., Wang, H., Sun, J., et al. (2018). Chicken interferon regulatory factor 1 (IRF1) involved in antiviral innate immunity via regulating IFN-beta production. *Dev Comp Immunol* 88(June)**,** 77-82. doi: 10.1016/j.dci.2018.07.003.

Liu, Y., Lin, R., and Olagnier, D. (2017). RIGulation of STING expression: at the crossroads of viral RNA and DNA sensing pathways. *Inflamm Cell Signal* 4(1)**,** e1491. doi: 10.14800/ics.1491.

Liu, Y.J. (2005). IPC: professional type 1 interferon-producing cells and plasmacytoid dendritic cell precursors. *Annu Rev Immunol* 23(3)**,** 275-306. doi: 10.1146/annurev.immunol.23.021704.115633.

Liu, Z.-S., Zhang, Z.-Y., Cai, H., Zhao, M., Mao, J., Dai, J., et al. (2018). RINCK-mediated monoubiquitination of cGAS promotes antiviral innate immune responses. *Cell & Bioscience* 8(1)**,** 35. doi: 10.1186/s13578-018-0233-3.

Loedige, I., Gaidatzis, D., Sack, R., Meister, G., and Filipowicz, W. (2013). The mammalian TRIM-NHL protein TRIM71/LIN-41 is a repressor of mRNA function. *Nucleic Acids Res* 41(1)**,** 518-532. doi: 10.1093/nar/gks1032.

Londt, B.Z., Nunez, A., Banks, J., Alexander, D.J., Russell, C., Richard-Londt, A.C., et al. (2010). The effect of age on the pathogenesis of a highly pathogenic avian influenza (HPAI) H5N1 virus in Pekin ducks (Anas platyrhynchos) infected experimentally. *Influenza Other Respir Viruses* 4(1)**,** 17-25. doi: 10.1111/j.1750-2659.2009.00116.x.

Loo, Y.-M., and Gale, M., Jr. (2011). Immune signaling by RIG-I-like receptors. *Immunity* 34(5)**,** 680-692. doi: 10.1016/j.immuni.2011.05.003.

Loye, J.E., and Zuk, M. (1991). "Bird-parasite interactions: ecology, evolution and behaviour," in *Bird-parasite interactions: ecology, evolution and behaviour*. Oxford University Press; Ornithology Series, 2).

Lu, C., Xing, Y., Cai, H., Shi, Y., Liu, J., and Huang, Y. (2019). Identification and analysis of long non-coding RNAs in response to H5N1 influenza viruses in duck (Anas platyrhynchos). *BMC Genomics* 20(1)**,** 36. doi: 10.1186/s12864-018-5422-2.

Lund, J.M., Alexopoulou, L., Sato, A., Karow, M., Adams, N.C., Gale, N.W., et al. (2004). Recognition of single-stranded RNA viruses by Toll-like receptor 7. *Proc Natl Acad Sci U S A* 101(15)**,** 5598-5603. doi: 10.1073/pnas.0400937101.

Ma, W., Brenner, D., Wang, Z., Dauber, B., Ehrhardt, C., Högner, K., et al. (2010). The NS segment of an H5N1 highly pathogenic avian influenza virus (HPAIV) is sufficient to alter replication efficiency, cell tropism, and host range of an H7N1 HPAIV. *Journal of virology* 84(4)**,** 2122-2133. doi: 10.1128/JVI.01668-09.

Ma, X., Yang, T., Luo, Y., Wu, L., Jiang, Y., Song, Z., et al. (2019). TRIM28 promotes HIV-1 latency by SUMOylating CDK9 and inhibiting P-TEFb. *Elife* 8. doi: 10.7554/eLife.42426.

MacDonald, M.R., Xia, J., Smith, A.L., and Magor, K.E. (2008). The duck toll like receptor 7: genomic organization, expression and function. *Mol Immunol* 45(7)**,** 2055-2061. doi: 10.1016/j.molimm.2007.10.018.

Magor, K.E., Miranzo Navarro, D., Barber, M.R., Petkau, K., Fleming-Canepa, X., Blyth, G.A., et al. (2013). Defense genes missing from the flight division. *Dev Comp Immunol* 41(3)**,** 377-388. doi: 10.1016/j.dci.2013.04.010.

Malkinson, M., Perk, K., and Weisman, Y. (1981). Reovirus infection of young Muscovy ducks (Cairina moschata). *Avian Pathol* 10(4)**,** 433-440. doi: 10.1080/03079458108418493.

Maller Schulman, B.R., Liang, X., Stahlhut, C., DelConte, C., Stefani, G., and Slack, F.J. (2008). The let-7 microRNA target gene, Mlin41/Trim71 is required for mouse embryonic survival and neural tube closure. *Cell Cycle* 7(24)**,** 3935-3942. doi: 10.4161/cc.7.24.7397.

Malur, M., Gale, M., Jr., and Krug, R.M. (2012). LGP2 downregulates interferon production during infection with seasonal human influenza A viruses that activate interferon regulatory factor 3. *J Virol* 86(19)**,** 10733-10738. doi: 10.1128/JVI.00510-12.

Mar, K.B., Rinkenberger, N.R., Boys, I.N., Eitson, J.L., McDougal, M.B., Richardson, R.B., et al. (2018). LY6E mediates an evolutionarily conserved enhancement of virus infection by targeting a late entry step. *Nature Communications* 9(1)**,** 3603. doi: 10.1038/s41467-018-06000-y.

Marín, I. (2012). Origin and diversification of TRIM ubiquitin ligases. *PLOS ONE* 7(11)**,** e50030. doi: 10.1371/journal.pone.0050030.

Martin, M. (2011). Cutadapt removes adapter sequences from high-throughput sequencing reads. *EMBnet.journal; Vol 17, No 1: Next Generation Sequencing Data Analysis*. doi: 10.14806/ej.17.1.200.

Masroori, N., Merindol, N., and Berthoux, L. (2016). The interferon-induced antiviral protein PML (TRIM19) promotes the restriction and transcriptional silencing of lentiviruses in a context-specific, isoform-specific fashion. *Retrovirology* 13(1)**,** 19. doi: 10.1186/s12977-016-0253-1.

Massiah, M.A. (2019). *Zinc-Binding B-Box Domains with RING Folds Serve Critical Roles in the Protein Ubiquitination Pathways in Plants and Animals.* London: IntechOpen.

Massiah, M.A., Simmons, B.N., Short, K.M., and Cox, T.C. (2006). Solution structure of the RBCC/TRIM B-box1 domain of human MID1: B-box with a RING. *J Mol Biol* 358(2)**,** 532-545. doi: 10.1016/j.jmb.2006.02.009.

Meng, J., Yao, Z., He, Y., Zhang, R., Zhang, Y., Yao, X., et al. (2017). ARRDC4 regulates enterovirus 71-induced innate immune response by promoting K63 polyubiquitination of MDA5 through TRIM65. *Cell Death Dis* 8(6)**,** e2866. doi: 10.1038/cddis.2017.257.

Meng, S., Yang, L., Xu, C., Qin, Z., Xu, H., Wang, Y., et al. (2011). Recombinant chicken interferon-alpha inhibits H9N2 avian influenza virus replication in vivo by oral administration. *J Interferon Cytokine Res* 31(7)**,** 533-538. doi: 10.1089/jir.2010.0123.

Meroni, G. (2012). Genomics and evolution of the TRIM gene family. *Adv Exp Med Biol* 770**,** 1-9. doi: 10.1007/978-1-4614-5398-7_1.

Metzger, M.B., Pruneda, J.N., Klevit, R.E., and Weissman, A.M. (2014). RING-type E3 ligases: Master manipulators of E2 ubiquitin-conjugating enzymes and ubiquitination. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research* 1843(1)**,** 47-60. doi: https://doi.org/10.1016/j.bbamcr.2013.05.026.

Meyer, M., Gaudieri, S., Rhodes, D.A., and Trowsdale, J. (2003). Cluster of TRIM genes in the human MHC class I region sharing the B30.2 domain. *Tissue Antigens* 61(1)**,** 63-71. doi: 10.1034/j.1399-0039.2003.610105.x.

Meyerson, N.R., Zhou, L., Guo, Y.R., Zhao, C., Tao, Y.J., Krug, R.M., et al. (2017). Nuclear TRIM25 specifically targets influenza virus ribonucleoproteins to block the onset of

RNA chain elongation. *Cell Host Microbe* 22(5)**,** 627-638 e627. doi: 10.1016/j.chom.2017.10.003.

Minh, B.Q., Nguyen, M.A.T., and von Haeseler, A. (2013). Ultrafast approximation for phylogenetic bootstrap. *Molecular Biology and Evolution* 30(5)**,** 1188-1195. doi: 10.1093/molbev/mst024.

Miranda, C., Roccato, E., Raho, G., Pagliardini, S., Pierotti, M.A., and Greco, A. (2006). The TFG protein, involved in oncogenic rearrangements, interacts with TANK and NEMO, two proteins involved in the NF-kappaB pathway. *J Cell Physiol* 208(1)**,** 154-160. doi: 10.1002/jcp.20644.

Miranzo-Navarro, D., and Magor, K.E. (2014). Activation of duck RIG-I by TRIM25 is independent of anchored ubiquitin. *PLoS One* 9(1)**,** e86968. doi: 10.1371/journal.pone.0086968.

Miyashita, M., Oshiumi, H., Matsumoto, M., and Seya, T. (2011). DDX60, a DEXD/H box helicase, is a novel antiviral factor promoting RIG-I-like receptor-mediated signaling. *Molecular and cellular biology* 31(18)**,** 3802-3819. doi: 10.1128/MCB.01368-10.

Mizushima, N. (2007). Autophagy: process and function. *Genes Dev* 21(22)**,** 2861-2873. doi: 10.1101/gad.1599207.

Mohan, T., Deng, L., and Wang, B.-Z. (2017). CCL28 chemokine: An anchoring point bridging innate and adaptive immunity. *International Immunopharmacology* 51**,** 165-170. doi: https://doi.org/10.1016/j.intimp.2017.08.012.

Morris, K.M., Hindle, M.M., Boitard, S., Burt, D.W., Danner, A.F., Eory, L., et al. (2020). The quail genome: insights into social behaviour, seasonal biology and infectious disease response. *BMC Biol* 18(1)**,** 14. doi: 10.1186/s12915-020-0743-4.

Muroi, M., and Tanamoto, K. (2008). TRAF6 distinctively mediates MyD88- and IRAK-1-induced activation of NF-kappaB. *J Leukoc Biol* 83(3)**,** 702-707. doi: 10.1189/jlb.0907629.

Nabi, G., Wang, Y., Lü, L., Jiang, C., Ahmad, S., Wu, Y., et al. (2021). Bats and birds as viral reservoirs: A physiological and ecological perspective. *Sci Total Environ* 754**,** 142372. doi: 10.1016/j.scitotenv.2020.142372.

Nanduri, S., Carpick, B.W., Yang, Y., Williams, B.R., and Qin, J. (1998). Structure of the double-stranded RNA-binding domain of the protein kinase PKR reveals the molecular

basis of its dsRNA-mediated activation. *EMBO J* 17(18)**,** 5458-5465. doi: 10.1093/emboj/17.18.5458.

Napolitano, L.M., and Meroni, G. (2012). TRIM family: Pleiotropy and diversification through homomultimer and heteromultimer formation. *IUBMB Life* 64(1)**,** 64-71. doi: 10.1002/iub.580.

Narayan, K., Waggoner, L., Pham, S.T., Hendricks, G.L., Waggoner, S.N., Conlon, J., et al. (2014). TRIM13 is a negative regulator of MDA5-mediated type I interferon production. *Journal of Virology* 88(18)**,** 10748. doi: 10.1128/JVI.02593-13.

Nathan, J.A., Kim, H.T., Ting, L., Gygi, S.P., and Goldberg, A.L. (2013). Why do cellular proteins linked to K63-polyubiquitin chains not associate with proteasomes? *Embo j* 32(4)**,** 552-565. doi: 10.1038/emboj.2012.354.

Nenasheva, V.V., Nikitenko, N.A., Stepanenko, E.A., Makarova, I.V., Andreeva, L.E., Kovaleva, G.V., et al. (2021). Human TRIM14 protects transgenic mice from influenza A viral infection without activation of other innate immunity pathways. *Genes & Immunity* 22(1)**,** 56-63. doi: 10.1038/s41435-021-00128-6.

Nerren, J.R., He, H., Genovese, K., and Kogut, M.H. (2010). Expression of the avian-specific toll-like receptor 15 in chicken heterophils is mediated by gram-negative and gram-positive bacteria, but not TLR agonists. *Veterinary immunology and immunopathology* 136(1-2)**,** 151-156. doi: 10.1016/j.vetimm.2010.02.017.

Nguyen, L.-T., Schmidt, H.A., von Haeseler, A., and Minh, B.Q. (2014). IQ-TREE: A fast and effective stochastic algorithm for estimating maximum-likelihood phylogenies. *Molecular Biology and Evolution* 32(1)**,** 268-274. doi: 10.1093/molbev/msu300.

Nisole, S., Maroui, M.A., Mascle, X., Aubry, M., and Chelbi-Alix, M. (2013). Differential roles of PML isoforms. *Frontiers in Oncology* 3. doi: 10.3389/fonc.2013.00125.

Nisole, S., Stoye, J.P., and Saib, A. (2005). TRIM family proteins: retroviral restriction and antiviral defence. *Nat Rev Microbiol* 3(10)**,** 799-808. doi: 10.1038/nrmicro1248.

Niu, Y., Ma, H., Ding, Y., Li, Z., Sun, Y., Li, M., et al. (2019). The pathogenicity of duck hepatitis A virus types 1 and 3 on ducklings. *Poultry Science* 98(12)**,** 6333-6339. doi: https://doi.org/10.3382/ps/pez455.

Niyonzima, N., Freeley, S., Arbore, G., Le Friec, G., Lappegård, K.T., Mollnes, T.E., et al. (2017). The intracellular C5 system is critical to DAMP sensing and cellular responses in human monocytes. *The Journal of Immunology* 198(1 Supplement)**,** 75.78.

Noguchi, K., Okumura, F., Takahashi, N., Kataoka, A., Kamiyama, T., Todo, S., et al. (2011). TRIM40 promotes neddylation of IKKγ and is downregulated in gastrointestinal cancers. *Carcinogenesis* 32(7)**,** 995-1004. doi: 10.1093/carcin/bgr068.

Nooroong, P., Watthanadirek, A., Minsakorn, S., Poolsawat, N., Junsiri, W., Srionrod, N., et al. (2022). Molecular genetic diversity and bioinformatic analysis of Leucocytozoon sabrazesi based on the mitochondrial genes cytb, coxI and coxIII and co-infection of Plasmodium spp. *Parasite* 29**,** 22. doi: 10.1051/parasite/2022022.

Okamoto, M., Tsukamoto, H., Kouwaki, T., Seya, T., and Oshiumi, H. (2017). Recognition of viral RNA by pattern recognition receptors in the induction of innate immunity and excessive inflammation during respiratory viral infections. *Viral Immunol* 30(6)**,** 408-420. doi: 10.1089/vim.2016.0178.

Oliveros, J.C. (2007). *An interactive tool for comparing lists with Venn's diagrams.* [Online]. Available: https://bioinfogp.cnb.csic.es/tools/venny/index.html [Accessed 2022].

Olsen, B., Munster, V.J., Wallensten, A., Waldenstrom, J., Osterhaus, A.D., and Fouchier, R.A. (2006). Global patterns of influenza a virus in wild birds. *Science* 312(5772)**,** 384-388. doi: 10.1126/science.1122438.

Onoguchi, K., Yoneyama, M., Takemura, A., Akira, S., Taniguchi, T., Namiki, H., et al. (2007). Viral infections activate types I and III interferon genes through a common mechanism. *J Biol Chem* 282(10)**,** 7576-7581. doi: 10.1074/jbc.M608618200.

Opitz, B., Rejaibi, A., Dauber, B., Eckhard, J., Vinzing, M., Schmeck, B., et al. (2007). IFNβ induction by influenza A virus is mediated by RIG-I which is regulated by the viral NS1 protein. *Cellular Microbiology* 9(4)**,** 930-938. doi: 10.1111/j.1462-5822.2006.00841.x.

Oshiumi, H. (2020). Recent advances and contradictions in the study of the individual roles of ubiquitin ligases that regulate RIG-I-like receptor-mediated antiviral innate immune responses. *Front Immunol* 11**,** 1296. doi: 10.3389/fimmu.2020.01296.

Oshiumi, H., Matsumoto, M., Hatakeyama, S., and Seya, T. (2009). Riplet/RNF135, a RING finger protein, ubiquitinates RIG-I to promote interferon-β induction during the early

phase of viral infection. *Journal of Biological Chemistry* 284(2)**,** 807-817. doi: https://doi.org/10.1074/jbc.M804259200.

Oshiumi, H., Miyashita, M., Inoue, N., Okabe, M., Matsumoto, M., and Seya, T. (2010). The ubiquitin ligase Riplet is essential for RIG-I-dependent innate immune responses to RNA virus infection. *Cell Host Microbe* 8(6)**,** 496-509. doi: 10.1016/j.chom.2010.11.008.

Oshiumi, H., Miyashita, M., Matsumoto, M., and Seya, T. (2013). A distinct role of Riplet-mediated K63-Linked polyubiquitination of the RIG-I repressor domain in human antiviral innate immune responses. *PLoS Pathog* 9(8)**,** e1003533. doi: 10.1371/journal.ppat.1003533.

Oshiumi, H., Miyashita, M., Okamoto, M., Morioka, Y., Okabe, M., Matsumoto, M., et al. (2015). DDX60 Is Involved in RIG-I-Dependent and Independent Antiviral Responses, and Its Function Is Attenuated by Virus-Induced EGFR Activation. *Cell Rep* 11(8)**,** 1193-1207. doi: 10.1016/j.celrep.2015.04.047.

Oven, I., Resman Rus, K., Dušanić, D., Benčina, D., Keeler, C.L., and Narat, M. (2013). Diacylated lipopeptide from Mycoplasma synoviae mediates TLR15 induced innate immune responses. *Veterinary Research* 44(1)**,** 99. doi: 10.1186/1297-9716-44-99.

Ozato, K., Shin, D.M., Chang, T.H., and Morse, H.C., 3rd (2008). TRIM family proteins and their emerging roles in innate immunity. *Nat Rev Immunol* 8(11)**,** 849-860. doi: 10.1038/nri2413.

Pal, K., and Grover, P.L. (1983). A simple method for the removal of contaminating fibroblasts from cultures of rat mammary epithelial cells. *Cell Biol Int Rep* 7(10)**,** 779-783. doi: 10.1016/0309-1651(83)90181-9.

Pan, C., Zhao, H., Shen, L., and Sheng, J. (2011). Molecular characterization and expression pattern of tripartite motif protein 39 in Gallus gallus with a complete PRY/SPRY domain. *International journal of molecular sciences* 12(6)**,** 3797-3809. doi: 10.3390/ijms12063797.

Pang, I.K., Pillai, P.S., and Iwasaki, A. (2013). Efficient influenza A virus replication in the respiratory tract requires signals from TLR7 and RIG-I. *Proceedings of the National Academy of Sciences***,** 201303275. doi: 10.1073/pnas.1303275110.

Pantin-Jackwood, M., Swayne, D.E., Smith, D., and Shepherd, E. (2013). Effect of species, breed and route of virus inoculation on the pathogenicity of H5N1 highly pathogenic

influenza (HPAI) viruses in domestic ducks. *Vet Res* 44(1)**,** 62. doi: 10.1186/1297-9716-44-62.

Pantin-Jackwood, M.J., Smith, D.M., Wasilenko, J.L., Cagle, C., Shepherd, E., Sarmento, L., et al. (2012). Effect of age on the pathogenesis and innate immune responses in Pekin ducks infected with different H5N1 highly pathogenic avian influenza viruses. *Virus Research* 167(2)**,** 196-206. doi: https://doi.org/10.1016/j.virusres.2012.04.015.

Papp, Z., Clark, R.G., Parmley, E.J., Leighton, F.A., Waldner, C., and Soos, C. (2017). The ecology of avian influenza viruses in wild dabbling ducks (*Anas spp*.) in Canada. *PLoS One* 12(5)**,** e0176297. doi: 10.1371/journal.pone.0176297.

Park, H.H. (2018). Structure of TRAF Family: Current understanding of receptor recognition. *Frontiers in Immunology* 9.

Parmley, E.J., Bastien, N., Booth, T.F., Bowes, V., Buck, P.A., Breault, A., et al. (2008). Wild bird influenza survey, Canada, 2005. *Emerg Infect Dis* 14(1)**,** 84-87. doi: 10.3201/eid1401.061562.

Patil, G., Xu, L., Wu, Y., Song, K., Hao, W., Hua, F., et al. (2020). TRIM41-mediated ubiquitination of nucleoprotein limits vesicular stomatitis virus infection. *Viruses* 12(2). doi: 10.3390/v12020131.

Patil, G., Zhao, M., Song, K., Hao, W., Bouchereau, D., Wang, L., et al. (2018). TRIM41-mediated ubiquitination of nucleoprotein limits influenza A virus infection. *J Virol* 92(16). doi: 10.1128/JVI.00905-18.

Peisley, A., Wu, B., Xu, H., Chen, Z.J., and Hur, S. (2014). Structural basis for ubiquitin-mediated antiviral signal activation by RIG-I. *Nature* 509(7498)**,** 110-114. doi: 10.1038/nature13140.

Peng, C., Zhao, C., Wang, P.F., Yan, L.L., Fan, S.G., and Qiu, L.H. (2021). Identification of a TRIM32 from *Penaeus monodon* is involved in autophagy and innate immunity during white spot syndrome virus infection. *Dev Comp Immunol* 123**,** 104169. doi: 10.1016/j.dci.2021.104169.

Peng, X., Wen, Y., Zha, L., Zhuang, J., Lin, L., Li, X., et al. (2020). TRIM45 suppresses the development of non-small cell lung cancer. *Curr Mol Med* 20(4)**,** 299-306. doi: 10.2174/1566524019666191017143833.

Perera, P.-Y., Lichy, J.H., Waldmann, T.A., and Perera, L.P. (2012). The role of interleukin-15 in inflammation and immune responses to infection: implications for its therapeutic use. *Microbes and infection* 14(3)**,** 247-261. doi: 10.1016/j.micinf.2011.10.006.

Perera, S., Mankoo, B., and Gautel, M. (2012). Developmental regulation of MURF E3 ubiquitin ligases in skeletal muscle. *J Muscle Res Cell Motil* 33(2)**,** 107-122. doi: 10.1007/s10974-012-9288-7.

Pérez-Rodríguez, A., de la Puente, J., Onrubia, A., and Pérez-Tris, J. (2013). Molecular characterization of haemosporidian parasites from kites of the genus Milvus (Aves: Accipitridae). *International Journal for Parasitology* 43(5)**,** 381-387. doi: https://doi.org/10.1016/j.ijpara.2012.12.007.

Perlas, A., Argilaguet, J., Bertran, K., Sánchez-González, R., Nofrarías, M., Valle, R., et al. (2021). Dual host and pathogen RNA-seq analysis unravels chicken genes potentially involved in resistance to highly pathogenic avian influenza virus infection. *Frontiers in Immunology* 12.

Peterson, A.T., Vieglais, D.A., and Andreasen, J.K. (2003). Migratory birds modeled as critical transport agents for West Nile Virus in North America. *Vector Borne Zoonotic Dis* 3(1)**,** 27-37. doi: 10.1089/153036603765627433.

Philbin, V.J., Iqbal, M., Boyd, Y., Goodchild, M.J., Beal, R.K., Bumstead, N., et al. (2005). Identification and characterization of a functional, alternatively spliced Toll-like receptor 7 (TLR7) and genomic disruption of TLR8 in chickens. *Immunology* 114(4)**,** 507-521. doi: 10.1111/j.1365-2567.2005.02125.x.

Phuong do, Q., Dung, N.T., Jorgensen, P.H., Handberg, K.J., Vinh, N.T., and Christensen, J.P. (2011). Susceptibility of Muscovy (*Cairina Moschata*) and mallard ducks (*Anas Platyrhynchos*) to experimental infections by different genotypes of H5N1 avian influenza viruses. *Vet Microbiol* 148(2-4)**,** 168-174. doi: 10.1016/j.vetmic.2010.09.007.

Pichlmair, A., Schulz, O., Tan, C.P., Näslund, T.I., Liljeström, P., Weber, F., et al. (2006). RIG-I-mediated antiviral responses to single-stranded RNA bearing 5'-phosphates. *Science (New York, N.Y.)* 314(5801)**,** 997-1001. doi: 10.1126/science.1132998.

Pippig, D.A., Hellmuth, J.C., Cui, S., Kirchhofer, A., Lammens, K., Lammens, A., et al. (2009). The regulatory domain of the RIG-I family ATPase LGP2 senses double-stranded RNA. *Nucleic Acids Res* 37(6)**,** 2014-2025. doi: 10.1093/nar/gkp059.

Pirhonen, J., Sareneva, T., Julkunen, I., and Matikainen, S. (2001). Virus infection induces proteolytic processing of IL-18 in human macrophages via caspase-1 and caspase-3 activation. *Eur J Immunol* 31(3)**,** 726-733. doi: 10.1002/1521-4141(200103)31:3<726::aid-immu726>3.0.co;2-5.

Plank, C., Zelphati, O., and Mykhaylyk, O. (2011). Magnetically enhanced nucleic acid delivery. Ten years of magnetofection-progress and prospects. *Adv Drug Deliv Rev* 63(14-15)**,** 1300-1331. doi: 10.1016/j.addr.2011.08.002.

Poole, E., Groves, I., MacDonald, A., Pang, Y., Alcami, A., and Sinclair, J. (2009). Identification of TRIM23 as a cofactor involved in the regulation of NF-kappaB by human cytomegalovirus. *J Virol* 83(8)**,** 3581-3590. doi: 10.1128/jvi.02072-08.

Pothlichet, J., Meunier, I., Davis, B.K., Ting, J.P., Skamene, E., von Messling, V., et al. (2013). Type I IFN triggers RIG-I/TLR3/NLRP3-dependent inflammasome activation in influenza A virus infected cells. *PLoS Pathog* 9(4)**,** e1003256. doi: 10.1371/journal.ppat.1003256.

Prabhu, N., Ho, A.W., Wong, K.H., Hutchinson, P.E., Chua, Y.L., Kandasamy, M., et al. (2013). Gamma interferon regulates contraction of the influenza virus-specific CD8 T cell response and limits the size of the memory population. *J Virol* 87(23)**,** 12510-12522. doi: 10.1128/JVI.01776-13.

Prakash, A., and Levy, D.E. (2006). Regulation of IRF7 through cell type-specific protein stability. *Biochem Biophys Res Commun* 342(1)**,** 50-56. doi: 10.1016/j.bbrc.2006.01.122.

Price, P.J.R., Bánki, Z., Scheideler, A., Stoiber, H., Verschoor, A., Sutter, G., et al. (2015). Complement component C5 recruits neutrophils in the absence of C3 during respiratory infection with modified vaccinia virus Ankara. *The Journal of Immunology* 194(3)**,** 1164. doi: 10.4049/jimmunol.1301410.

Proost, P., De Wolf-Peeters, C., Conings, R., Opdenakker, G., Billiau, A., and Van Damme, J. (1993). Identification of a novel granulocyte chemotactic protein (GCP-2) from human tumor cells. In vitro and in vivo comparison with natural forms of GRO, IP-10, and IL-8. *The Journal of Immunology* 150(3)**,** 1000.

Qi, H., Xia, F.-N., Xiao, S., and Li, J. (2022). TRAF proteins as key regulators of plant development and stress responses. *Journal of Integrative Plant Biology* 64(2)**,** 431-448. doi: https://doi.org/10.1111/jipb.13182.

Qian, W., Wei, X., Li, Y., Guo, K., Zou, Z., Zhou, H., et al. (2018). Duck interferon regulatory factor 1 acts as a positive regulator in duck innate antiviral response. *Dev Comp Immunol* 78**,** 1-13. doi: 10.1016/j.dci.2017.09.004.

Qiao, Y., Zhou, Y., Song, C., Zhang, X., and Zou, Y. (2020). MID1 and MID2 regulate cell migration and epithelial-mesenchymal transition via modulating Wnt/β-catenin signaling. *Ann Transl Med* 8(16)**,** 1021. doi: 10.21037/atm-20-5583.

Qin, B., Xiao, T., Ding, C., Deng, Y., Lv, Z., and Su, J. (2021). Genome-wide identification and expression analysis of potential antiviral tripartite motif proteins (TRIMs) in grass carp (Ctenopharyngodon idella). *Biology (Basel)* 10(12). doi: 10.3390/biology10121252.

Qin, Y., Liu, Q., Tian, S., Xie, W., Cui, J., and Wang, R.F. (2016). TRIM9 short isoform preferentially promotes DNA and RNA virus-induced production of type I interferon by recruiting GSK3β to TBK1. *Cell Res* 26(5)**,** 613-628. doi: 10.1038/cr.2016.27.

Qiu, Y., Shen, Y., Li, X., Ding, C., and Ma, Z. (2008). Molecular cloning and functional characterization of a novel isoform of chicken myeloid differentiation factor 88 (MyD88). *Developmental and Comparative Immunology* 32(12)**,** 1522-1530. doi: 10.1016/j.dci.2008.05.016.

Quimby, B.B., Yong-Gonzalez, V., Anan, T., Strunnikov, A.V., and Dasso, M. (2006). The promyelocytic leukemia protein stimulates SUMO conjugation in yeast. *Oncogene* 25(21)**,** 2999-3005. doi: 10.1038/sj.onc.1209335.

Rahman, S.A., Hassan, S.S., Olival, K.J., Mohamed, M., Chang, L.Y., Hassan, L., et al. (2010). Characterization of Nipah virus from naturally infected Pteropus vampyrus bats, Malaysia. *Emerg Infect Dis* 16(12)**,** 1990-1993. doi: 10.3201/eid1612.091790.

Rajsbaum, R., Albrecht, R.A., Wang, M.K., Maharaj, N.P., Versteeg, G.A., Nistal-Villán, E., et al. (2012). Species-specific inhibition of RIG-I ubiquitination and IFN induction by the influenza A virus NS1 protein. *PLOS Pathogens* 8(11)**,** e1003059. doi: 10.1371/journal.ppat.1003059.

Rajsbaum, R., Garcia-Sastre, A., and Versteeg, G.A. (2014). TRIMmunity: the roles of the TRIM E3-ubiquitin ligase family in innate antiviral immunity. *J Mol Biol* 426(6)**,** 1265-1284. doi: 10.1016/j.jmb.2013.12.005.

Rajsbaum, R., Stoye, J.P., and O'Garra, A. (2008). Type I interferon-dependent and -independent expression of tripartite motif proteins in immune cells. *Eur J Immunol* 38(3)**,** 619-630. doi: 10.1002/eji.200737916.

Ran, J.S., Jin, J., Zhang, X.X., Wang, Y., Ren, P., Li, J.J., et al. (2018). Molecular characterization, expression and functional analysis of chicken STING. *Int J Mol Sci* 19(12)**,** 1-19. doi: 10.3390/ijms19123706.

Ran, Y., Zhang, J., Liu, L.L., Pan, Z.Y., Nie, Y., Zhang, H.Y., et al. (2016). Autoubiquitination of TRIM26 links TBK1 to NEMO in RLR-mediated innate antiviral immune response. *J Mol Cell Biol* 8(1)**,** 31-43. doi: 10.1093/jmcb/mjv068.

Ranaware, P.B., Mishra, A., Vijayakumar, P., Gandhale, P.N., Kumar, H., Kulkarni, D.D., et al. (2016). Genome wide host gene expression analysis in chicken lungs infected with avian influenza viruses. *PLoS One* 11(4)**,** e0153671. doi: 10.1371/journal.pone.0153671.

Rappole, J.H., Derrickson, S.R., and Hubálek, Z. (2000). Migratory birds and spread of West Nile virus in the Western Hemisphere. *Emerg Infect Dis* 6(4)**,** 319-328. doi: 10.3201/eid0604.000401.

Ravelomanantsoa, N.A.F., Guth, S., Andrianiaina, A., Andry, S., Gentles, A., Ranaivoson, H.C., et al. (2020). The zoonotic potential of bat-borne coronaviruses. *Emerging Topics in Life Sciences* 4(4)**,** 365-381. doi: 10.1042/ETLS20200097.

Reed, K.D., Meece, J.K., Henkel, J.S., and Shukla, S.K. (2003). Birds, migration and emerging zoonoses: west nile virus, lyme disease, influenza A and enteropathogens. *Clin Med Res* 1(1)**,** 5-12. doi: 10.3121/cmr.1.1.5.

Reuter, A., Soubies, S., Härtle, S., Schusser, B., Kaspers, B., Staeheli, P., et al. (2014). Antiviral activity of lambda interferon in chickens. *Journal of Virology* 88(5)**,** 2835. doi: 10.1128/JVI.02764-13.

Reymond, A., Meroni, G., Fantozzi, A., Merla, G., Cairo, S., Luzi, L., et al. (2001). Reymond et al. - 2001 - The tripartite motif family identifies cell compartments. 20(9).

Reynes, J.M., Counor, D., Ong, S., Faure, C., Seng, V., Molia, S., et al. (2005). Nipah virus in Lyle's flying foxes, Cambodia. *Emerg Infect Dis* 11(7)**,** 1042-1047. doi: 10.3201/eid1107.041350.

Rice, P., Longden, I., and Bleasby, A. (2000). EMBOSS: the European Molecular Biology Open Software Suite. *Trends Genet* 16(6)**,** 276-277. doi: 10.1016/s0168-9525(00)02024-2.

Robinson, M.D., McCarthy, D.J., and Smyth, G.K. (2010). edgeR: a Bioconductor package for differential expression analysis of digital gene expression data. *Bioinformatics* 26(1)**,** 139-140. doi: 10.1093/bioinformatics/btp616.

Roebroek, A.J.M., Gordts, P.L.S.M., and Reekmans, S. (2011). "Knock-In Approaches," in *Transgenic Mouse Methods and Protocols,* eds. M.H. Hofker & J. van Deursen. (Totowa, NJ: Humana Press), 257-275.

Rohaim, M.A., Santhakumar, D., Naggar, R.F.E., Iqbal, M., Hussein, H.A., and Munir, M. (2018). Chickens Expressing IFIT5 Ameliorate Clinical Outcome and Pathology of Highly Pathogenic Avian Influenza and Velogenic Newcastle Disease Viruses. *Front Immunol* 9(SEP)**,** 2025. doi: 10.3389/fimmu.2018.02025.

Rohde, F., Schusser, B., Hron, T., Farkasova, H., Plachy, J., Hartle, S., et al. (2018). Characterization of chicken tumor necrosis factor-alpha, a long missed cytokine in birds. *Front Immunol* 9(APR)**,** 605. doi: 10.3389/fimmu.2018.00605.

Rom, O., and Reznick, A.Z. (2016). The role of E3 ubiquitin-ligases MuRF-1 and MAFbx in loss of skeletal muscle mass. *Free Radic Biol Med* 98**,** 218-230. doi: 10.1016/j.freeradbiomed.2015.12.031.

Rong, E., Hu, J., Yang, C., Chen, H., Wang, Z., Liu, X., et al. (2018). Broad-spectrum antiviral functions of duck interferon-induced protein with tetratricopeptide repeats (AvIFIT). *Dev Comp Immunol* 84**,** 71-81. doi: 10.1016/j.dci.2018.02.007.

Rong, E., Wang, X., Chen, H., Yang, C., Hu, J., Liu, W., et al. (2018). Molecular mechanisms for the adaptive switching between the OAS/RNase L and OASL/RIG-I pathways in birds and mammals. *Front Immunol* 9(1398)**,** 1398. doi: 10.3389/fimmu.2018.01398.

Rose, K.M., Spada, S.J., Broeckel, R., McNally, K.L., Hirsch, V.M., Best, S.M., et al. (2021). From capsids to complexes: Expanding the role of TRIM5α in the restriction of divergent RNA viruses and elements. *Viruses* 13(3). doi: 10.3390/v13030446.

Rose, R., Golosova, O., Sukhomlinov, D., Tiunov, A., and Prosperi, M. (2019). Flexible design of multiple metagenomics classification pipelines with UGENE. *Bioinformatics* 35(11)**,** 1963-1965. doi: 10.1093/bioinformatics/bty901.

Rothenfusser, S., Goutagny, N., DiPerna, G., Gong, M., Monks, B.G., Schoenemeyer, A., et al. (2005). The RNA helicase Lgp2 inhibits TLR-independent sensing of viral replication by

retinoic acid-inducible gene-I. *J Immunol* 175(8)**,** 5260-5268. doi: 10.4049/jimmunol.175.8.5260.

RStudio Team (2020). "RStudio: Integrated Development Environment for R". (Boston, MA: RStudio, Inc.).

Ruby, T., Bed'Hom, B., Wittzell, H., Morin, V., Oudin, A., and Zoorob, R. (2005). Characterisation of a cluster of TRIM-B30.2 genes in the chicken MHC B locus. *Immunogenetics* 57(1-2)**,** 116-128. doi: 10.1007/s00251-005-0770-x.

Runstadler, J.A., Happ, G.M., Slemons, R.D., Sheng, Z.M., Gundlach, N., Petrula, M., et al. (2007). Using RRT-PCR analysis and virus isolation to determine the prevalence of avian influenza virus infections in ducks at Minto Flats State Game Refuge, Alaska, during August 2005. *Arch Virol* 152(10)**,** 1901-1910. doi: 10.1007/s00705-007-0994-1.

Saito, L.B., Diaz-Satizabal, L., Evseev, D., Fleming-Canepa, X., Mao, S., Webster, R.G., et al. (2018). IFN and cytokine responses in ducks to genetically similar H5N1 influenza A viruses of varying pathogenicity. *J Gen Virol* 99(4)**,** 464-474. doi: 10.1099/jgv.0.001015.

Salomon, R., Franks, J., Govorkova, E.A., Ilyushina, N.A., Yen, H.-L., Hulse-Post, D.J., et al. (2006). The polymerase complex genes contribute to the high virulence of the human H5N1 influenza virus isolate A/Vietnam/1203/04. *The Journal of experimental medicine* 203(3)**,** 689-697. doi: 10.1084/jem.20051938.

Samukawa, S., Yoshimi, R., Kirino, Y., and Nakajima, H. (2021). The PRY/SPRY domain of pyrin/TRIM20 interacts with β(2)-microglobulin to promote inflammasome formation. *Sci Rep* 11(1)**,** 23613. doi: 10.1038/s41598-021-03073-6.

Sanchez, J.G., Okreglicka, K., Chandrasekaran, V., Welker, J.M., Sundquist, W.I., and Pornillos, O. (2014). The tripartite motif coiled-coil is an elongated antiparallel hairpin dimer. *Proceedings of the National Academy of Sciences of the United States of America* 111(7)**,** 2494-2499. doi: 10.1073/pnas.1318962111.

Santhakumar, D., Rohaim, M., Hussein, H.A., Hawes, P., Ferreira, H.L., Behboudi, S., et al. (2018). Chicken interferon-induced protein with tetratricopeptide repeats 5 antagonizes replication of RNA viruses. *Sci Rep* 8(1)**,** 6794. doi: 10.1038/s41598-018-24905-y.

Santhakumar, D., Rubbenstroth, D., Martinez-Sobrido, L., and Munir, M. (2017). Avian interferons and their antiviral effectors. *Front Immunol* 8(JAN)**,** 49. doi: 10.3389/fimmu.2017.00049.

Sardiello, M., Cairo, S., Fontanella, B., Ballabio, A., and Meroni, G. (2008). Genomic analysis of the TRIM family reveals two groups of genes with distinct evolutionary properties. *BMC Evol Biol* 8(1)**,** 225. doi: 10.1186/1471-2148-8-225.

Sarkar, S.N., Bandyopadhyay, S., Ghosh, A., and Sen, G.C. (1999). Enzymatic characteristics of recombinant medium isozyme of 2'-5' oligoadenylate synthetase. *J Biol Chem* 274(3)**,** 1848-1855. doi: 10.1074/jbc.274.3.1848.

Sarkar, S.N., Ghosh, A., Wang, H.W., Sung, S.S., and Sen, G.C. (1999). The nature of the catalytic domain of 2'-5'-oligoadenylate synthetases. *J Biol Chem* 274(36)**,** 25535-25542. doi: 10.1074/jbc.274.36.25535.

Sato, M., Hata, N., Asagiri, M., Nakaya, T., Taniguchi, T., and Tanaka, N. (1998). Positive feedback regulation of type I IFN genes by the IFN-inducible transcription factor IRF-7. *FEBS Lett* 441(1)**,** 106-110. doi: 10.1016/s0014-5793(98)01514-2.

Sato, S., Sugiyama, M., Yamamoto, M., Watanabe, Y., Kawai, T., Takeda, K., et al. (2003). Toll/IL-1 receptor domain-containing adaptor inducing IFN-beta (TRIF) associates with TNF receptor-associated factor 6 and TANK-binding kinase 1, and activates two distinct transcription factors, NF-kappa B and IFN-regulatory factor-3, in the Toll-like receptor signaling. *J Immunol* 171(8)**,** 4304-4310. doi: 10.4049/jimmunol.171.8.4304.

Satoh, T., Kato, H., Kumagai, Y., Yoneyama, M., Sato, S., Matsushita, K., et al. (2010). LGP2 is a positive regulator of RIG-I- and MDA5-mediated antiviral responses. *Proceedings of the National Academy of Sciences of the United States of America* 107(4)**,** 1512-1517. doi: 10.1073/pnas.0912986107.

Sawyer, S.L., Emerman, M., and Malik, H.S. (2007). Discordant evolution of the adjacent antiretroviral genes TRIM22 and TRIM5 in mammals. *PLOS Pathogens* 3(12)**,** e197. doi: 10.1371/journal.ppat.0030197.

Sawyer, S.L., Wu, L.I., Emerman, M., and Malik, H.S. (2005). Positive selection of primate TRIM5alpha identifies a critical species-specific retroviral restriction domain. *Proc Natl Acad Sci U S A* 102(8)**,** 2832-2837. doi: 10.1073/pnas.0409853102.

Scherer, M., Schilling, E.M., and Stamminger, T. (2017). The Human CMV IE1 protein: An offender of PML nuclear bodies. *Adv Anat Embryol Cell Biol* 223**,** 77-94. doi: 10.1007/978-3-319-53168-7_4.

Schmidt, A., Schwerd, T., Hamm, W., Hellmuth, J.C., Cui, S., Wenzel, M., et al. (2009). 5'-triphosphate RNA requires base-paired structures to activate antiviral signaling via RIG-I. *Proceedings of the National Academy of Sciences of the United States of America* 106(29)**,** 12067-12072. doi: 10.1073/pnas.0900971106.

Schroder, K., Hertzog, P.J., Ravasi, T., and Hume, D.A. (2004). Interferon-gamma: an overview of signals, mechanisms and functions. *J Leukoc Biol* 75(2)**,** 163-189. doi: 10.1189/jlb.0603252.

Schusser, B., Reuter, A., von der Malsburg, A., Penski, N., Weigend, S., Kaspers, B., et al. (2011). Mx is dispensable for interferon-mediated resistance of chicken cells against influenza A virus. *J Virol* 85(16)**,** 8307-8315. doi: 10.1128/JVI.00535-11.

Seo, S.U., Kwon, H.J., Song, J.H., Byun, Y.H., Seong, B.L., Kawai, T., et al. (2010). MyD88 signaling is indispensable for primary influenza A virus infection but dispensable for secondary infection. *J Virol* 84(24)**,** 12713-12722. doi: 10.1128/JVI.01675-10.

Shannon, P., Markiel, A., Ozier, O., Baliga, N.S., Wang, J.T., Ramage, D., et al. (2003). Cytoscape: a software environment for integrated models of biomolecular interaction networks. *Genome Res* 13(11)**,** 2498-2504. doi: 10.1101/gr.1239303.

Shapira, S.D., Gat-Viks, I., Shum, B.O., Dricot, A., de Grace, M.M., Wu, L., et al. (2009). A physical and regulatory map of host-influenza interactions reveals pathways in H1N1 infection. *Cell* 139(7)**,** 1255-1267. doi: 10.1016/j.cell.2009.12.018.

Sharma, V., Verma, S., Seranova, E., Sarkar, S., and Kumar, D. (2018). Selective autophagy and xenophagy in infection and disease. *Frontiers in Cell and Developmental Biology* 6.

Shaw, M.L., and Stertz, S. (2018). Role of Host Genes in Influenza Virus Replication. *Curr Top Microbiol Immunol* 419**,** 151-189. doi: 10.1007/82_2017_30.

Shen, Y., Li, N.L., Wang, J., Liu, B., Lester, S., and Li, K. (2012). TRIM56 is an essential component of the TLR3 antiviral signaling pathway. *J Biol Chem* 287(43)**,** 36404-36413. doi: 10.1074/jbc.M112.397075.

Shi, M., Cho, H., Inn, K.S., Yang, A., Zhao, Z., Liang, Q., et al. (2014). Negative regulation of NF-κB activity by brain-specific TRIpartite Motif protein 9. *Nat Commun* 5**,** 4820. doi: 10.1038/ncomms5820.

Shi, W., and Gao, G.F. (2021). Emerging H5N8 avian influenza viruses. *Science* 372(6544)**,** 784. doi: 10.1126/science.abg6302.

Shi, Y., Hu, S., Duan, W., Ding, T., and Zhao, Z. (2019). The distinct evolutionary properties of the tripartite motif-containing protein 39 in the Chinese softshell turtle based on its structural and functional characterization. *Developmental & Comparative Immunology* 99**,** 103407. doi: https://doi.org/10.1016/j.dci.2019.103407.

Shiina, T., Briles, W.E., Goto, R.M., Hosomichi, K., Yanagiya, K., Shimizu, S., et al. (2007). Extended gene map reveals tripartite motif, C-type lectin, and Ig superfamily type genes within a subregion of the chicken MHC-B affecting infectious disease. *J Immunol* 178(11)**,** 7162-7172. doi: 10.4049/jimmunol.178.11.7162.

Shinya, K., Okamura, T., Sueta, S., Kasai, N., Tanaka, M., Ginting, T.E., et al. (2011). Toll-like receptor pre-stimulation protects mice against lethal infection with highly pathogenic influenza viruses. *Virology Journal* 8(1)**,** 97. doi: 10.1186/1743-422X-8-97.

Short, K.M., and Cox, T.C. (2006). Subclassification of the RBCC/TRIM superfamily reveals a novel motif necessary for microtubule binding. *Journal of Biological Chemistry* 281(13)**,** 8970-8980. doi: https://doi.org/10.1074/jbc.M512755200.

Sievers, F., Wilm, A., Dineen, D., Gibson, T.J., Karplus, K., Li, W., et al. (2011). Fast, scalable generation of high-quality protein multiple sequence alignments using Clustal Omega. *Molecular systems biology* 7**,** 539-539. doi: 10.1038/msb.2011.75.

Silverman, R.H., and Weiss, S.R. (2014). Viral phosphodiesterases that antagonize double-stranded RNA signaling to RNase L by degrading 2-5A. *J Interferon Cytokine Res* 34(6)**,** 455-463. doi: 10.1089/jir.2014.0007.

Simao, F.A., Waterhouse, R.M., Ioannidis, P., Kriventseva, E.V., and Zdobnov, E.M. (2015). BUSCO: assessing genome assembly and annotation completeness with single-copy orthologs. *Bioinformatics* 31(19)**,** 3210-3212. doi: 10.1093/bioinformatics/btv351.

Sivaramakrishnan, G., Sun, Y., Rajmohan, R., and Lin, V.C. (2009). B30.2/SPRY domain in tripartite motif-containing 22 is essential for the formation of distinct nuclear bodies. *FEBS Lett* 583(12)**,** 2093-2099. doi: 10.1016/j.febslet.2009.05.036.

Slowik, V., and Apte, U. (2017). Leukocyte cell-derived chemotaxin-2: It's role in pathophysiology and future in clinical medicine. *Clinical and translational science* 10(4)**,** 249-259. doi: 10.1111/cts.12469.

Smietanka, K., Minta, Z., Wyrostek, K., Jóźwiak, M., Olszewska, M., Domańska-Blicharz, A.K., et al. (2011). Susceptibility of pigeons to clade 1 and 2.2 high pathogenicity avian

influenza H5N1 virus. *Avian diseases* 55(1)**,** 106-112. doi: 10.1637/9514-090110-ResNote.1.

Smith, J., Smith, N., Yu, L., Paton, I.R., Gutowska, M.W., Forrest, H.L., et al. (2015). A comparative analysis of host responses to avian influenza infection in ducks and chickens highlights a role for the interferon-induced transmembrane proteins in viral resistance. *BMC Genomics* 16(1)**,** 574. doi: 10.1186/s12864-015-1778-8.

Smith, S.E., Gibson, M.S., Wash, R.S., Ferrara, F., Wright, E., Temperton, N., et al. (2013). Chicken interferon-inducible transmembrane protein 3 restricts influenza viruses and lyssaviruses in vitro. *J Virol* 87(23)**,** 12957-12966. doi: 10.1128/JVI.01443-13.

Sokawa, J., Shimizu, N., and Sokawa, Y. (1984). "Presence of (2•OE-5•OE)Oligoadenylate Synthetase in Avian Erythrocytes".

Sommereyns, C., Paul, S., Staeheli, P., and Michiels, T. (2008). IFN-lambda (IFN-lambda) is expressed in a tissue-dependent fashion and primarily acts on epithelial cells in vivo. *PLoS Pathog* 4(3)**,** e1000017. doi: 10.1371/journal.ppat.1000017.

Song, B., Javanbakht, H., Perron, M., Park, D.H., Stremlau, M., and Sodroski, J. (2005). Retrovirus restriction by TRIM5alpha variants from Old World and New World primates. *Journal of virology* 79(7)**,** 3930-3937. doi: 10.1128/JVI.79.7.3930-3937.2005.

Song, J., Feng, H., Xu, J., Zhao, D., Shi, J., Li, Y., et al. (2011). The PA protein directly contributes to the virulence of H5N1 avian influenza viruses in domestic ducks. *J Virol* 85(5)**,** 2180-2188. doi: 10.1128/jvi.01975-10.

Sparrer, K.M.J., Gableske, S., Zurenski, M.A., Parker, Z.M., Full, F., Baumgart, G.J., et al. (2017). TRIM23 mediates virus-induced autophagy via activation of TBK1. *Nat Microbiol* 2(11)**,** 1543-1557. doi: 10.1038/s41564-017-0017-2.

Spencer, J.A., Eliazer, S., Ilaria, R.L., Jr., Richardson, J.A., and Olson, E.N. (2000). Regulation of Microtubule Dynamics and Myogenic Differentiation by Murf, a Striated Muscle Ring-Finger Protein. *Journal of Cell Biology* 150(4)**,** 771-784. doi: 10.1083/jcb.150.4.771.

St. Paul, M., Mallick, A.I., Read, L.R., Villanueva, A.I., Parvizi, P., Abdul-Careem, M.F., et al. (2012). Prophylactic treatment with Toll-like receptor ligands enhances host immunity to avian influenza virus in chickens. *Vaccine* 30(30)**,** 4524-4531. doi: https://doi.org/10.1016/j.vaccine.2012.04.033.

Staeheli, P., Grob, R., Meier, E., Sutcliffe, J.G., and Haller, O. (1988). Influenza virus-susceptible mice carry Mx genes with a large deletion or a nonsense mutation. *Mol Cell Biol* 8(10)**,** 4518-4523. doi: 10.1128/mcb.8.10.4518.

Stewart, C.R., Bagnaud-Baule, A., Karpala, A.J., Lowther, S., Mohr, P.G., Wise, T.G., et al. (2012). Toll-like receptor 7 ligands inhibit influenza A infection in chickens. *J Interferon Cytokine Res* 32(1)**,** 46-51. doi: 10.1089/jir.2011.0036.

Stremlau, M., Owens, C.M., Perron, M.J., Kiessling, M., Autissier, P., and Sodroski, J. (2004). The cytoplasmic body component TRIM5alpha restricts HIV-1 infection in Old World monkeys. *Nature* 427(6977)**,** 848-853. doi: 10.1038/nature02343.

Sturm-Ramirez, K.M., Ellis, T., Bousfield, B., Bissett, L., Dyrting, K., Rehg, J.E., et al. (2004). Reemerging H5N1 influenza viruses in Hong Kong in 2002 are highly pathogenic to ducks. *Journal of virology* 78(9)**,** 4892-4901. doi: 10.1128/jvi.78.9.4892-4901.2004.

Sun, L., Dong, S., Ge, Y., Fonseca, J.P., Robinson, Z.T., Mysore, K.S., et al. (2019a). DiVenn: An interactive and integrated web-based visualization tool for comparing gene lists. *Frontiers in Genetics* 10(421). doi: 10.3389/fgene.2019.00421.

Sun, M., Li, S., Jin, S., Li, X., Xiang, J., and Li, F. (2022). A Novel TRIM9 protein promotes NF-κB activation through interacting with LvIMD in shrimp during WSSV infection. *Frontiers in Immunology* 13.

Sun, M., Li, S., Yu, K., Xiang, J., and Li, F. (2019b). An E3 ubiquitin ligase TRIM9 is involved in WSSV infection via interaction with β-TrCP. *Developmental & Comparative Immunology* 97**,** 57-63. doi: https://doi.org/10.1016/j.dci.2019.03.014.

Sun, N., Jiang, L., Ye, M., Wang, Y., Wang, G., Wan, X., et al. (2020). TRIM35 mediates protection against influenza infection by activating TRAF3 and degrading viral PB2. *Protein Cell* 11(12)**,** 894-914. doi: 10.1007/s13238-020-00734-6.

Suzuki, M., Watanabe, M., Nakamaru, Y., Takagi, D., Takahashi, H., Fukuda, S., et al. (2016). TRIM39 negatively regulates the NFκB-mediated signaling pathway through stabilization of Cactin. *Cellular and Molecular Life Sciences* 73(5)**,** 1085-1101. doi: 10.1007/s00018-015-2040-x.

Swatek, K.N., and Komander, D. (2016). Ubiquitin modifications. *Cell Res* 26(4)**,** 399-422. doi: 10.1038/cr.2016.39.

Tag-El-Din-Hassan, H.T., Morimatsu, M., and Agui, T. (2018). Functional analysis of duck, goose, and ostrich 2'-5'-oligoadenylate synthetase. *Infect Genet Evol* 62**,** 220-232. doi: 10.1016/j.meegid.2018.04.036.

Tag-El-Din-Hassan, H.T., Sasaki, N., Moritoh, K., Torigoe, D., Maeda, A., and Agui, T. (2012). The chicken 2 '-5 ' oligoadenylate synthetase A inhibits the replication of West Nile Virus. *Japanese Journal of Veterinary Research* 60(2-3)**,** 95-103.

Taieb, L., Ludwig, A., Ogden, N.H., Lindsay, R.L., Iranpour, M., Gagnon, C.A., et al. (2020). Bird species involved in West Nile virus epidemiological cycle in Southern Québec. *Int J Environ Res Public Health* 17(12). doi: 10.3390/ijerph17124517.

Tan, G., Xu, F., Song, H., Yuan, Y., Xiao, Q., Ma, F., et al. (2018). Identification of TRIM14 as a type I IFN-stimulated gene controlling hepatitis B virus replication by targeting HBx. *Front Immunol* 9**,** 1872. doi: 10.3389/fimmu.2018.01872.

Tanaka, Y., and Chen, Z.J. (2012). STING specifies IRF3 phosphorylation by TBK1 in the cytosolic DNA signaling pathway. *Sci Signal* 5(214)**,** ra20. doi: 10.1126/scisignal.2002521.

Tanji, K., Kamitani, T., Mori, F., Kakita, A., Takahashi, H., and Wakabayashi, K. (2010). TRIM9, a novel brain-specific E3 ubiquitin ligase, is repressed in the brain of Parkinson's disease and dementia with Lewy bodies. *Neurobiology of Disease* 38(2)**,** 210-218. doi: https://doi.org/10.1016/j.nbd.2010.01.007.

Taubenberger, J.K., and Kash, J.C. (2010). Influenza virus evolution, host adaptation, and pandemic formation. *Cell host & microbe* 7(6)**,** 440-451. doi: 10.1016/j.chom.2010.05.009.

Taylor, R.T., Lubick, K.J., Robertson, S.J., Broughton, J.P., Bloom, M.E., Bresnahan, W.A., et al. (2011). TRIM79alpha, an interferon-stimulated gene product, restricts tick-borne encephalitis virus replication by degrading the viral RNA polymerase. *Cell Host Microbe* 10(3)**,** 185-196. doi: 10.1016/j.chom.2011.08.004.

te Velthuis, A.J.W., Long, J.C., Bauer, D.L.V., Fan, R.L.Y., Yen, H.-L., Sharps, J., et al. (2018). Mini viral RNAs act as innate immune agonists during influenza virus infection. *Nature Microbiology* 3(11)**,** 1234-1242. doi: 10.1038/s41564-018-0240-5.

Teijaro, J.R., Walsh, K.B., Rice, S., Rosen, H., and Oldstone, M.B. (2014). Mapping the innate signaling cascade essential for cytokine storm during influenza virus infection. *Proc Natl Acad Sci U S A* 111(10)**,** 3799-3804. doi: 10.1073/pnas.1400593111.

Temperley, N.D., Berlin, S., Paton, I.R., Griffin, D.K., and Burt, D.W. (2008). Evolution of the chicken Toll-like receptor gene family: a story of gene gain and gene loss. *BMC Genomics* 9**,** 62. doi: 10.1186/1471-2164-9-62.

Thomas, P.G., Dash, P., Aldridge, J.R., Jr., Ellebedy, A.H., Reynolds, C., Funk, A.J., et al. (2009). The intracellular sensor NLRP3 mediates key innate and healing responses to influenza A virus via the regulation of caspase-1. *Immunity* 30(4)**,** 566-575. doi: 10.1016/j.immuni.2009.02.006.

Thompson, M.G., Dittmar, M., Mallory, M.J., Bhat, P., Ferretti, M.B., Fontoura, B.M., et al. (2020). Viral-induced alternative splicing of host genes promotes influenza replication. *eLife* 9**,** e55500. doi: 10.7554/eLife.55500.

Thompson, M.G., and Lynch, K.W. (2019). Functional and Mechanistic Interplay of Host and Viral Alternative Splicing Regulation during Influenza Infection. *Cold Spring Harb Symp Quant Biol* 84**,** 123-131. doi: 10.1101/sqb.2019.84.039040.

Thompson, M.G., Muñoz-Moreno, R., Bhat, P., Roytenberg, R., Lindberg, J., Gazzara, M.R., et al. (2018). Co-regulatory activity of hnRNP K and NS1-BP in influenza and human mRNA splicing. *Nature Communications* 9(1)**,** 2407. doi: 10.1038/s41467-018-04779-4.

Toka, F.N., Dunaway, K., Mielcarska, M., Smaltz, F., and Bossowska-Nowicka, M. (2017). Expression pattern of TRIM genes in bovine macrophages stimulated with PAMPs. The Journal of Immunology 198(1 Supplement), 129.127.

Towner, J.S., Amman, B.R., Sealy, T.K., Carroll, S.A., Comer, J.A., Kemp, A., et al. (2009). Isolation of genetically diverse Marburg viruses from Egyptian fruit bats. *PLoS Pathog* 5(7)**,** e1000536. doi: 10.1371/journal.ppat.1000536.

Tracz, M., and Bialek, W. (2021). Beyond K48 and K63: non-canonical protein ubiquitination. *Cellular & Molecular Biology Letters* 26(1)**,** 1. doi: 10.1186/s11658-020-00245-6.

Tsuchida, T., Zou, J., Saitoh, T., Kumar, H., Abe, T., Matsuura, Y., et al. (2010). The ubiquitin ligase TRIM56 regulates innate immune responses to intracellular double-stranded DNA. *Immunity* 33(5)**,** 765-776. doi: 10.1016/j.immuni.2010.10.013.

Tucker, M.E., and Benton, M.J. (1982). Triassic environments, climates and reptile evolution. *Palaeogeography, Palaeoclimatology, Palaeoecology* 40(4)**,** 361-379. doi: https://doi.org/10.1016/0031-0182(82)90034-7.

Uhlen, M., Fagerberg, L., Hallstrom, B.M., Lindskog, C., Oksvold, P., Mardinoglu, A., et al. (2015). Proteomics. Tissue-based map of the human proteome. *Science* 347(6220)**,** 1260419. doi: 10.1126/science.1260419.

UniProt Consortium, T. (2018). UniProt: the universal protein knowledgebase. *Nucleic Acids Research* 46(5)**,** 2699-2699. doi: 10.1093/nar/gky092.

Vainauska, D., Kozireva, S., Karpovs, A., Čistjakovs, M., and Bariševs, M. (2012). A novel approach for nucleic acid delivery into cancer cells. *Medicina (Kaunas)* 48(6)**,** 324-329.

Valiyeva, F., Jiang, F., Elmaadawi, A., Moussa, M., Yee, S.P., Raptis, L., et al. (2011). Characterization of the oncogenic activity of the novel TRIM59 gene in mouse cancer models. *Mol Cancer Ther* 10(7)**,** 1229-1240. doi: 10.1158/1535-7163.Mct-11-0077.

Valkiūnas, G. (2005). *Avian Malaria Parasites and Other Haemosporidia.* Boca Raton: CRC Press.

van Beuningen, S.F.B., Will, L., Harterink, M., Chazeau, A., van Battum, E.Y., Frias, C.P., et al. (2015). TRIM46 controls neuronal polarity and axon specification by driving the formation of parallel microtubule arrays. *Neuron* 88(6)**,** 1208-1226. doi: 10.1016/j.neuron.2015.11.012.

Van Brussel, K., and Holmes, E.C. (2022). Zoonotic disease and virome diversity in bats. *Current Opinion in Virology* 52**,** 192-202. doi: https://doi.org/10.1016/j.coviro.2021.12.008.

van der Aa, L.M., Levraud, J.-P., Yahmi, M., Lauret, E., Briolat, V., Herbomel, P., et al. (2009). A large new subset of TRIM genes highly diversified by duplication and positive selection in teleost fish. *BMC Biology* 7(1)**,** 7. doi: 10.1186/1741-7007-7-7.

van der Veen, A.G., Maillard, P.V., Schmidt, J.M., Lee, S.A., Deddouche-Grass, S., Borg, A., et al. (2018). The RIG-I-like receptor LGP2 inhibits Dicer-dependent processing of long double-stranded RNA and blocks RNA interference in mammalian cells. *EMBO J* 37(4). doi: 10.15252/embj.201797479.

van Gent, M., Sparrer, K.M.J., and Gack, M.U. (2018 TRIM proteins and their roles in antiviral host defenses. *Annu Rev Virol* 5(1)**,** 385-405. doi: 10.1146/annurev-virology-092917-043323.

Vanderven, H.A., Petkau, K., Ryan-Jean, K.E., Aldridge, J.R., Jr., Webster, R.G., and Magor, K.E. (2012). Avian influenza rapidly induces antiviral genes in duck lung and intestine. *Mol Immunol* 51(3-4)**,** 316-324. doi: 10.1016/j.molimm.2012.03.034.

Varga, Z.T., Grant, A., Manicassamy, B., and Palese, P. (2012). Influenza virus protein PB1-F2 inhibits the induction of type I interferon by binding to MAVS and decreasing mitochondrial membrane potential. *J Virol* 86(16)**,** 8359-8366. doi: 10.1128/jvi.01122-12.

Venuto, S., and Merla, G. (2019). E3 ubiquitin ligase TRIM proteins, cell cycle and mitosis. *Cells* 8(5). doi: 10.3390/cells8050510.

Versteeg, G.A., Rajsbaum, R., Sánchez-Aparicio, M.T., Maestre, A.M., Valdiviezo, J., Shi, M., et al. (2013). The E3-ligase TRIM family of proteins regulates signaling pathways triggered by innate immune pattern-recognition receptors. *Immunity* 38(2)**,** 384-398. doi: 10.1016/j.immuni.2012.11.013.

Vidana, B., Dolz, R., Busquets, N., Ramis, A., Sanchez, R., Rivas, R., et al. (2018). Transmission and immunopathology of the avian influenza virus A/Anhui/1/2013 (H7N9) human isolate in three commonly commercialized avian species. *Zoonoses Public Health* 65(3)**,** 312-321. doi: 10.1111/zph.12393.

Vince Caery, L.L.a.R.G. (2021). "RBGL: An interface to the BOOST graph library". (http://www.bioconductor.org).

Vu, H.T., Hong, Y., Truong, A.D., Lee, J., Lee, S., Song, K.-D., et al. (2021). Cytokine-cytokine receptor interactions in the highly pathogenic avian influenza H5N1 virus-infected lungs of genetically disparate Ri chicken lines. *Asian-Australas J Anim Sci* 0(0)**,** 0-0. doi: 10.5713/ab.21.0163.

Wagner, J.M., Roganowicz, M.D., Skorupka, K., Alam, S.L., Christensen, D., Doss, G., et al. (2016). Mechanism of B-box 2 domain-mediated higher-order assembly of the retroviral restriction factor TRIM5α. *Elife* 5. doi: 10.7554/eLife.16309.

Wallenhammar, A., Anandapadamanaban, M., Lemak, A., Mirabello, C., Lundström, P., Wallner, B., et al. (2017). Solution NMR structure of the TRIM21 B-box2 and

identification of residues involved in its interaction with the RING domain. *PLoS One* 12(7), e0181551. doi: 10.1371/journal.pone.0181551.

Wang, B., Chen, Y., Mu, C., Su, Y., Liu, R., Huang, Z., et al. (2015). Identification and expression analysis of the interferon-induced protein with tetratricopeptide repeats 5 (IFIT5) gene in duck (Anas platyrhynchos domesticus). *PLoS One* 10(3), e0121065. doi: 10.1371/journal.pone.0121065.

Wang, J., Liu, B., Wang, N., Lee, Y.M., Liu, C., and Li, K. (2011). TRIM56 is a virus- and interferon-inducible E3 ubiquitin ligase that restricts pestivirus infection. *J Virol* 85(8), 3733-3745. doi: 10.1128/jvi.02546-10.

Wang, J., Vasaikar, S., Shi, Z., Greer, M., and Zhang, B. (2017). WebGestalt 2017: a more comprehensive, powerful, flexible and interactive gene set enrichment analysis toolkit. *Nucleic Acids Research* 45(W1), W130-W137. doi: 10.1093/nar/gkx356.

Wang, L., Fu, B., Li, W., Patil, G., Liu, L., Dorf, M.E., et al. (2017). Comparative influenza protein interactomes identify the role of plakophilin 2 in virus restriction. *Nature Communications* 8(1), 13876. doi: 10.1038/ncomms13876.

Wang, L., Lu, K.C., Chen, G.L., Li, M., Zhang, C.Z., and Chen, Y.H. (2020). A Litopenaeus vannamei TRIM32 gene is involved in oxidative stress response and innate immunity. *Fish Shellfish Immunol* 107(Pt B), 547-555. doi: 10.1016/j.fsi.2020.11.002.

Wang, P., Benhenda, S., Wu, H., Lallemand-Breitenbach, V., Zhen, T., Jollivet, F., et al. (2018). RING tetramerization is required for nuclear body biogenesis and PML sumoylation. *Nat Commun* 9(1), 1277. doi: 10.1038/s41467-018-03498-0.

Wang, P., Zhao, W., Zhao, K., Zhang, L., and Gao, C. (2015). TRIM26 negatively regulates interferon-β production and antiviral response through polyubiquitination and degradation of nuclear IRF3. *PLoS Pathog* 11(3), e1004726. doi: 10.1371/journal.ppat.1004726.

Wang, S., Chen, Y., Li, C., Wu, Y., Guo, L., Peng, C., et al. (2016). TRIM14 inhibits hepatitis C virus infection by SPRY domain-dependent targeted degradation of the viral NS5A protein. *Scientific Reports* 6(1), 32336. doi: 10.1038/srep32336.

Wang, S., Yu, M., Liu, A., Bao, Y., Qi, X., Gao, L., et al. (2021). TRIM25 inhibits infectious bursal disease virus replication by targeting VP3 for ubiquitination and degradation. *PLoS Pathog* 17(9), e1009900. doi: 10.1371/journal.ppat.1009900.

Wang, W., Huang, Y., Yu, Y., Yang, Y., Xu, M., Chen, X., et al. (2016). Fish TRIM39 regulates cell cycle progression and exerts its antiviral function against iridovirus and nodavirus. *Fish & Shellfish Immunology* 50**,** 1-10. doi: https://doi.org/10.1016/j.fsi.2016.01.016.

Wang, W., Said, A., Wang, B., Qu, G., Xu, Q., Liu, B., et al. (2018). Establishment and evaluation of the goose embryo epithelial (GEE) cell line as a new model for propagation of avian viruses. *PLOS ONE* 13(3)**,** e0193876. doi: 10.1371/journal.pone.0193876.

Wang, W., Said, A., Wang, Y., Fu, Q., Xiao, Y., Lv, S., et al. (2016c). Establishment and characterization of duck embryo epithelial (DEE) cell line and its use as a new approach toward DHAV-1 propagation and vaccine development. *Virus Res* 213**,** 260-268. doi: 10.1016/j.virusres.2015.12.021.

Wang, X., Hinson, E.R., and Cresswell, P. (2007). The interferon-inducible protein viperin inhibits influenza virus release by perturbing lipid rafts. *Cell Host Microbe* 2(2)**,** 96-105. doi: 10.1016/j.chom.2007.06.009.

Wang, X.X., Lu, C., Rong, E.G., Hu, J.X., Xing, Y.L., Liu, Z.Y., et al. (2019). Identification of novel genes associated with duck OASL in response to influenza A virus. *Journal of Integrative Agriculture* 18(7)**,** 1451-1459. doi: 10.1016/S2095-3119(19)62685-7.

Wang, Y., Li, Y., Qi, X., Yuan, W., Ai, J., Zhu, C., et al. (2004). TRIM45, a novel human RBCC/TRIM protein, inhibits transcriptional activities of ElK-1 and AP-1. *Biochem Biophys Res Commun* 323(1)**,** 9-16. doi: 10.1016/j.bbrc.2004.08.048.

Wang, Y., Li, Z., Lu, Y., Hu, G., Lin, L., Zeng, L., et al. (2016). Molecular characterization, tissue distribution and expression, and potential antiviral effects of TRIM32 in the common carp (*Cyprinus carpio*).. *International Journal of Molecular Sciences* 17(10). doi: 10.3390/ijms17101693.

Wang, Y., Lian, Q., Yang, B., Yan, S., Zhou, H., He, L., et al. (2015). TRIM30α is a negative-feedback regulator of the intracellular DNA and DNA virus-triggered response by targeting STING. *PLoS Pathog* 11(6)**,** e1005012. doi: 10.1371/journal.ppat.1005012.

Wang, Y., Yan, S., Yang, B., Wang, Y., Zhou, H., Lian, Q., et al. (2015). TRIM35 negatively regulates TLR7- and TLR9-mediated type I interferon production by targeting IRF7. *FEBS Lett* 589(12)**,** 1322-1330. doi: 10.1016/j.febslet.2015.04.019.

Wang, Y., Yin, Y., Lan, X., Ye, F., Tian, K., Zhao, X., et al. (2017). Molecular characterization, expression of chicken TBK1 gene and its effect on IRF3 signaling pathway. *PLoS One* 12(5), e0177608. doi: 10.1371/journal.pone.0177608.

Watanabe, M., and Hatakeyama, S. (2017). TRIM proteins and diseases. *J Biochem* 161(2), 135-144. doi: 10.1093/jb/mvw087.

Waterhouse, A.M., Procter, J.B., Martin, D.M.A., Clamp, M., and Barton, G.J. (2009). Jalview Version 2—a multiple sequence alignment editor and analysis workbench. *Bioinformatics* 25(9), 1189-1191. doi: 10.1093/bioinformatics/btp033.

Weber, M., Sediri, H., Felgenhauer, U., Binzen, I., Bänfer, S., Jacob, R., et al. (2015). Influenza virus adaptation PB2-627K modulates nucleocapsid inhibition by the pathogen sensor RIG-I. *Cell host & microbe* 17(3), 309-319. doi: 10.1016/j.chom.2015.01.005.

Webster, R.G., Bean, W.J., Gorman, O.T., Chambers, T.M., and Kawaoka, Y. (1992). Evolution and ecology of influenza A viruses. *Microbiol Rev* 56(1), 152-179.

Webster, R.G., Yakhno, M., Hinshaw, V.S., Bean, W.J., and Murti, K.G. (1978). Intestinal influenza: replication and characterization of influenza viruses in ducks. *Virology* 84(2), 268-278. doi: 10.1016/0042-6822(78)90247-7.

Wei, L., Cui, J., Song, Y., Zhang, S., Han, F., Yuan, R., et al. (2014). Duck MDA5 functions in innate immunity against H5N1 highly pathogenic avian influenza virus infections. *Vet Res* 45(1), 66. doi: 10.1186/1297-9716-45-66.

Wei, L., Song, Y., Cui, J., Qu, N., Wang, N., Ouyang, G., et al. (2016). Cloning, characterization, and expression analysis of LGP2 cDNA from goose, Anser cygnoides. *Poult Sci* 95(10), 2290-2296. doi: 10.3382/ps/pew162.

Wei, X., Qian, W., Sizhu, S., Li, Y., Guo, K., Jin, M., et al. (2018). Negative regulation of interferon-beta production by alternative splicing of tumor necrosis factor receptor-associated factor 3 in ducks. *Front Immunol* 9(MAR), 409. doi: 10.3389/fimmu.2018.00409.

Wei, X., Qian, W., Sizhu, S., Shi, L., Jin, M., and Zhou, H. (2016). Molecular cloning and functional analysis of the duck TIR domain-containing adaptor inducing IFN-beta (TRIF) gene. *Dev Comp Immunol* 65, 369-376. doi: 10.1016/j.dci.2016.08.006.

Wei, Y., Zhou, H., Wang, A., Sun, L., Wang, M., Jia, R., et al. (2016). TRIM25 Identification in the Chinese Goose: Gene Structure, Tissue Expression Profiles, and Antiviral Immune

Responses In Vivo and In Vitro. *Biomed Res Int* 2016**,** 1403984. doi: 10.1155/2016/1403984.

Weinert, C., Morger, D., Djekic, A., Grütter, M.G., and Mittl, P.R.E. (2015). Crystal structure of TRIM20 C-terminal coiled-coil/B30.2 fragment: implications for the recognition of higher order oligomers. *Scientific Reports* 5(1)**,** 10819. doi: 10.1038/srep10819.

Weiss, I.D., Wald, O., Wald, H., Beider, K., Abraham, M., Galun, E., et al. (2010). IFN-gamma treatment at early stages of influenza virus infection protects mice from death in a NK cell-dependent manner. *J Interferon Cytokine Res* 30(6)**,** 439-449. doi: 10.1089/jir.2009.0084.

Weissensteiner, M.H., and Suh, A. (2019). "Repetitive DNA: The dark matter of avian genomics," in *Avian Genomics in Ecology and Evolution: From the Lab into the Wild,* ed. R.H.S. Kraus. (Cham: Springer International Publishing), 93-150.

Wettere, A.J.V. (2020). *Leucocytozoonosis in Poultry* [Online]. https://www.merckvetmanual.com/: Merck Veterinary Manual Available: https://www.merckvetmanual.com/poultry/bloodborne-organisms/leucocytozoonosis-in-poultry [Accessed July 16 2022].

Wheaton, S., Lambourne, M.D., Sarson, A.J., Brisbin, J.T., Mayameei, A., and Sharif, S. (2007). Molecular cloning and expression analysis of chicken MyD88 and TRIF genes. *DNA Seq* 18(6)**,** 480-486. doi: 10.1080/10425170701295856.

Wickham, H. (2016). "ggplot2: Elegant Graphics for Data Analysis. ". Singer-Verlag New York).

Wille, M., and Holmes, E.C. (2020). Wild birds as reservoirs for diverse and abundant gamma- and deltacoronaviruses. *FEMS Microbiology Reviews* 44(5)**,** 631-644. doi: 10.1093/femsre/fuaa026.

Williams, F.P., Haubrich, K., Perez-Borrajero, C., and Hennig, J. (2019). Emerging RNA-binding roles in the TRIM family of ubiquitin ligases. *Biological Chemistry* 400(11)**,** 1443-1464. doi: doi:10.1515/hsz-2019-0158.

Woo, J.S., Suh, H.Y., Park, S.Y., and Oh, B.H. (2006). Structural basis for protein recognition by B30.2/SPRY domains. *Mol Cell* 24(6)**,** 967-976. doi: 10.1016/j.molcel.2006.11.009.

Wozniakowski, G., Samorek-Salamonowicz, E., and Gawel, A. (2014). Occurrence of reovirus infection in Muscovy ducks (Cairina moschata) in south western Poland. *Pol J Vet Sci* 17(2)**,** 299-305. doi: 10.2478/pjvs-2014-0041.

Wu, B., and Hur, S. (2015a). How RIG-I like receptors activate MAVS. *Curr Opin Virol* 12**,** 91-98. doi: 10.1016/j.coviro.2015.04.004.

Wu, B., Peisley, A., Tetrault, D., Li, Z., Egelman, E.H., Magor, K.E., et al. (2014). Molecular imprinting as a signal-activation mechanism of the viral RNA sensor RIG-I. *Mol Cell* 55(4)**,** 511-523. doi: 10.1016/j.molcel.2014.06.010.

Wu, S., Zhang, J., Huang, J., Li, W., Liu, Z., He, Z., et al. (2019). Immune-Related Gene Expression in Ducks Infected With Waterfowl-Origin H5N6 Highly Pathogenic Avian Influenza Viruses. *Front Microbiol* 10(February)**,** 1782. doi: 10.3389/fmicb.2019.01782.

Wu, S., Zhang, J., Xue, Q., Liu, J., Huang, B., He, Z., et al. (2020). Duck TRIM32 functions in IFN-β signaling against the Infection of H5N6 highly pathogenic avian influenza virus. *Front Immunol* 11**,** 377. doi: 10.3389/fimmu.2020.00377.

Wu, W., Wang, X., Zhang, W., Tian, L., Booth, J.L., Duggan, E.S., et al. (2018). RIG-I Signaling via MAVS Is Dispensable for Survival in Lethal Influenza Infection In Vivo. *Mediators Inflamm* 2018**,** 6808934. doi: 10.1155/2018/6808934.

Wu, W., Zhang, W., Duggan, E.S., Booth, J.L., Zou, M.H., and Metcalf, J.P. (2015). RIG-I and TLR3 are both required for maximum interferon induction by influenza virus in human lung alveolar epithelial cells. *Virology* 482**,** 181-188. doi: 10.1016/j.virol.2015.03.048.

Wu, X., Liu, K., Jia, R., Pan, Y., Wang, M., Chen, S., et al. (2020). Duck IFIT5 differentially regulates Tembusu virus replication and inhibits virus-triggered innate immune response. *Cytokine* 133**,** 155161. doi: 10.1016/j.cyto.2020.155161.

Wu, X., Wang, J., Wang, S., Wu, F., Chen, Z., Li, C., et al. (2019). Inhibition of influenza A virus replication by TRIM14 via its multifaceted protein-protein interaction with NP. *Front Microbiol* 10(FEB)**,** 344. doi: 10.3389/fmicb.2019.00344.

Wynne, C., Lazzari, E., Smith, S., McCarthy, E.M., J, N.G., Kallal, L.E., et al. (2014). TRIM68 negatively regulates IFN-β production by degrading TRK fused gene, a novel driver of IFN-β downstream of anti-viral detection systems. *PLoS One* 9(7)**,** e101503. doi: 10.1371/journal.pone.0101503.

Xiang, C., Huang, M., Xiong, T., Rong, F., Li, L., Liu, D.X., et al. (2020). Transcriptomic analysis and functional characterization reveal the duck interferon regulatory factor 1 as an important restriction factor in the replication of Tembusu virus. *Front Microbiol* 11**,** 2069. doi: 10.3389/fmicb.2020.02069.

Xiao, Y., Evseev, D., Stevens, C.A., Moghrabi, A., Miranzo-Navarro, D., Fleming-Canepa, X., et al. (2020). Influenza PB1-F2 inhibits avian MAVS signaling. *Viruses* 12(4). doi: 10.3390/v12040409.

Xiao, Y., Reeves, M.B., Caulfield, A.F., Evseev, D., and Magor, K.E. (2018). The core promoter controls basal and inducible expression of duck retinoic acid inducible gene-I (RIG-I). *Mol Immunol* 103(September)**,** 156-165. doi: 10.1016/j.molimm.2018.09.002.

Xing, J., Weng, L., Yuan, B., Wang, Z., Jia, L., Jin, R., et al. (2016). Identification of a role for TRIM29 in the control of innate immunity in the respiratory tract. *Nat Immunol* 17(12)**,** 1373-1380. doi: 10.1038/ni.3580.

Xing, J., Zhang, A., Minze, L.J., Li, X.C., and Zhang, Z. (2018 TRIM29 negatively regulates the Type I IFN production in response to RNA virus. *J Immunol* 201(1)**,** 183-192. doi: 10.4049/jimmunol.1701569.

Xing, J., Zhang, A., Zhang, H., Wang, J., Li, X.C., Zeng, M.-S., et al. (2017). TRIM29 promotes DNA virus infections by inhibiting innate immune response. *Nature Communications* 8(1)**,** 945. doi: 10.1038/s41467-017-00101-w.

Xu, L., Yu, D., Fan, Y., Liu, Y.-P., and Yao, Y.-G. (2019). Evolutionary selection on MDA5 and LGP2 in the chicken preserves antiviral competence in the absence of RIG-I. *Journal of Genetics and Genomics* 46(10)**,** 499-503. doi: https://doi.org/10.1016/j.jgg.2019.10.001.

Xu, L., Yu, D., Fan, Y., Peng, L., Wu, Y., and Yao, Y.G. (2016). Loss of RIG-I leads to a functional replacement with MDA5 in the Chinese tree shrew. *Proc Natl Acad Sci U S A* 113(39)**,** 10950-10955. doi: 10.1073/pnas.1604939113.

Xue, B., Li, H., Guo, M., Wang, J., Xu, Y., Zou, X., et al. (2018). TRIM21 promotes innate immune response to RNA viral infection through Lys27-linked polyubiquitination of MAVS. *J Virol* 92(14). doi: 10.1128/jvi.00321-18.

Yamagoe, S., Yamakawa, Y., Matsuo, Y., Minowada, J., Mizuno, S., and Suzuki, K. (1996). Purification and primary amino acid sequence of a novel neutrophil chemotactic factor

LECT2. *Immunology Letters* 52(1)**,** 9-13. doi: https://doi.org/10.1016/0165-2478(96)02572-2.

Yamamoto, M., Sato, S., Hemmi, H., and Hoshino, K. (2003). Role of adaptor TRIF in the MyD88-independent Toll-like receptor signaling pathway: *Science* 301(5633)**,** 640-643.

Yamamoto, Y., Nakamura, K., Yamada, M., and Mase, M. (2012). Limited susceptibility of pigeons experimentally inoculated with H5N1 highly pathogenic avian influenza viruses. *The Journal of veterinary medical science* 74(2)**,** 205-208. doi: 10.1292/jvms.11-0312.

Yan, H.-Y., Wang, H.-Q., Zhong, M., Wu, S., Yang, L., Li, K., et al. (2021). PML suppresses influenza virus replication by promoting FBXW7 expression. *Virologica Sinica*. doi: 10.1007/s12250-021-00399-3.

Yan, J., Li, Q., Mao, A.P., Hu, M.M., and Shu, H.B. (2014). TRIM4 modulates type I interferon induction and cellular antiviral response by targeting RIG-I for K63-linked ubiquitination. *J Mol Cell Biol* 6(2)**,** 154-163. doi: 10.1093/jmcb/mju005.

Yang, B., Wang, J., Wang, Y., Zhou, H., Wu, X., Tian, Z., et al. (2013). Novel function of Trim44 promotes an antiviral response by stabilizing VISA. *J Immunol* 190(7)**,** 3613-3619. doi: 10.4049/jimmunol.1202507.

Yang, C., Zhao, X., Sun, D., Yang, L., Chong, C., Pan, Y., et al. (2016). Interferon alpha (IFNα)-induced TRIM22 interrupts HCV replication by ubiquitinating NS5A. *Cellular & Molecular Immunology* 13(1)**,** 94-102. doi: 10.1038/cmi.2014.131.

Yang, D., Li, N.L., Wei, D., Liu, B., Guo, F., Elbahesh, H., et al. (2019). The E3 ligase TRIM56 is a host restriction factor of Zika virus and depends on its RNA-binding activity but not miRNA regulation, for antiviral function. *PLOS Neglected Tropical Diseases* 13(6)**,** e0007537. doi: 10.1371/journal.pntd.0007537.

Yang, L., Liu, X., Zhang, L., Li, X., Zhang, X., Niu, G., et al. (2022). Porcine TRIM21 Enhances Porcine Circovirus 2 Infection and Host Immune Responses, But Inhibits Apoptosis of PCV2-Infected Cells. Viruses 14(1). doi: 10.3390/v14010156.

Yang, H.L., Feng, Z.Q., Zeng, S.Q., Li, S.M., Zhu, Q., and Liu, Y.P. (2015). Molecular cloning and expression analysis of TRAF3 in chicken. *Genet Mol Res* 14(2)**,** 4408-4419. doi: 10.4238/2015.April.30.14.

Yang, Q., Liu, T.T., Lin, H., Zhang, M., Wei, J., Luo, W.W., et al. (2017). TRIM32-TAX1BP1-dependent selective autophagic degradation of TRIF negatively regulates TLR3/4-

mediated innate immune responses. *PLoS Pathog* 13(9)**,** e1006600. doi: 10.1371/journal.ppat.1006600.

Yang, Q., Zhao, J., Chen, D., and Wang, Y. (2021). E3 ubiquitin ligases: styles, structures and functions. *Molecular Biomedicine* 2(1)**,** 23. doi: 10.1186/s43556-021-00043-2.

Yang, W., Gu, Z., Zhang, H., and Hu, H. (2020). To TRIM the Immunity: From Innate to Adaptive Immunity. *Frontiers in Immunology* 11.

Yang, Y., Huang, Y., Yu, Y., Zhou, S., Wang, S., Yang, M., et al. (2016). Negative regulation of the innate antiviral immune response by TRIM62 from orange spotted grouper. *Fish Shellfish Immunol* 57**,** 68-78. doi: 10.1016/j.fsi.2016.08.035.

Yao, Q., Fischer, K.P., Arnesen, K., Tyrrell, D.L., and Gutfreund, K.S. (2014). Molecular cloning, expression and characterization of Pekin duck interferon-λ. *Gene* 548(1)**,** 29-38. doi: https://doi.org/10.1016/j.gene.2014.06.066.

Ye, J., Yu, M., Zhang, K., Liu, J., Wang, Q., Tao, P., et al. (2015). Tissue-specific expression pattern and histological distribution of NLRP3 in Chinese yellow chicken. *Vet Res Commun* 39(3)**,** 171-177. doi: 10.1007/s11259-015-9641-6.

Ye, W., Hu, M.M., Lei, C.Q., Zhou, Q., Lin, H., Sun, M.S., et al. (2017) TRIM8 negatively regulates TLR3/4-mediated innate immune response by blocking TRIF-TBK1 interaction. *J Immunol* 199(5)**,** 1856-1864. doi: 10.4049/jimmunol.1601647.

Yoneyama, M., Kikuchi, M., Matsumoto, K., Imaizumi, T., Miyagishi, M., Taira, K., et al. (2005). Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. *J Immunol* 175(5)**,** 2851-2858. doi: 10.4049/jimmunol.175.5.2851.

Yoneyama, M., Kikuchi, M., Natsukawa, T., Shinobu, N., Imaizumi, T., Miyagishi, M., et al. (2004). The RNA helicase RIG-I has an essential function in double-stranded RNA-induced innate antiviral responses. *Nat Immunol* 5(7)**,** 730-737. doi: 10.1038/ni1087.

Yu, J.W., Wu, J., Zhang, Z., Datta, P., Ibrahimi, I., Taniguchi, S., et al. (2006). Cryopyrin and pyrin activate caspase-1, but not NF-κB, via ASC oligomerization. *Cell Death & Differentiation* 13(2)**,** 236-249. doi: 10.1038/sj.cdd.4401734.

Yu, L., and Liu, P. (2021). Cytosolic DNA sensing by cGAS: regulation, function, and human diseases. *Signal Transduct Target Ther* 6(1)**,** 170. doi: 10.1038/s41392-021-00554-y.

Yu, Y., Huang, X., Liu, J., Zhang, J., Hu, Y., Yang, Y., et al. (2017). Fish TRIM32 functions as a critical antiviral molecule against iridovirus and nodavirus. *Fish & Shellfish Immunology* 60**,** 33-43. doi: https://doi.org/10.1016/j.fsi.2016.11.036.

Yu, Z., Li, X., Yang, M., Huang, J., Fang, Q., Jia, J., et al. (2021). TRIM41 is required to innate antiviral response by polyubiquitinating BCL10 and recruiting NEMO. *Signal Transduction and Targeted Therapy* 6(1)**,** 90. doi: 10.1038/s41392-021-00477-8.

Yuk, S.S., Lee, D.H., Park, J.K., Tseren-Ochir, E.O., Kwon, J.H., Noh, J.Y., et al. (2016). Pre-immune state induced by chicken interferon gamma inhibits the replication of H1N1 human and H9N2 avian influenza viruses in chicken embryo fibroblasts. *Virol J* 13(1)**,** 71. doi: 10.1186/s12985-016-0527-1.

Zanchetta, M.E., and Meroni, G. (2019). Emerging roles of the TRIM E3 ubiquitin ligases MID1 and MID2 in cytokinesis. *Frontiers in Physiology* 10.

Zha, J., Han, K.J., Xu, L.G., He, W., Zhou, Q., Chen, D., et al. (2006). The Ret finger protein inhibits signaling mediated by the noncanonical and canonical IkappaB kinase family members. *J Immunol* 176(2)**,** 1072-1080. doi: 10.4049/jimmunol.176.2.1072.

Zhang, B., Liu, X., Chen, W., and Chen, L. (2013). IFIT5 potentiates anti-viral response through enhancing innate immune signaling pathways. *Acta Biochim Biophys Sin (Shanghai)* 45(10)**,** 867-874. doi: 10.1093/abbs/gmt088.

Zhang, J., Hu, M.M., Wang, Y.Y., and Shu, H.B. (2012). TRIM32 protein modulates type I interferon induction and cellular antiviral response by targeting MITA/STING protein for K63-linked ubiquitination. *J Biol Chem* 287(34)**,** 28646-28655. doi: 10.1074/jbc.M112.362608.

Zhang, J., Zhang, C., Cui, J., Ou, J., Han, J., Qin, Y., et al. (2017). TRIM45 functions as a tumor suppressor in the brain via its E3 ligase activity by stabilizing p53 through K63-linked ubiquitination. *Cell Death Dis* 8(5)**,** e2831. doi: 10.1038/cddis.2017.149.

Zhang, M., Song, K., Li, C., Chen, Z., Ding, C., and Liu, G. (2015). Molecular cloning of Peking duck Toll-like receptor 3 (duTLR3) gene and its responses to reovirus infection. *Virol J* 12(1)**,** 207. doi: 10.1186/s12985-015-0434-x.

Zhang, S., Guo, J.T., Wu, J.Z., and Yang, G. (2013). Identification and characterization of multiple TRIM proteins that inhibit hepatitis B virus transcription. *PLoS One* 8(8)**,** e70001. doi: 10.1371/journal.pone.0070001.

Zhang, W., Chen, S., Mahalingam, S., Wang, M., and Cheng, A. (2017). An updated review of avian-origin Tembusu virus: a newly emerging avian Flavivirus. *J Gen Virol* 98(10)**,** 2413-2420. doi: 10.1099/jgv.0.000908.

Zhang, Z., Bao, M., Lu, N., Weng, L., Yuan, B., and Liu, Y.J. (2013). The E3 ubiquitin ligase TRIM21 negatively regulates the innate immune response to intracellular double-stranded DNA. *Nat Immunol* 14(2)**,** 172-178. doi: 10.1038/ni.2492.

Zhang, Z., Jia, Y., Almeida, P., Mank, J.E., van Tuinen, M., Wang, Q., et al. (2018). Whole-genome resequencing reveals signatures of selection and timing of duck domestication. *GigaScience* 7(4). doi: 10.1093/gigascience/giy027.

Zhang, Z., Yuan, B., Bao, M., Lu, N., Kim, T., and Liu, Y.J. (2011). The helicase DDX41 senses intracellular DNA mediated by the adaptor STING in dendritic cells. *Nat Immunol* 12(10)**,** 959-965. doi: 10.1038/ni.2091.

Zhang, Z., Zou, T., Hu, X., and Jin, H. (2015). Type III interferon gene expression in response to influenza virus infection in chicken and duck embryonic fibroblasts. *Mol Immunol* 68(2 Pt C)**,** 657-662. doi: 10.1016/j.molimm.2015.10.013.

Zhao, C., Jia, M., Song, H., Yu, Z., Wang, W., Li, Q., et al. (2017). The E3 ubiquitin ligase TRIM40 attenuates antiviral immune responses by targeting MDA5 and RIG-I. *Cell Rep* 21(6)**,** 1613-1623. doi: 10.1016/j.celrep.2017.10.020.

Zhao, C., Peng, C., Wang, P., Yan, L., Fan, S., and Qiu, L. (2021). Identification of a shrimp E3 ubiquitin ligase TRIM50-like involved in restricting white spot syndrome virus proliferation by its mediated autophagy and ubiquitination. *Front Immunol* 12**,** 682562. doi: 10.3389/fimmu.2021.682562.

Zhao, L., Hao, Y., Song, Z., Fan, Y., and Li, S. (2021). TRIM37 negatively regulates inflammatory responses induced by virus infection via controlling TRAF6 ubiquitination. *Biochem Biophys Res Commun* 556**,** 87-92. doi: 10.1016/j.bbrc.2021.03.147.

Zhao, W., Wang, L., Zhang, M., Yuan, C., and Gao, C. (2012). E3 ubiquitin ligase tripartite motif 38 negatively regulates TLR-mediated immune responses by proteasomal degradation of TNF receptor-associated factor 6 in macrophages. *J Immunol* 188(6)**,** 2567-2574. doi: 10.4049/jimmunol.1103255.

Zheng, Q., Hou, J., Zhou, Y., Yang, Y., Xie, B., and Cao, X. (2015). Siglec1 suppresses antiviral innate immune response by inducing TBK1 degradation via the ubiquitin ligase TRIM27. *Cell Res* 25(10), 1121-1136. doi: 10.1038/cr.2015.108.

Zheng, W., and Satta, Y. (2018). Functional evolution of avian RIG-I-like receptors. *Genes* 9(9). doi: 10.3390/genes9090456.

Zhong, B., Yang, Y., Li, S., Wang, Y.Y., Li, Y., Diao, F., et al. (2008). The adaptor protein MITA links virus-sensing receptors to IRF3 transcription factor activation. *Immunity* 29(4), 538-550. doi: 10.1016/j.immuni.2008.09.003.

Zhong, Z., Ji, Y., Fu, Y., Liu, B., and Zhu, Q. (2015). Molecular characterization and expression analysis of the duck viperin gene. *Gene* 570(1), 100-107. doi: 10.1016/j.gene.2015.06.003.

Zhou, J.-r., Liu, J.-h., Li, H.-m., Zhao, Y., Cheng, Z., Hou, Y.-m., et al. (2020). Regulatory effects of chicken TRIM25 on the replication of ALV-A and the MDA5-mediated type I interferon response. *Veterinary Research* 51(1), 145. doi: 10.1186/s13567-020-00870-1.

Zhou, X., Michal, J.J., Zhang, L., Ding, B., Lunney, J.K., Liu, B., et al. (2013). Interferon induced IFIT family genes in host antiviral defense. *Int J Biol Sci* 9(2), 200-208. doi: 10.7150/ijbs.5613.

Zhou, Z., Jia, X., Xue, Q., Dou, Z., Ma, Y., Zhao, Z., et al. (2014). TRIM14 is a mitochondrial adaptor that facilitates retinoic acid-inducible gene-I-like receptor-mediated innate immune response. *Proc Natl Acad Sci U S A* 111(2), E245-254. doi: 10.1073/pnas.1316941111.

Zhou, Z., Li, M., Cheng, H., Fan, W., Yuan, Z., Gao, Q., et al. (2018). An intercross population study reveals genes associated with body size and plumage color in ducks. *Nature Communications* 9(1), 2648. doi: 10.1038/s41467-018-04868-4.

Zhu, J., Zhang, Y., Ghosh, A., Cuevas, R.A., Forero, A., Dhar, J., et al. (2014). Antiviral activity of human OASL protein is mediated by enhancing signaling of the RIG-I RNA sensor. *Immunity* 40(6), 936-948. doi: 10.1016/j.immuni.2014.05.007.

Zipfel, P.F., and Skerka, C. (2009). Complement regulators and inhibitory proteins. *Nature Reviews Immunology* 9(10), 729-740. doi: 10.1038/nri2620.

Zou, J., Chang, M., Nie, P., and Secombes, C.J. (2009). Origin and evolution of the RIG-I like RNA helicase gene family. *BMC Evol Biol* 9(1), 85. doi: 10.1186/1471-2148-9-85.

APPENDIX

A1 Duck cell line development

A1.1 Introduction

There are currently no publicly available duck cell lines derived from mallard ducks (*Anas platyrhynchos*). There is an immortalized Muscovy duck (*Cairina moschata*) cell line derived from Muscovy embryonic retina or somites (Jordan et al., 2009). These cell lines were created by transfecting the E1A and E1B genes from human adenovirus serotype 5 into Muscovy cells from either 8-day old or 3-week-old embryos. Muscovy ducks are more susceptible to IAV than mallard-type ducks (Pantin-Jackwood et al., 2013). Additionally, these cells were specifically selected for their ability to propagate virus for vaccine production (Jordan et al., 2009) and as such have limited immunological protection against viral replication and may not be as relevant to studying host protection against viral infection. There is also an immortalized Pekin duck stem-cell line (EB66) that is only available under licensing agreements with the current (2022) patent-holder Valneva (https://valneva.com/). Additionally, this line is derived from embryos "at the developmental stage around oviposition" (Patent: https://patents.google.com/patent/EP1985305A1/en). In chickens, the 10th day of embryonic development is when the first signs of immune function begin appearing, with days 11 and 12 when B and T-cells first appear in the embryo (Davison, 2003). The 18th day of development is when the developing embryo is considered to be immunocompetent. The EB66 cell line may not have transcriptional activity of either intrinsic immune genes or genes which control regulation of immune gene pathways to study.

This side project had four main goals: The first goal was to establish a protocol to harvest duck embryonic fibroblast from Pekin duck embryos. The second goal was to establish a protocol to purify duck embryonic epithelial cells from Pekin duck embryos, the third goal was to attempt to select for spontaneously immortalized DEE cell lines from 11- and 12-day-old (do) Pekin duck embryos. Finally, the fourth goal was to establish an efficient transfection method in

DEF cells. These goals are written as a detailed methods section with minimal results and discussion.

A1.2 Establishing a protocol to harvest DEF cells

The following protocol is a modified version of a protocol provided by Maple Leaf farms titled "Duck embryonic fibroblast tissue harvest for toxiscreen". The protocol has been adapted from collecting and combining fibroblasts from many embryos, to collecting fibroblast cultures from single embryos. The age of embryos, how to extract embryos from eggs, general homogenization and incubation of mixed cultures were kept from the Maple leaf farms protocol. Changes include the growth media and antibiotics used, temperature used to grow fibroblast cultures, tools, and volumes of reagents used. Also, speed and time of centrifugation was also changed. Changes and adaptations to the original protocol are listed below.

Materials required:

(Materials changed or added from the Maple Leaf farms protocol are indicated in **bold**.)

Part 1

- Pekin duck eggs, incubated at 37-39°C for 12 days
- Autoclaved scissors, scalpel handle, tweezers (Between 2x and 3x of each)
- Power Gen 125 homogenizer (Fisher Scientific)
- Autoclaved homogenizer attachments for the Power Gen 125 (Fisher Scientific) mechanical homogenizer (1x per egg, +1)
- Autoclaved glass petri dishes (1/2 dish per egg, +1)
- **Autoclaved large beaker filled with cold 1x PBS**
- Autoclaved medium beaker filled with cold 1x PBS
- **Autoclaved medium beaker filled with 75% EtOH**
- Autoclaved medium beaker, empty
- **100µm cell strainer (Corning, 1x per egg, +1)**
- Autoclave bags and discard
- **Sealed bag of 50mL conicals (Corning)**
- Hank's balanced salt solution (HBSS) (Gibco)
- 2.5% Trypsin in EDTA (Gibco)
- Sterile wrapped scalpel blades

Part 2

- **100 µm cell strainer (Corning, 1x per egg, +1)**
- **40 µm cell strainer (Corning, 1x per egg, +1)**
- **DMEM + 20% FBS (500 mL)**
- **DMEM with no serum added (500 mL)**
- **DMEM (Gibco) + 10% FBS (Gibco) + Primocin (100 µg/mL, Invivogen) (1000 mL)**

Set up:

Before placing eggs in the incubator, wash and scrub eggs with distilled water, and wipe down with 75% EtOH. Make sure there is no fecal or other foreign material on eggs before placing in the incubator. Eggs should be incubated on an egg rotator in the incubator with the air space facing upwards. Ensure there is sufficient humidity during the entire incubation process by placing several large pans full of distilled H_2O in the incubator. Humidity should be at least 55%. Eggs should be candled and marked before beginning the procedure.

To candle the egg: Hold the egg against a bright light source (Illuminator or flashlight on cell phone) and look for blood vessels, yolk and embryo. Embryo will appear as a dark shadow, sometimes with eye spots visible and may be twitching or moving slightly at 12 do. If it is hard to see an embryo it may be in the center of the egg, rocking the egg back and forth gently will sometimes help with visualization. Mark any eggs without visible blood vessels or yolk as non-fertilized, and the eggs with visible embryos, blood vessels and yolk as fertilized. Do not mark egg as unfertilized if you don't see an embryo as it could be hiding. Do not open any clearly unfertilized eggs in the hood as they will smell terrible. The air space should be carefully marked on the egg with a pencil.

Setting up the hood: All utensils should be wrapped in tin foil, clearly labeled, and autoclaved before use. In the cell culture hood, spray entire hood down well with 75% EtOH. Once hood is dry, place an autoclave down over workspace. Set up a waste bin with an autoclave bag inside, and fill beakers with PBS or EtOH. Place all items needed for the first half of the protocol in the hood (see above). Remove all utensils from tinfoil in the hood and discard the tinfoil. Set up maximum number of conicals you will need for part 1 of the protocol. Once all necessary utensils are in the hood, turn on the UV for at least 20 minutes.

(Parts of protocol changed or added from the Maple Leaf farms protocol are indicated by two stars **.)

Part 1:

1. Spray entire hood with 75 % EtOH again. Before placing eggs in hood, wrap an egg carton with saran wrap, spray with 75 % EtOH (**). Place eggs on saran wrap and wipe with 75 % EtOH. Make sure eggs are cleaned well before placing eggs into the hood. Once eggs are in the hood place the HBSS and 2.5 % trypsin in the hood. Ensure that conicals and utensils are within reaching distance, and that your arm will not have to reach across the embryos to grab anything.
2. Label 3x conical per egg. In the first conical, add 5 mL of Hanks' salt solution (**). Place a 100 μm cell strainer on the second conical (**). Add 10 mL of Hank's salt solution to the third conical (**).
3. Using a clean pair of tweezers, following the marked outline of the air space, carefully tweeze along the marked outline to remove the top of the egg. Ensure that the eggshell isn't falling into the egg. Remove the top of the egg and as much of the sides of the egg as possible, without the contents of the egg spilling out. Using a new clean pair of tweezers, carefully remove the white membrane and discard it (**). There should be a clearly visible embryo nestled between the yolk and the inner shell. Before removing the embryo, place a new clean glass petri dish beside the egg. Using new tweezers, carefully grab the embryo and pull it out of the egg. It is delicate and may fall apart. If the embryo falls onto the surface of anything but the clean petri dish it must be discarded. As soon as the embryo is out of the egg, remove the head and ensure the heart is not beating (clearly visible through the chest cavity). If the heart is still beating, quickly remove more of the neck stem (**). Discard head into the waste container. The empty egg can be discarded in the waste container.
4. To collect fibroblasts, remove appendages, tail stub and internal organs. The appendages and tail stub are easiest to tweeze off. To remove organs, scoop in the direction from where the neck was located to where the tail stub was located. Most organs should come out. If anything remains in the trunk that is not a uniform fleshy pink colour, remove it and discard into the waste container. Place the trunk in the first labeled conical with

Hank's salt solution (**). Continue extracting embryos from any other eggs in the same way.

5. Once all embryos have been harvested, strain the trunk and Hank's solution from the first conical into the cell strainer (**). Using fresh tweezers, carefully remove trunk from cell strainer and place into fresh glass petri dish. Using a new scalpel blade, mince the trunk into small pieces. Transfer the mined trunk to the third labeled conical with 10mL of Hank's solution in it (**).
6. Remove the homogenizer tip from tin foil and attach to the homogenizer (**). Place tip into the conical with the minced trunk, ensuring the small hole at bottom of the homogenizer tip is below the liquid line. Turning the homogenizer to the number 3 setting, homogenize trunks 3x for 10 seconds each, moving homogenizer around in the conical while it is running (**). If there are still many large pieces in the mixture, homogenize for 10 seconds at a time until only small pieces are visible. Homogenize each trunk. Clean homogenizer tips in 75 % EtOH and PBS before placing them off to the side. Only use one tip per embryo; if the tip touches anything else in the hood, discard and use a fresh one.
7. Once all trunks are homogenized in the HBSS solution, add 3.75 mL of 2.5 % Trypsin to each conical (**). Top up each conical to the 15mL line using fresh Hank's salt solution, invert and shake lightly to mix (**). Incubate each conical at room temperature on the rotator for 30 minutes (**). Every 10 minutes give the conical a quick shake to get rid of gelatinous clumps.
8. Turn on the refrigerated centrifuge and set it to 8 °C.
9. While incubation is happening, clean up the hood. All equipment from part 1 can be removed, surfaces sprayed liberally with 75 % EtOH and wiped down. Ensure waste is autoclaved immediately, otherwise, it will quickly start to smell. All dishware and utensils can be washed with Sparkleen (Fisher Scientific), scrubbed vigorously to remove residual tissue and viscera, and rinsed well in distilled H_2O. Set up hood for part 2.

Note: To wash used tweezers and utensils, place the soiled utensil in the 75 % EtOH beaker for several minutes, wash in the PBS beaker, and place into an empty beaker to dry.

Part 2:

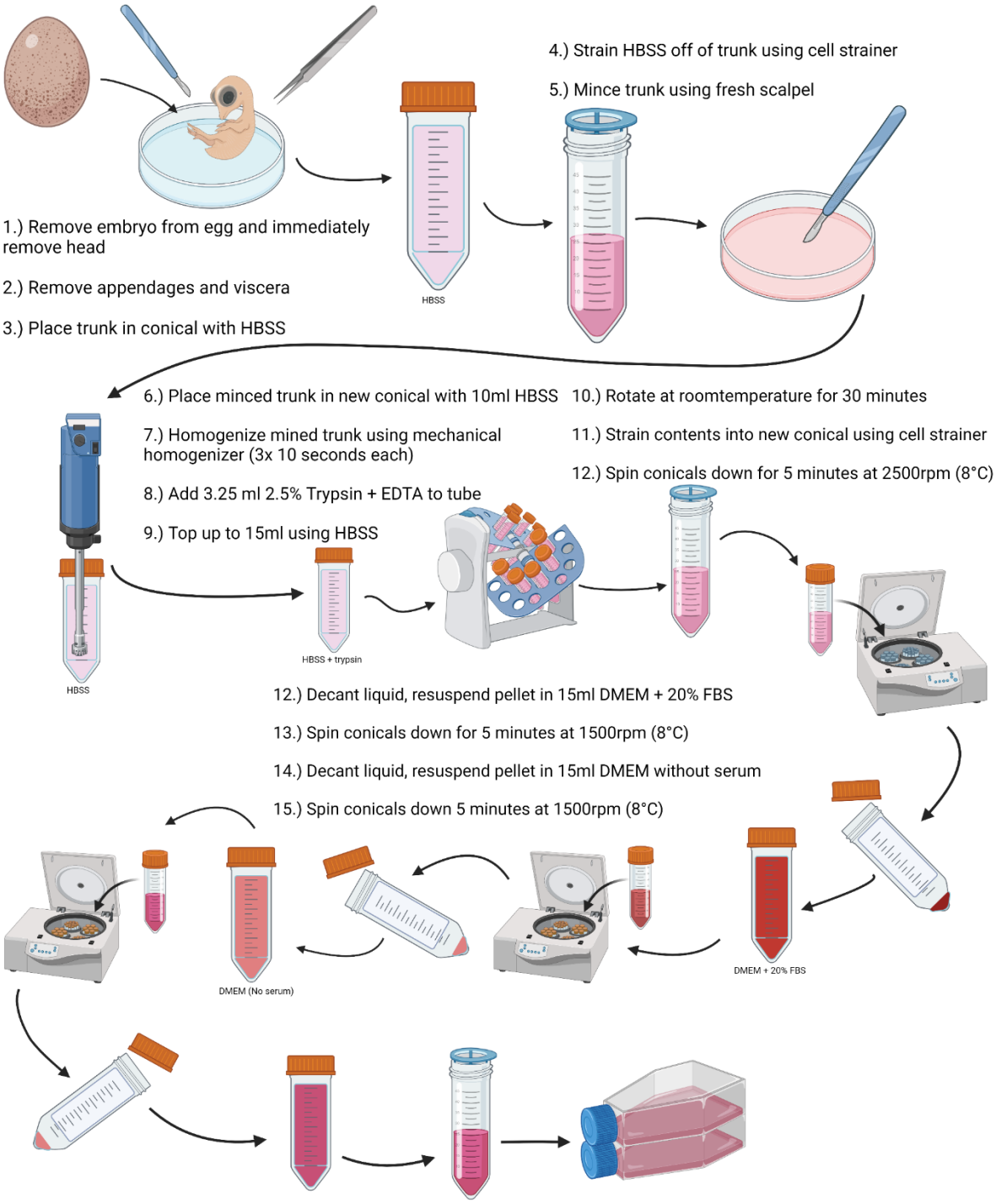
1. After homogenized trunks have been incubated, strain the large pieces out using 100 µm cell strainers into a fresh labeled 50mL conical (**). Spin down at 2000 rpm for 5 minutes at 8 °C in the refrigerated centrifuge (**).
2. Very carefully, decant the liquid, ensuring to keep the loose, white, fluffy-appearing precipitate and pellet. To remove residual trypsin, add 15 mL of cold DMEM + 20 % FBS, shake lightly to resuspend the pellet and spin down again at 1500rpm for 5 minutes at 8 °C (**). Again, decant the liquid, reserving the pellet (Carefully, it is very delicate and will break apart easily). Resuspend the pellet in 15 mL cold DMEM without serum added. Spin down again at 1500 rpm for 5 minutes at 8 °C (**).
3. Carefully decant the liquid from the pellet again. Add 40mL pre-warmed DMEM + 10 % FBS + Primocin and resuspend the pellet (**). Filter this suspension into a fresh 50 mL conical using a 40 µm cell strainer (**). Discard the cell strainer. Using 2x T75 flasks (Corning) add 20 mL of this mixture to each flask, label and place in the 39 °C incubator (**). To remove dead cells and debris, add fresh DMEM + 10 % FBS + Primocin 24 hours later (**).
4. These fibroblasts will usually undergo a slight lag period before they start growing. Generally, after 48-72 hours the cells will start growing rapidly, so they need to be checked under the microscope everyday. Once cells reach ~90 % confluency, they should be passed once to remove dead or non-replicating cells (**).
5. Cells should then be frozen and stored in liquid nitrogen until needed. DEF cells appear to recover best when the freezing media is DMEM + 10 % FBS + Primocin + 10 % DMSO at a density of ~$1.5x10^6$ cells/mL (**). This density is slightly higher than that which we use to freeze our immortalized cells ($1x10^6$ cells/mL), as there is a higher rate of dead cells during the thawing process for DEF cells (**).

Notes:

1. DEF cells tend to form “skin-like” layers when they start growing fast, presumed to be sheets of cells connected with extracellular matrix or other excretory products, that are resistant to trypsinization. If these cells are very clumpy or are in sheets, make sure to vigorously pipette the trypsinized cell solution up and down and use a 40 µm cell strainer so only single cells are present in cryovials. Cells should be pre-frozen before being stored in liquid nitrogen using Mr.Frosty 1 °C freezing containers (Nalgene) in the -80 °C

freezer. Additionally, before discarding any stock flasks of DEF cells, ensure cells that have been frozen will grow by adding 1mL of previously frozen cells to 10 mL of DMEM + 10 % FBS in a T25 flask (Corning), incubating said flask for 2 hours at 39 °C and checking under microscope that cells have adhered and are stretching out.

2. DEF cells should be passed every 2-3 days at a density of 1:5 (Early passages) or 1:3 (8> passages) using 0.25 % Trypsin + EDTA (Gibco). When using DEF cells for transfection experiments, monitor cell doubling times. After 10 passages the cells start to grow much slower and usually completely stop replicating by passage 20. Transfection efficiency is usually best between passages 3-12. As these cells are from individual embryos and are a mix of fibroblasts and other unknown cell types, some stocks may have better transfection efficiency. Make note of which cells have the best transfection efficiency.
3. Extra effort should be taken to follow the best cell culture practice with DEF cells, as they are unforgiving to poor culturing techniques. Do not let cells grow past ~95% confluency and do not pass at lower cell densities to prolong the growth period between passages as this will decrease transfection efficiency. At later passages there will be more debris in flasks, extra wash steps are often needed with PBS to get rid of this debris.
4. As these cultures are derived from whole trunks of embryos, and much of the viscera is often indistinguishable between the trunk and developing organs, there will be contamination of these cultures with other cells. You may see epithelial-like cells and polynuclear “giant-cells”. Both giant-cells and the epithelial like-cells will be overgrown by fibroblast cells within one or two passages.



1.) Remove embryo from egg and immediately remove head

2.) Remove appendages and viscera

3.) Place trunk in conical with HBSS

4.) Strain HBSS off of trunk using cell strainer

5.) Mince trunk using fresh scalpel

6.) Place minced trunk in new conical with 10ml HBSS

7.) Homogenize mined trunk using mechanical homogenizer (3x 10 seconds each)

8.) Add 3.25 ml 2.5% Trypsin + EDTA to tube

9.) Top up to 15ml using HBSS

10.) Rotate at roomtemperature for 30 minutes

11.) Strain contents into new conical using cell strainer

12.) Spin conicals down for 5 minutes at 2500rpm (8°C)

12.) Decant liquid, resuspend pellet in 15ml DMEM + 20% FBS

13.) Spin conicals down for 5 minutes at 1500rpm (8°C)

14.) Decant liquid, resuspend pellet in 15ml DMEM without serum

15.) Spin conicals down 5 minutes at 1500rpm (8°C)

16.) Decant liquid, resuspend pellet in 40ml DMEM + 10% FBS + primocin

17.) Strain contents into new conical using cell strainer

18.) Add 20ml to T75 flask (x2)

19.) Incubate cells at 39°C for 24 hours

20.) Change media after 24 hours

Figure A1.1. Quick protocol for harvesting DEF cells from 12-day old Pekin duck embryos.

A1.3 Establishing a protocol to harvest and purify DEE cells

The protocol I established for DEE cell harvesting and separation was originally based on the protocols outlined by Wang and colleagues (Wang et al., 2016; Wang et al., 2018). I modified and changed the protocol used to purify DEE cells in our lab due to: A.) The original DEE stocks were highly contaminated by nonepithelial-like cells (visually appeared to be fibroblasts, myocytes, and other unknown cell types) and B.) Finding some discrepancies in the literature regarding DEE and GEE cell line establishment. In this section, I will briefly outline what methods I tried and the results as well as the current protocol I have established for growing pure cultures of DEE cells.

Initial harvesting protocol:

1. At part A1.4 of goal 1, distinguishable organs were separated from trunks and stored in 10 mL Hank's balanced salt solution. These organs were minced using a scalpel or homogenized using the mechanical homogenizer.
2. Embryonic organs were washed 3x in PBS by spinning the organs down at 2000 rpm for 5 mins at 8 °C, adding 10 mL of fresh HBSS and spinning again 2x times. HBSS was removed from the conical, and 15 mL of DMEM + 10 % FBS + Primocin (DEF media) was added to the conical. Conicals were placed in the incubator (39 °C + 5 % CO_2) and incubated for 2 hours.
3. After the incubation mined or homogenized organs were washed 2x in PBS (2000 rpm for 5 mins at 8 °C). The pellet was resuspended in 10 mL DMEM + 10 % FBS + Primocin and added to a fresh T75 flask.
4. To remove cell debris and non-adherent cells, 24 hours after seeding, flasks were washed 3x with PBS. Media was changed to DMEM + 5 % FBS + Primocin (DEE media). The DEF media was necessary for the recovery of organoids, while the DEE media is utilized to select epithelial cells by slowing down fibroblast growth.
5. Cells and organoids were allowed to recover for 2-7 days before proceeding to the epithelial selection phase. Cells and organoids were passed and/or digested before adherent cells reached confluency.

Epithelial purification method 1:

This method was previously published as the methodology employed in the development of both DEE and goose embryonic epithelial (GEE) cell line development (Wang et al., 2016; Wang et al., 2018). Type I collagenase (1 mg/mL in PBS, Gibco) was warmed to 37 °C before use. Collagenase type I was added to cell/organoid cultures and incubated for 5 minutes. The reaction was quenched using ice-cold PBS. Detached cells and organoids were spun in a desktop centrifuge at 2000 rpm for 5 minutes, the supernatant was removed, and the pellet was washed 2x in PBS. 1.5 mL DMEM + 5 % + Primocin was used to resuspend the pellet, and the resulting mixture was filtered using a 40 µm cell strainer (Corning). The filtered mixture was then added to a T25 with 8.5 mL of fresh DEE media. The collagenase digestion was repeated 3-7 more times, as suggested by Wang et al in their publications on the development of DEE and GEE cell lines (Wang et al., 2016; Wang et al., 2018), however even after collagenase digestion #5, there were still many fibroblast-like cells present.

Epithelial purification method 2:

In separation method #1, fibroblast contamination of epithelial cell cultures was a visible and consistent problem. To remove fibroblast cells from these epithelial cell cultures two different methods of selection were tested:

The first method was established in 1983 to remove fibroblast contamination from rat mammary epithelial cells (Pal et al., 1983). Mixed cultures of mammary epithelial cells and fibroblasts were incubated in HBSS for 2-3 hours, at which point contaminating fibroblasts detached and washed away. Using cultures of duck embryonic cells with visible foci of DEE, DEF, and other unknown duck embryonic cells I attempted to purify DEE cells. First, I marked suspected epithelial and fibroblast cell foci on the plates, then I added HBSS to these mixed cultures and placed them in the incubator (39 °C + 5 % CO_2). I visualized the cells under the microscope in 30-minute increments. After 30 minutes, many different types of duck epithelial cells started to ball up and detach. Visually, there was no difference in the amount of epithelial and fibroblast cell detachment. By 1 hour, almost all the cells in the dish had either balled up or detached. This suggested that selective starvation was not an appropriate method for removing DEF contamination from DEE cells.

The second method tested selective trypsinization of the mixed cell cultures. In this technique, cells are trypsinized for a short duration, and ideally fibroblasts detach before

epithelial cells. I tested 0.25 %, 0.05 % and 0.01 % trypsin in EDTA on the cells for 5-minute incubations. The 0.25 % trypsin concentration caused almost all cell types to detach, while the 0.05 % trypsin mixture caused ~95 % of the fibroblasts to detach, however clumps of epithelial-like cells also detached at this concentration. The 0.01 % trypsin concentration caused about 85 % of the fibroblasts to detach while only the outer cells in epithelial cell colonies were starting to detach. While using 0.01 % trypsin in EDTA to selectively remove fibroblasts was useful in removing many of the attached fibroblasts, there was still a significant fibroblast contamination problem after several days of growth.

The final, and most successful method I tested to remove fibroblast contamination from epithelial cell cultures was to utilize selective trypsinization and pre-plating methods in conjunction. Pre-plating isolates cells based on how fast they re-adhere to culture dishes after trypsinization. Our DEF cultures begin to adhere to cell culture flasks within 10 minutes after being trypsinized and passed to a new flask or after being thawed from liquid nitrogen storage. Following the protocol suggested by García-Posadas and colleagues (2013), I selectively trypsinized mixed cultures of duck embryonic cells derived from embryonic organs using 0.01 % trypsin + EDTA for 5 mins. After the 5 minutes of incubation, DEE media was added to the well to stop trypsinization, and the plate was gently tapped to remove detached cells. Cells were washed with PBS then digested in 0.25 % trypsin + EDTA until all cells were detached. The trypsinization reaction was stopped by adding DEE media. The cell suspension was then filtered using a 40µm cell strainer and was then added to a fresh T75 flask.

I initially tested incubating the cells for 1, 2 and 3 hours at 39 °C + 5 % CO_2. After the different incubation periods, the supernatant and floating cells were removed and added to a new T75 flask. 24 and 72 hours later, each flask was inspected to determine where the most visibly identifiable epithelial cells were located. I found the most epithelial cell colonies in the flask which contained the supernatant from the 2-hour pre-plating incubation. The flasks which contained the cells which adhered after 1-hour incubation in contained mostly fibroblast-like cells, while the flask which contained adherent cells from the 2-hour incubation contained more visible epithelial-like cell colonies upon expansion.

Further testing found that three rounds of selective trypsinization and pre-plating was sufficient to remove fibroblast contamination from epithelial cell cultures.

A1.4 Spontaneous immortalization of DEE cells

I attempted to spontaneously immortalize DEE cells based on the protocols published by Wang and colleagues documenting the spontaneous immortalization of both DEE and GEE cell types (Wang et al., 2016; Wang et al., 2018). Unfortunately, the immortalized cells in these publications were never added to a public repository for cells, so cannot be purchased.

To obtain epithelial-like cells, I harvested organs from 6 total Pekin duck embryos, 3 from 11 do embryos and 3 from 12 do embryos. All organs for each individual embryo were combined. Dishes were labeled 11-1, 11-2, 11-3, 12-1, 12-2, and 12-3 to identify the individual embryo these organs came from (First number is how many days old, second number is individual embryo number). Many of the resulting cell cultures from the embryonic organs formed mixed populations of cells (Figure A1.4.1A). Organoids which had "beating" or "squishing" movement were also present. I used collagenase type I to digest these mixed cell cultures 3-7 times.

As mentioned in section A1.2 in epithelial cell purification method 1, many of the cells that continued to grow following the epithelial cell purification using collagenase type I appeared to be fibroblast-like in morphology (Figure A1.4.1B).

There were distinguishable populations of epithelial-like cells present in these mixtures (Figure A1.4.1C). I attempted to separate these populations using cloning discs (Bel-Art) soaked in 0.25 % trypsin. Cloning disks were placed on patches of cells with epithelial-like morphology for 3 minutes before being removed. While the cloning disks did pick up these epithelial-like cells, there was still fibroblast cell contamination evident. Additionally, the use of cloning discs caused the rest of the well to dry out, effectively killing any cells without a disc. Cells attached to cloning discs were transferred to a single-well in a 24-well plate (Corning) and allowed to grow and expand for 7 days.

Once populations from cloning disks were confluent, they were passed into a 6-well plate and allowed to expand. When cells reached confluency, they were trypsinized, counted, and seeded at a density of 2 cells/well into a 96-well plate. Wells were marked if cells expanded from a single cell and had epithelial-like morphology. Marked wells were expanded into a well from a 24-well plate, then into a well of a 6-well plate, before being moved to a T25 flask.

All cells were selectively passed as they began arresting growth. If cells doubled once a week, they were passed at a ratio of 1:2 once a week. If cells did not double or have any visible

change in density during the week they were passed at least once a week at 100%. Passing the cells even once they stopped growing appeared to increase survivability of cultures, as those that were not passed once a week once cells arrested appeared to die much quicker.

As I was sorting for single cell clones, I also continued passing the parent cell populations. The cells from embryo 12-3 stopped growing much quicker than all other cell populations. The cells from 11-1 and 11-3 survived for the most amount of time, with the parent 11-3 population surviving until about passage 50, and the single cell clone derived from 11-3 called “Gandalf the white” surviving for 15 passages after the initial cell sort. Many of my initial clones were unfortunately lost due to what I assume was soap contamination in the cell culture glassware. Those that survived the soap contamination did not grow quite as nicely afterwards, however my cultures were all around passage 30 at that time, so may have been entering senescence anyways.

All cells went through dramatic phases of what appeared to be necrosis and apoptosis. When cells were dying the media would be full of cell debris. I made sure to wash this cell debris off when changing media.

While I did not succeed in getting any spontaneously immortalized duck epithelial cells, I did make some key observations if someone were to try this again in the future:

1.) Do not stop passing cells, even once they have stopped growing. Even when cells have become senescent, pass 100% of cells at least once a week. I noticed that when I didn’t pass cells (In order to try to conserve populations) they never started to grow again. Populations which I passed regularly would spontaneously start to grow again.

2.) Cell debris also seems to be a problem. As cells gain chromosomal abnormalities, some die by necrosis releasing contents into the media. It seemed to help the cells survive if gave them extra washes in between media changes to get rid of as much of this debris as possible.

3.) The longest-lived cells appeared to change morphology dramatically between passages. The clone named “Gandalf the white” would have epithelial-like morphology, until it was passed, then the cells that adhered would suddenly not have cell to cell adhesion and would

appear more motile. Many of the end populations looked quite different from their starting parent populations.

4.) Do not use conditioned media from DEF cells to try to extend growth. This seemed to cause cell growth arrest.

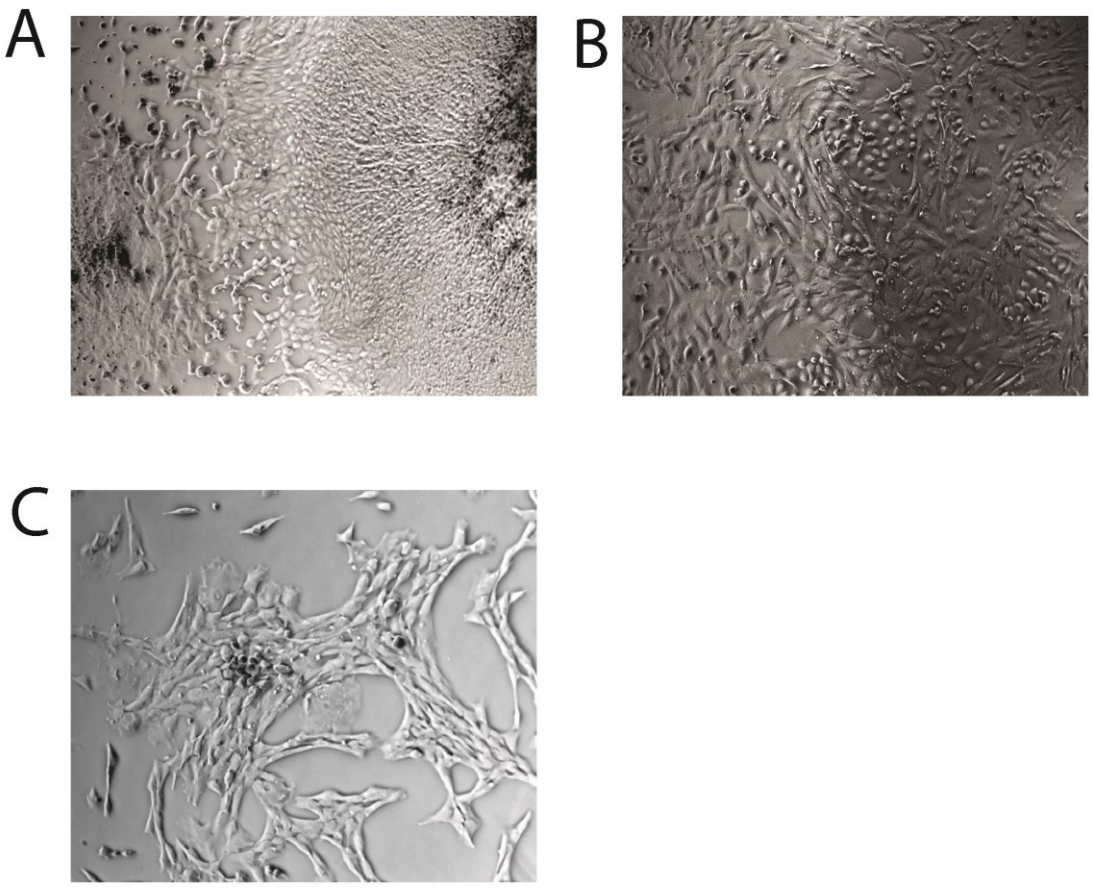


Figure A1.4.1. DEE cells that are purified using the collagenase type I protocol have fibroblast contamination. Mixed populations of DEE cells (A). A collagenase type I digested

population showing severe fibroblast contamination in the culture (B). Cells expanded from single cell clones of cells exhibiting epithelial-like morphology (C).

A1.5: Establishing an efficient magnetofection protocol for DEF cells

A1.5.1: Introduction

The most used transfection reagent used in our lab was Lipofectamine™ 2000, which has very poor transfection rates in DEF cells. Here I detail the protocols I modified and tested out to obtain transfection rates comparable to those in immortalized cell lines, such as DF-1 cells.

A1.5.2: Methods

A1.3.2.1 DEF growth and maintenance

DEF cells were grown and maintained in a cell culture incubator at 39 °C + 5 % CO_2. DEF cells are grown in DMEM (Gibco) + 10 % FBS (Gibco) (DEF media) without the addition of any antibiotics. DEF cells are passed 2 times after thawing from liquid nitrogen storage and are only used until ~12 passages or until they significantly reduce doubling time (Are not ready to pass after ~2 days of growth).

A1.3.2.3 Lipofection methods

To determine which combination of transfection reagents provided the highest transfection efficiency, 0.25 % Trypsin + EDTA (Gibco) was used to trypsinized DEF cells. Cells were seeded into 24-well plates at a density of $1x10^6$ cells/well and immediately transfected (Reverse transfection method). The transfection reagents tested were Lipofectamine™ 2000 (Invitrogen), Lipofectamine™ LTX with PLUS reagent (Invitrogen) and FuGENE® HD (Promega). A total of 500 ng *Renilla* luciferase expression plasmid (Promega) DNA was used per well. Single transfection reagents were added at a 2:1 reagent: DNA ratio. FuGENE® HD was mixed with either Lipofectamine™ 2000 or Lipofectamine™ LTX (With or without the PLUS reagent) at a ratio of 1:1 each reagent to total DNA ratio (ie. 1 µL Lipofectamine™ LTX: 1 µL FuGENE® HD: 1 µg DNA). PLUS reagent was added to Lipofectamine™ LTX mixtures at a 1:1 reagent to DNA ratio. Briefly, transfection reagents were added to Opti-MEM™ (50 µL/well) and mixed well. DNA with or without PLUS reagent was added to a separate tube containing Opti-MEM™ (50 µL/well) and mixed well by pipetting up and down. Tubes containing transfection reagents and DNA were immediately mixed, incubated at room temperature for 5 minutes then added dropwise to wells containing freshly seeded DEF cells. After 24-hours media

was removed from wells and 100 µL of 1x passive lysis buffer (Promega) was added to each well. DEF cells were lysed in passive lysis buffer for 1 hour. After the incubation period, the lysate was added to 100 µL of LAR II buffer (Promega). All luminescence measurements were completed using a the GloMax 20/20 Luminometer (Promega). Total *Renilla* luciferase luminescence was recorded for each well.

A1.3.2.3 Magnetofection methods

To determine if magnetofection could increase transfection efficiency of the transfection reagents DEF cells were seeded at a density of $0.75x10^6$ cells/well and allowed to grow for 24 hours. Lipofectamine™ 2000, Lipofectamine™ 3000, Lipofectamine™ LTX, FuGENE® HD or JetOPTIMUS® (Polyplus transfection) were used alone or in combination with CombiMag. 500 ng of *Renilla* control expression vector was added at a 2:1 ratio with each of the transfection reagents in Opti-MEM™ (100 µL total/well, as described above). One transfection reaction was performed according to manufacturer's instructions, while a duplicate transfection reaction was added to a tube containing CombiMag transfection reagent (Oz BioSciences) at a 1:1 CombiMag: DNA ratio. Transfection reagents and CombiMag were incubated at room temperature for 20 minutes before being added to a 24-well plate containing DEF cells and fresh DEF media (DMEM + 10 % FBS). 24-well plates were incubated for 20 minutes at room temperature on a Super Magnetic Plate (Oz BioSciences) before being returned to the cell culture incubator (39 °C + 5 % CO_2) for 24-hours. Lysis and analysis of *Renilla* luminescence proceeded as described above.

To determine which combination of transfection reagents increased transfection efficiency the most when used in conjunction with CombiMag, DEF cells were seeded as described above and allowed to grow for 24-hours before transfection or magnetofection. FuGENE® HD was mixed with either Lipofectamine™ LTX, Lipofectamine™ 3000 or JetOPTIMUS® at a 1:1:1 transfection reagent: transfection reagent: DNA ratio.

A1.5.3: Results and discussion

As there is no immortalized duck cell line available for the experiments performed in this thesis, I needed to transiently transfect primary DEF cells for many of the experiments. Initial transfection experiments with DEF cells utilized the standard Lipofectamine™ 2000 protocol that I use for DF-1 transfection in the lab. Lipofectamine™ 2000 does not efficiently transfect DEF

cells, with transfection rates of GFP producing plasmids only reaching transfection efficiency rates of approximately 1-3% (Figure A1.5.1).

To increase transfection rates, I followed the protocol for transfecting primary fibroblasts and hepatocytes put forth by Ishiguro and colleagues (Ishiguro et al., 2017). This protocol uses a combination of the transfection reagents Lipofectamine™ LTX and FuGENE® HD. The hypothesis is that primary cells are a heterogeneous population, and different lipofection reagents will have varying efficiencies the various cells present, so by mixing these reagents you increase the chances of more cells being successfully transfected

Using a luminometer I measured *Renilla* luminescence after transfecting primary DEF cells with either Lipofectamine™ 2000, Lipofectamine™ LTX, Lipofectamine™ LTX with PLUS reagent, FuGENE® HD, FuGENE® HD with Lipofectamine™ 2000 (With or without PLUS reagent) and FuGENE® HD with Lipofectamine™ LTX (With or without the PLUS reagent). I found that by mixing Lipofectamine™ LTX and FuGENE® HD, I could increase *Renilla* luminescence by approximately 4 times in DEF cells (Figure A1.3.2). This increase corresponded to a maximum transfection efficiency of approximately 5%. (Figure A1.5.3). Magnetofection is a transfection method that utilizes magnetic nanoparticles to deliver nucleic acid into cells in a more efficient manner than lipofection based transfection methods (Plank et al., 2011). CombiMag is an iron oxide nanoparticle developed for the efficient delivery of nucleic acid into cells (Vainauska et al., 2012). Plasmid DNA is first incubated with lipofection or cationic based transfection reagent. The DNA-transfection reagent complexes are incubated with CombiMag, associating the plasmid-DNA and transfection reagent with the nanoparticles. The resulting nano-particle-DNA complex is then added to cells, and a magnetic field is applied to force physical interaction between the nanoparticles and cell surfaces (Huth et al., 2003). The sedimentation of magnetofection particles on the surface of the cell enhances endocytic uptake of nucleic acids, and subsequent delivery of plasmid DNA to the nucleus. Initial testing of using CombiMag with various transfection reagents demonstrated that CombiMag increased transfection efficiency by at least two times in all reagents tested except for Lipofectamine™ LTX, where no increase in transfection efficiency was seen (Data not shown).

To determine if CombiMag could increase transfection efficiency of mixed transfection reagents, I measured *Renilla* luminescence in DEF cells transfected using the combination of FuGENE and Lipofectamine™ 3000, Lipofectamine™ LTX with PLUS reagent or

JetOPTIMUS®. When used with CombiMag transfection reagent, the mixtures of FuGENE® HD with JetOPTIMUS® or Lipofectamine™ 3000 increased relative *Renilla* luminescence by almost 10 times above that of Lipofectamine™ LTX mixed with FuGENE® HD and CombiMag (Figure A1.3.4). I confirmed these results by transiently transfecting DEF cells with pUB-GFP (Addgene plasmid #11155), a GFP expression plasmid under the control of constitutively active human ubiquitin C promoter and visualizing them 24-hours post transfection. Visual inspection confirms that the addition of CombiMag increases transfection efficiency, however the overall transfection rate is higher when using CombiMag in conjunction with JetOPTIMUS® and FuGENE® HD than when CombiMag is used in conjunction with Lipofectamine™ 3000 and JetOPTIMUS® (Figure A1.5.5). It appears that the FuGENE® HD and JetOPTIMUS® mixture increases transfection efficiencys overall, while the mixture of Lipofectamine™ 3000 and JetOPTIMUS® has a less efficient transfection efficiency but appears to transfect more plasmid into individual cells (Indicated by brighter fluorescing cells in the images). When analyzed visually, Lipofectamine™ 3000 appears to cause specifically fewer DEF cells to become transfected than FuGENE® HD, in which there was a greater overall transfection efficiency. However, the cells transfected using Lipofectamine™ 3000 always glow brighter under fluorescence microscopy compared to the cells transfected using FuGENE® HD, indicating more plasmid is present in these cells.

The reagents FuGENE® HD plus JetOPTIMUS® mixed with CombiMag were found to have the highest transfection efficiency out of all the treatments tested. A step-by-step protocol can be found in figure A1.5.6.

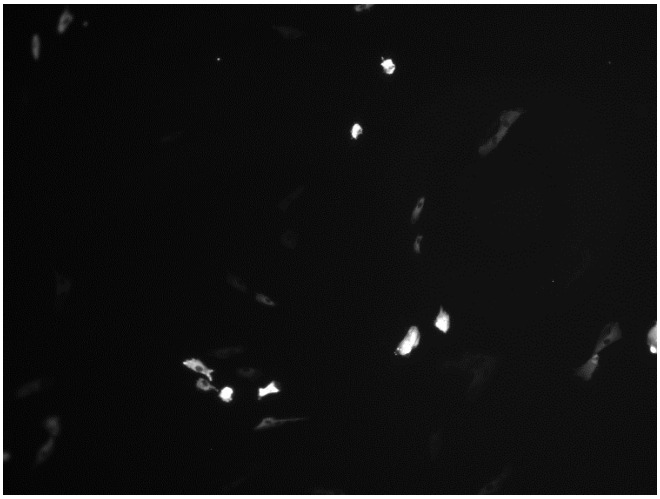


Figure A1.5.1. DEF cells transiently expressing pUBC-GFP 24-hours post transfection using Lipofectamine 2000.

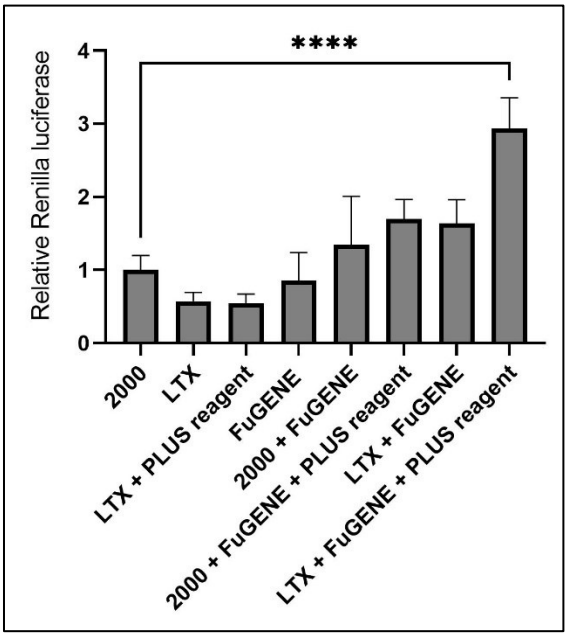


Figure A1.5.2. Transfection reagents Lipofectamine™ LTX and PLUS reagents mixed with FuGENE HD increases transfection efficiency of DEF cells compared to lipofectamine alone.

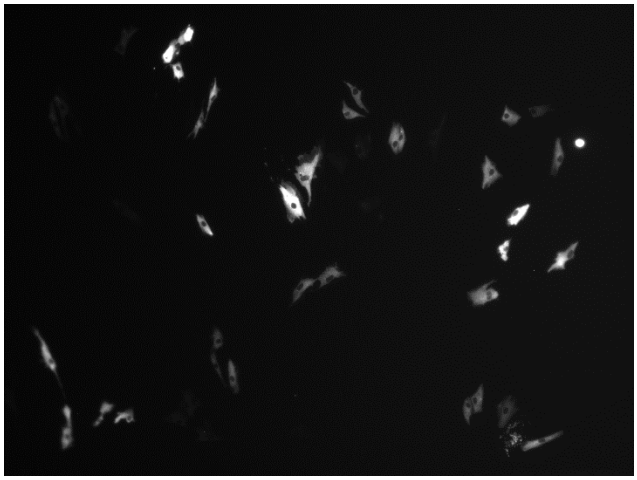


Figure A1.5.3. DEF cells transiently expressing pUBC-GFP 24-hours post transfection using a combination of Lipofectamine LTX with PLUS reagent, and FuGENE HD.

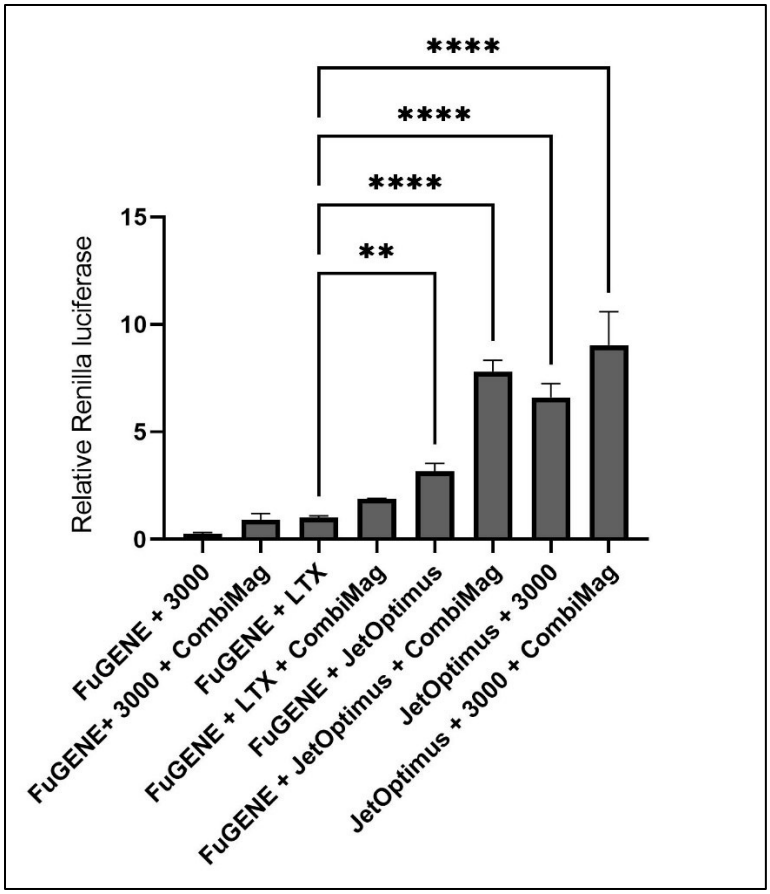


Figure A1.5.4. Magnetofection increases transfection efficiency when mixing transfection reagent in primary DEF cells. DEF cells were transiently transfected with pUBC-GFP plasmids

for 24-hours, before being lysed and luminescence analyzed on a luminometer. Relative *Renilla* luminescence normalized to FuGENE + LTX without CombiMag.

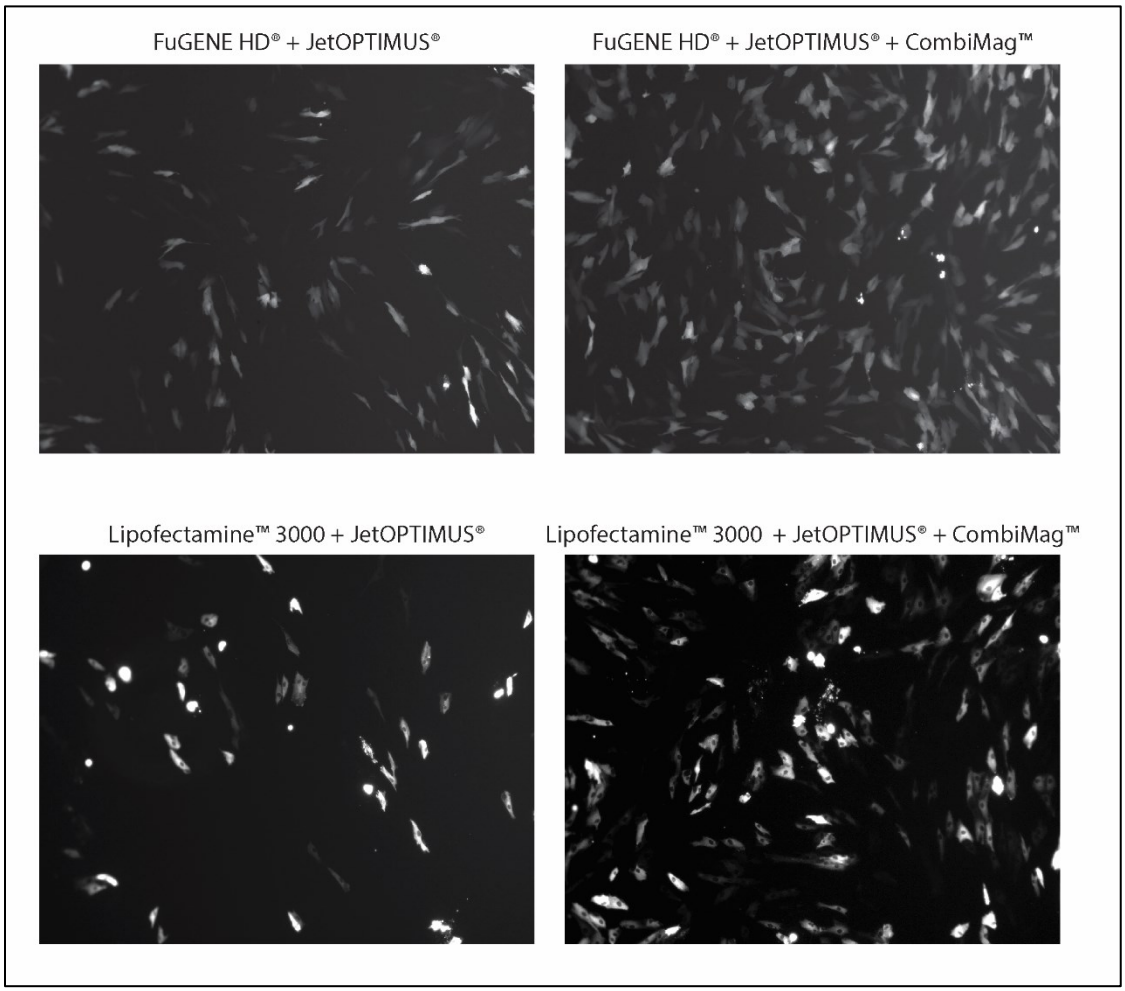


Figure A1.5.5. FuGENE HD + JetOPTIMUS + CombiMag magnetofection has a higher visual transfection efficiency than Lipofectamine 3000 + JetOPTIMUS + CombiMag.

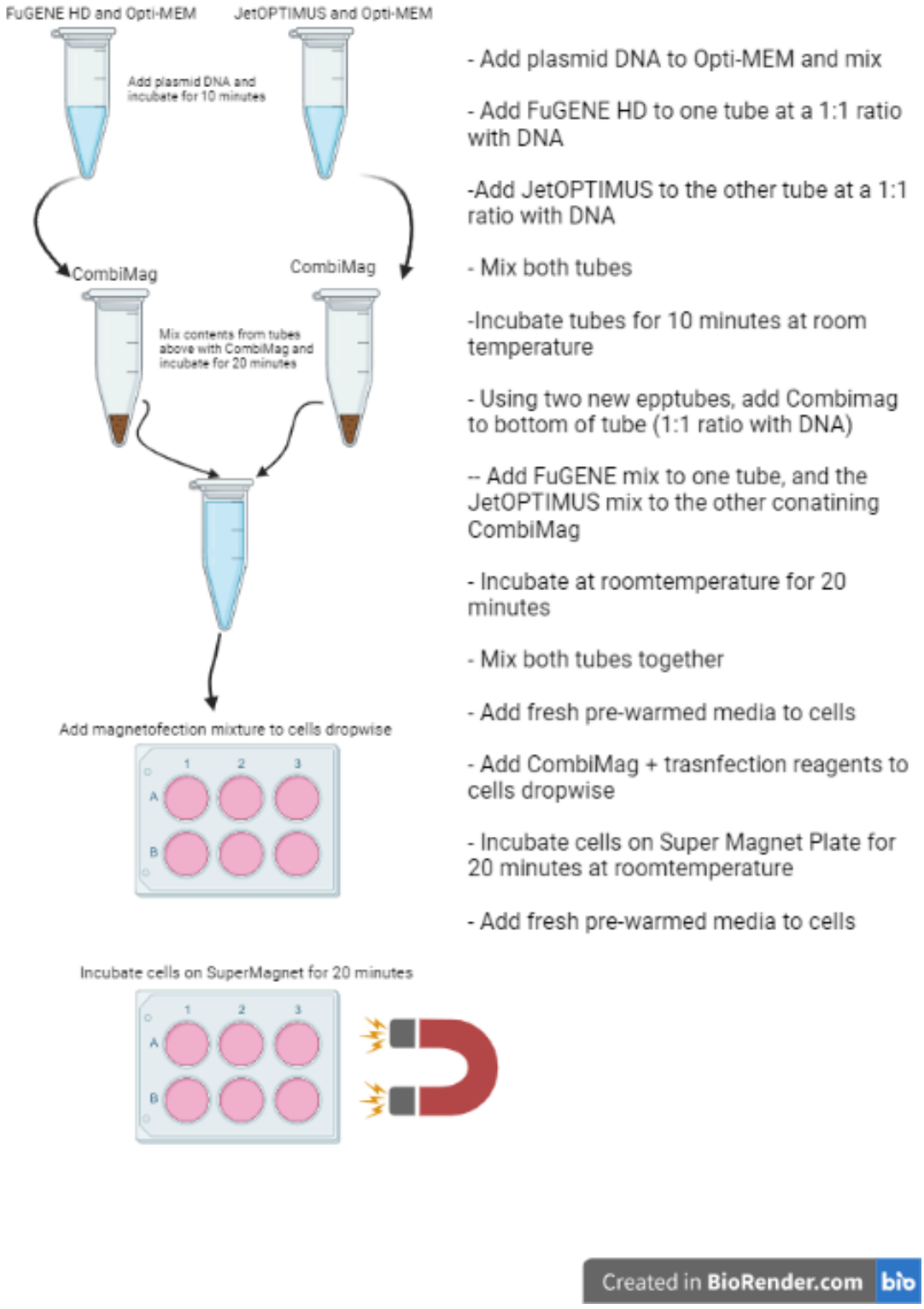


Figure A1.5.6. Summary for DEF magnetofection protocol.

A2 Identification of *Leucocytozoon spp*. in sick Pekin and Muscovy ducks

A2.1 Introduction

Birds are host to an incredibly diverse and vast array of haemosporidian parasites, often referred to generically as "bird malaria". However, the blood parasites infecting birds are not just *Plasmodium spp*. (Vectored through mosquitos/ *Culicidae*), but also *Haemoproteus spp*. (Vectored by biting midges/*Culicoides* and louse flies/*Hippoboscidae*) and *Leucocytozoon spp.* (Vectored by black flies/ *Simulium*) (Loye et al., 1991; Valkiūnas, 2005).

We acquire the Pekin duck eggs used for embryonic fibroblast and epithelial cell harvesting from a farm called "Duckscetera" located in Bonnyville, Alberta. They were having increased livestock mortality due to a pathogen present only in the summer months and reported that this unknown infection was much worse during years with higher-than-normal precipitation. The owners of Duckscetera also noted that the infection seemed to be more fatal in Pekin and Muscovy duck, but not Call duck or various species of chicken and geese. Worried that it was avian influenza, they sent tissue samples to the provincial lab and were informed that infection was likely caused by an apicomplexan parasite, but the report did not name a species. Without a species of parasite noted a treatment plan could not be implemented by the farm veterinarian. Duckscetera asked if we could assist in diagnosing the infection.

A2.2 Materials and methods

To determine what blood parasite was infecting and killing juvenile Pekin and Muscovy ducks in Bonnyville, Alberta during unseasonably wet summer months, three Pekin and one Muscovy duck were humanely euthanized by the owners of Duckscetera in Bonnyville Alberta. The Muscovy duck and two of the Pekin ducks were visibly ill with neurological symptoms (Head tilting, blindness and uncontrollable shaking) and one of the Pekin ducks was euthanized due to a broken leg but had no symptoms of infection (control). The injured Pekin duck was 4-weeks old, while the two infected Pekin ducks were 3- and 4-weeks old. The infected Muscovy duck was approximately 5-8 weeks old. The Muscovy duck was euthanized a day prior to the Pekin ducks, and all tissues from the Muscovy duck (Brain, liver, blood and intestine) were stored at -20°C. All tissues collected from Pekin ducks (Lung, brain, spleen, liver and blood) were kept on dry ice for approximately 5 hours before being stored at -80°C. One sample of

blood from the Pekin ducks was kept on ice for approximately 5 hours before being smeared and dried on glass slides.

To visually identify parasites present in blood samples, blood smears were stained using PROTOCOL™ Wright-Giemsa Stain Solutions (FisherScientific) using the manufacturer's instructions. Slides were visualized using a microscope under 40x magnification, and images were obtained using a Samsung Galaxy S10+ cell phone. Images were edited for clarity using Adobe photoshop.

To test DNA samples for the presence of haemosporidian parasites, DNA samples were extracted from all tissues using DNeasy Blood & Tissue kit (Qiagen). Nested PCR was performed using the PCR primers previously defined by Pérez-Rodríguez and colleagues (Pérez-Rodríguez et al., 2013) and primers sequences used can be found in Table 1. These primers are designed to amplify the cytochrome b (*cytb*) gene of common haemosporidian parasites which normally infect birds (Including: *Plasmodium spp*., *Haemoproteus spp*., and *Leucocytozoon spp*.) (Pérez-Rodríguez et al., 2013). The First PCR reaction was performed using 60 ng DNA, 0.2 µm primers (IDT), 200 µM of dNTPS (ThermoFisher), 1x taq buffer (New England BioLabs), 1 U taq DNA polymerase (New England BioLabs), and nuclease free H_2O and were run in a thermocycler. The first PCR reaction conditions were 1 cycle of denaturation (95 °C for 3 minutes) followed by 25 cycles of denaturation (95 °C for 30 seconds), annealing (50 °C for 30 seconds) and extension (68 °C for 30 seconds). A final extension step (68 °C for 5 minutes) was followed by a holding step at 4 °C. The second internal amplification (Nested) PCR used 1 µL from the first reaction in place of the DNA and used the same recipe. The nested PCR used 35 cycles in the thermocycler. The second PCR was visualized on a 1 % agarose gel stained with ethidium bromide. Resulting bands were gel extracted using a PuroSPIN™ Gel Extraction Kit (Luna Nanotech), placed into a pCR 2.1 TOPO TA vector using a TOPO™ TA Cloning™ Kit for Subcloning (Invitrogen). Five clones from each infected duck were sent for Sanger sequencing.

To identify the species of parasite present, sequences were submitted to the BLASTn search database (www.ncbi.com). To visualize clones, these same sequences were aligned using MAFFT and alignments were visualized using JalView (Waterhouse et al., 2009).

A2.3 Results and discussion

To visually identify if any of the Pekin ducks sampled had blood parasites present, blood smears were stained with Wright-Geimsa stain and inspected at 40x resolution under the microscope (Figure A2.1). The Pekin duck without visible illness did not have any distinctive parasitic infection in the slides visualized, and only regular nucleated red blood cells (RBS) were visualized (Figure A2.1A). The blood smears from the 3-week-old Pekin duck presenting with illness had visible blood parasites present, likely Leucocytozoon, in both elongate and round shape (Figure A2.1B). Similarly, the blood smears from the 4-week-old Pekin duck had parasitic gametocytes present (Figure A2.1C). *Leucocytozoon spp*. are apicomplexan blood parasites of birds, with over 100 species of *Leucocytozoon* documented in as many different species of birds (Valkiūnas, 2005). Leucocytozoon gametocytes are often present in blood smears of infected birds in erythrocytes, mononuclear leukocytes and macrophages. *Leucocytozoon spp*. are thought to be vectored by biting flies belonging to the genus Simuliinae (Colloquially known as blackflies). The most commonly associated *Leucocytozoon spp.* in waterfowl such as ducks and geese is *Leucocytozoon simondi* (Valkiūnas, 2005; Wettere, 2020). While this parasite is known to have caused die-offs in wild Canada geese (*Branta canadensis maxima*) (Herman et al., 1975), it is more commonly fatal in young domestic ducks such as the Pekin duck (Khan et al., 1968).

To confirm the type of haemosporidian blood parasite present in the ducks is *Leucocytozoon*, nested PCR was performed on blood, liver and brain samples. All visibly ill ducks had positive bands from the PCR (Figure A2.2), while the control Pekin duck (The duck without visible illness) did not have amplification products from the PCR. The PCR amplicons from the ill ducks were sequenced, and BLAST analysis determined the *ctyb* gene fragment was from *Leucocytozoon spp.,* closest in similarity to *Leucocytozoon spp*. found in ducks and geese and is likely *Leucocytozoon simondi*.

To investigate if all infected ducks were infected by a single Leucocytozoon strain, we aligned the *cytb* sequences cloned from each duck. Interestingly, with only four to five clones successfully sequenced from each bird, we found polymorphisms in the sequences. The Muscovy duck had 6 differences in the DNA sequence (Figure A2.3A) which corresponded to 3 different translations (A2.3B). Likewise, the *Leucocytozoon cytb* sequences obtained from the 3-week-old infected Pekin had 5 base differences (Figure A2.4A) which corresponded to three different translations (Figure A2.4B). Finally, the *cytb* clones found in the 4-week-old infected Pekin duck

had the most differences in the DNA sequencing, with a count of 27 differences between the DNA sequences (Figure A2.5A), most of which were silent mutations which corresponded to four different translations of the *cytb* sequence (Figure A2.5B). While some of these SNPs could be a result of PCR error, most of the changes happen at similar positions, or they change specific amino acids in the sequences suggesting these differences are allelic variation or the result of different species of subtypes of *Leucocytozoon* infecting the same bird.

To determine if the *cytb* sequences amplified from different ducks clustered together, we aligned all nucleotide sequences together and created a Neighbor-joining tree. The sequences amplified from the infected Muscovy duck clustered together, while the sequences from both Pekin ducks clustered together, albeit with more distance between sequences (Figure A2.6). While this tree would suggest that the Muscovy and Pekin ducks were infected by different strains of parasite, or that one variation of the parasite was more successful in Muscovy than Pekin duck, an n=4-5 is not high enough to say definitively. To finish this work, more *cytb* clones from each infected bird should be amplified (n=25). Other *Leucocytozoon* specific genes from *Leucocytozoon spp.* could also be amplified and clones to investigate differences. Other studies into Leucocytozoon spp. genetic diversity in birds have also investigated cytochrome c oxidase I and II (*coxI* and *coxII*, respectively) (Nooroong et al., 2022). Additionally, the ducks used for sequencing in this study were all domestic species. To obtain a better understanding of Leucocytozoon diversity, blood samples from wild ducks could be sampled for haemosporidian parasites and sequenced.

The preliminary results of this study have found Leucocytozoon spp. (Most likely *Leucocytozoon simondi*) responsible for the abnormally high mortality of young Pekin and Muscovy ducks in Bonnyville Alberta.

Table A2.1. Primers used for nested PCR of haemosporidian parasite cytochrome b amplification. Primer combination defined by Perez-Rodríguez et all 2013.

Primer name	Sequence	Orientation	Target
Plas1F	5′-GAGAATTATGGAGTGGATGGTG-3′	Forward	Outer cytochrome b
HaemNR3	5′-ATAGAAAGATAAGAAATACCATTC-3′	Reverse	Outer cytochrome b
3760F	5′-GAGTGGATGGTGTTTTAGAT-3′	Forward	Internal cytochrome b
HaemJR4	5′-GAAATACCATTCTGGAACAATATG-3′	Reverse	Internal cytochrome b

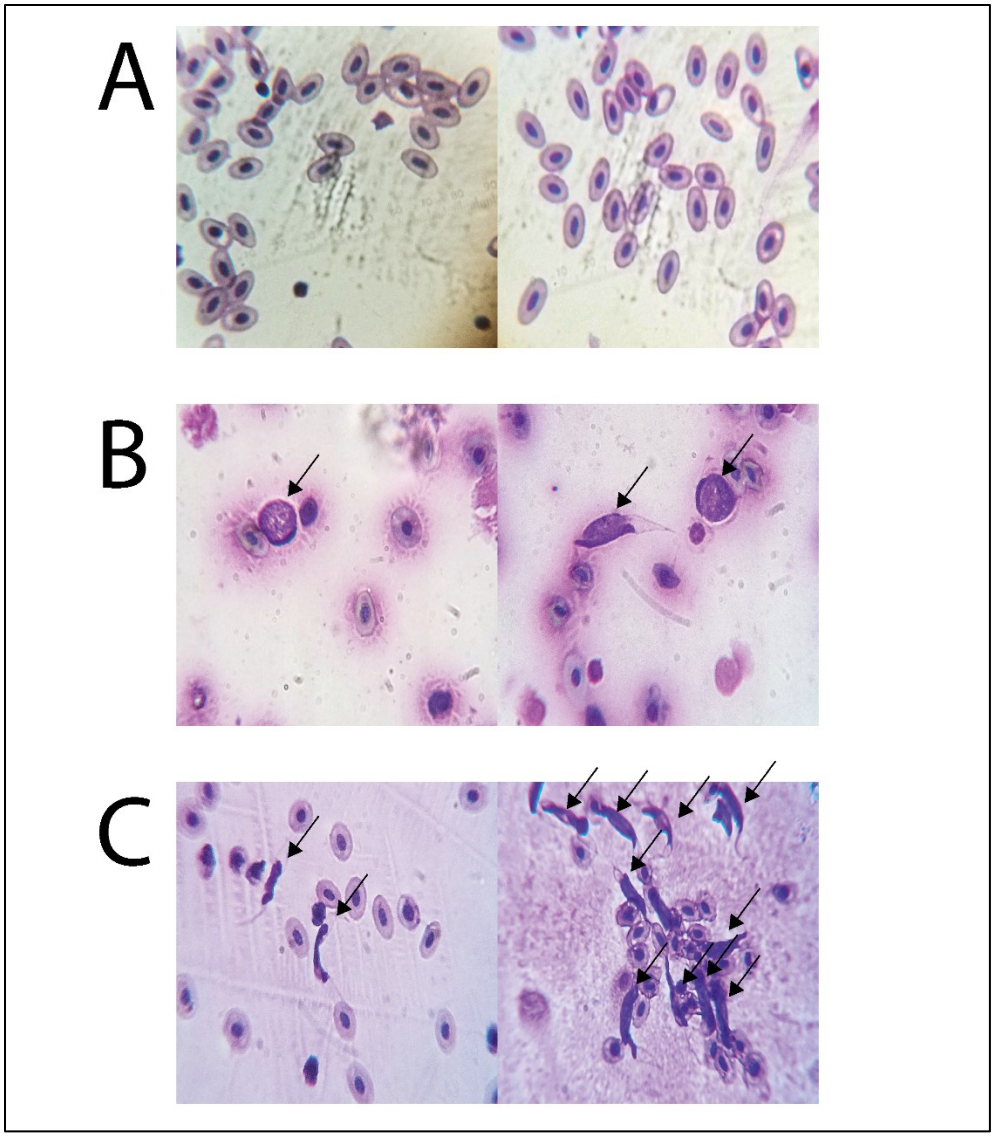


Figure A2.1. Blood smears from 3- and 4-week old Pekin ducks show *Leucocytozoon ssp*. Blood samples were obtained from recently euthanized 3- and 4-week old Pekin ducks presenting with severe and debilitating illness. Blood was smeared on glass slides and stained with H&E before visualization under 40x magnification using a microscope, images were taken using a Samsung Galaxy S10 cell phone. Arrows point to *Leukocytozoon* ssp. Gametocytes in both elongate and round form present in blood

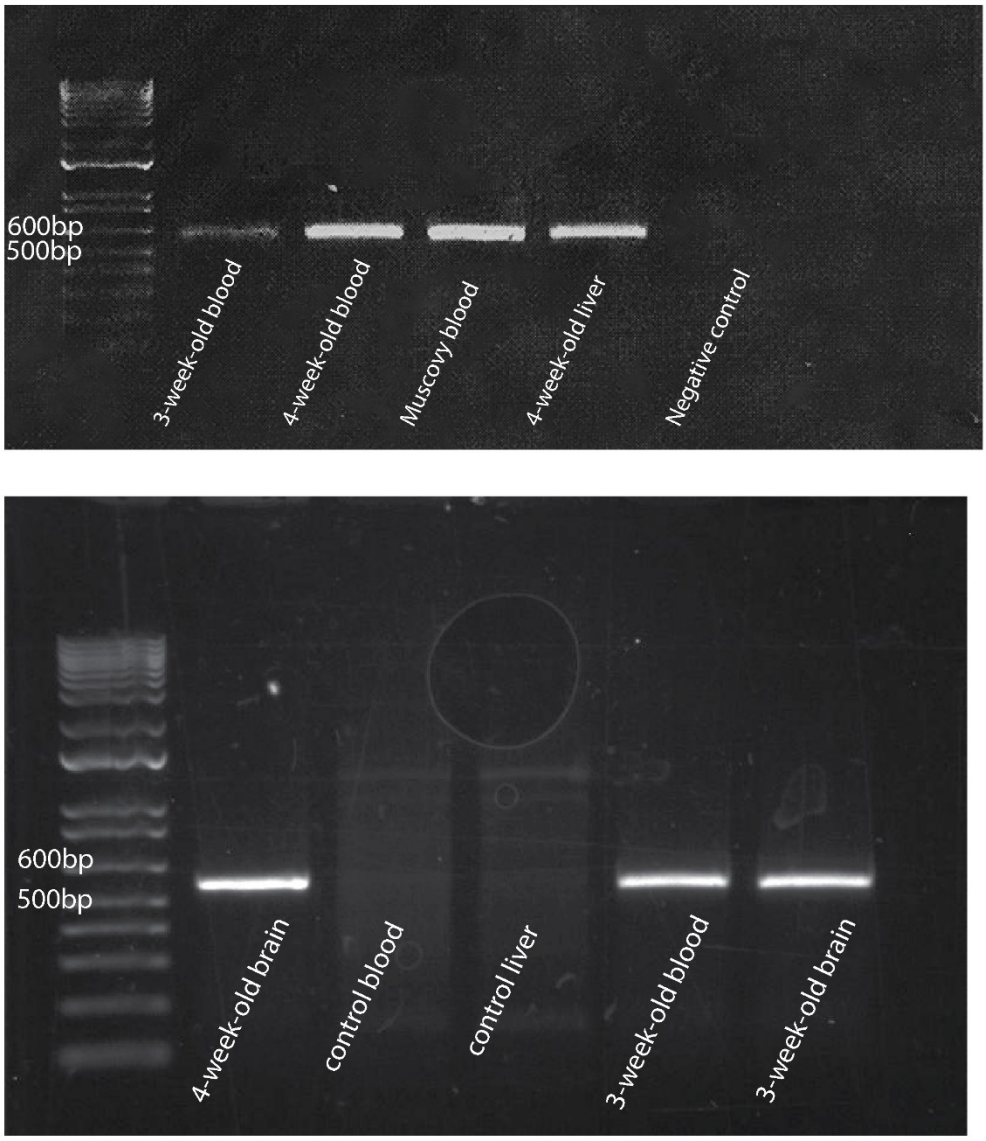
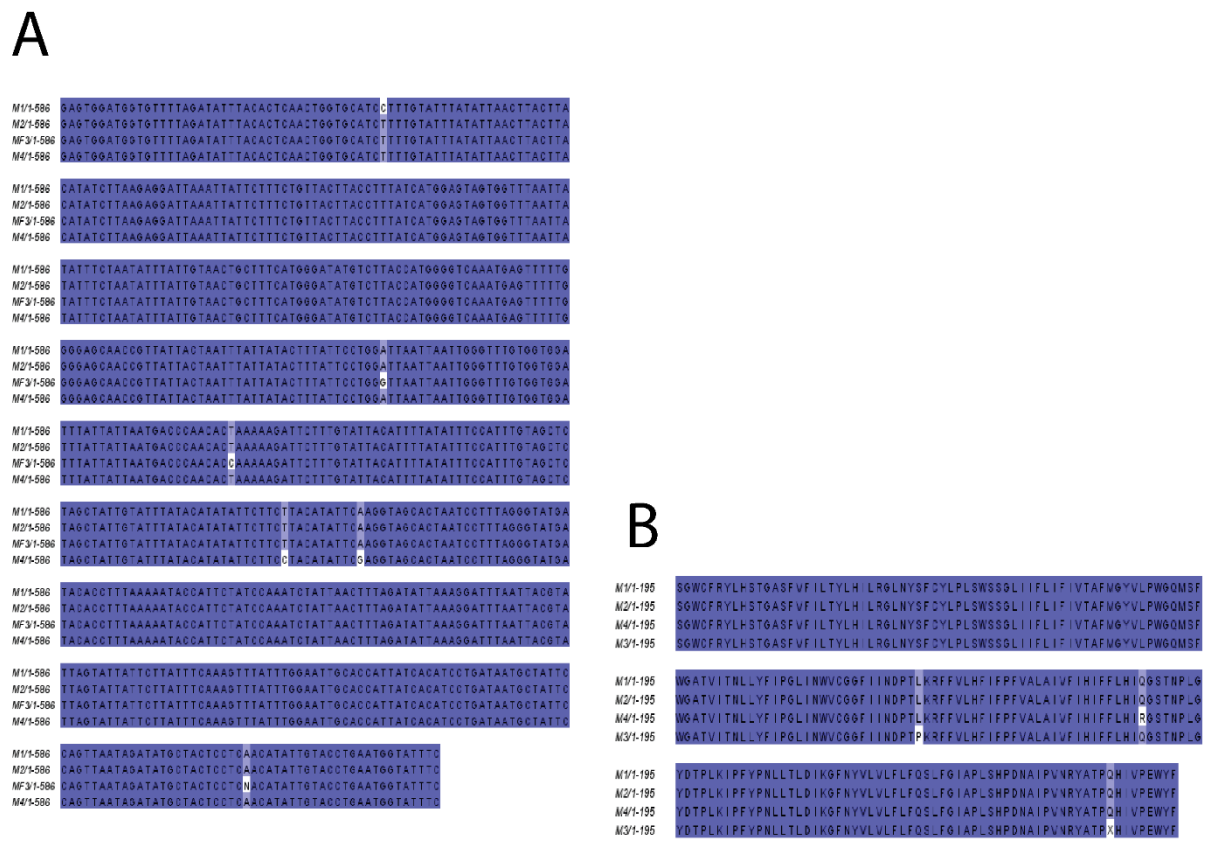


Figure A2.2. Nested PCR confirms presence of haemosporidian parasite cytochrome b gene in visibly ill Pekin and Muscovy ducks. Nested PCR was performed on DNA obtained from two visibly ill Pekin ducks (3- and 4-week-old), one visibly ill Muscovy duck and one healthy Pekin duck (control). Expected band size of the second (nested) PCR fragment was ~542bp. Control birds did not appear to have any parasites present in either blood or liver.



.

Figure A2.3. Single nucleotide or amino acid changes in *Leucocytozoon simondi cytb* sequences from Muscovy duck. Five clones resulting from nested PCR to detect *Leucocytozoon cytb* in liver from sick Muscovy duck were sequenced using Sanger sequencing. The resulting four clones positive for *cytb* were aligned using the MAFFT websever and visualized using JalView (A) or translated into amino acid (B) and aligned and visualized using MAFFT and JalView.

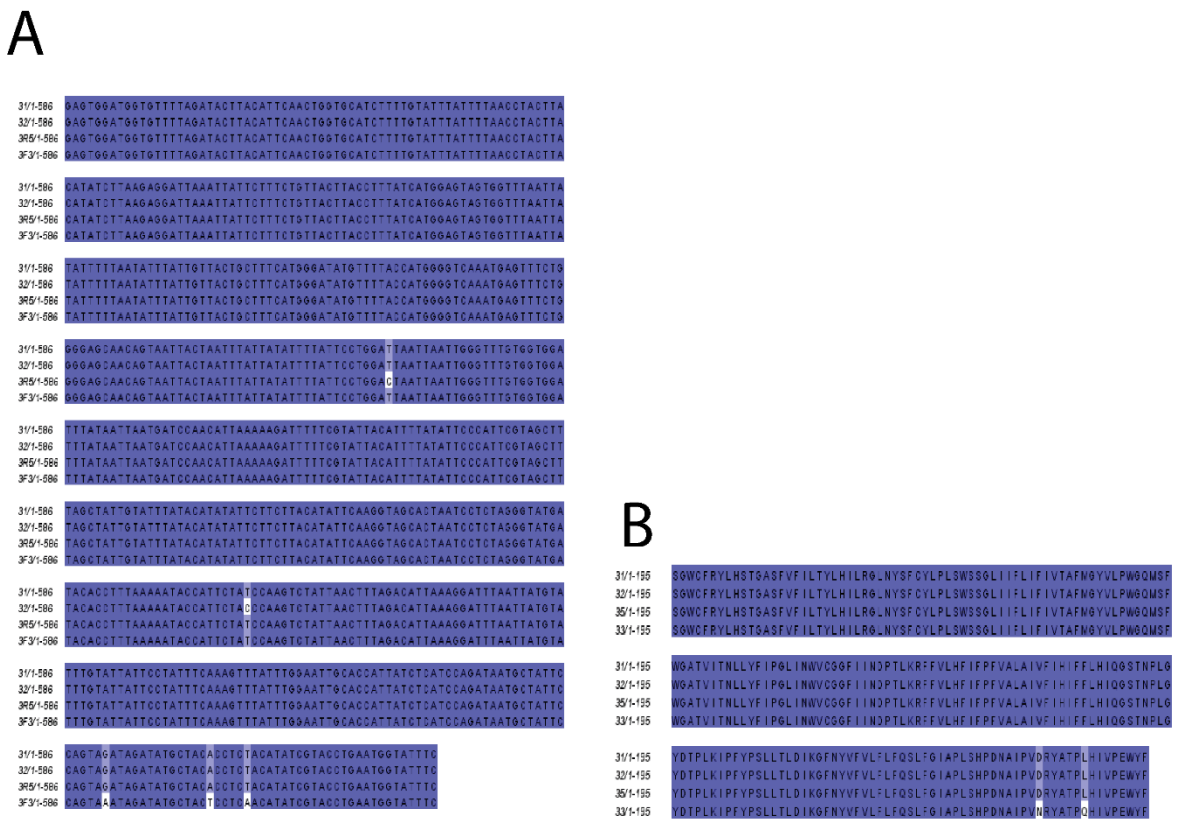


Figure A2.4. Single nucleotide or amino acid changes in *Leucocytozoon simondi cytb* sequences from A 3-week old Pekin duck. Five clones resulting from nested PCR to detect *Leucocytozoon cytb* in liver from sick Muscovy duck were sequenced using Sanger sequencing. The resulting four clones positive for *cytb* were aligned using the MAFFT webserver and visualized using JalView (A) or translated into amino acid (B) and aligned and visualized using MAFFT and JalView.

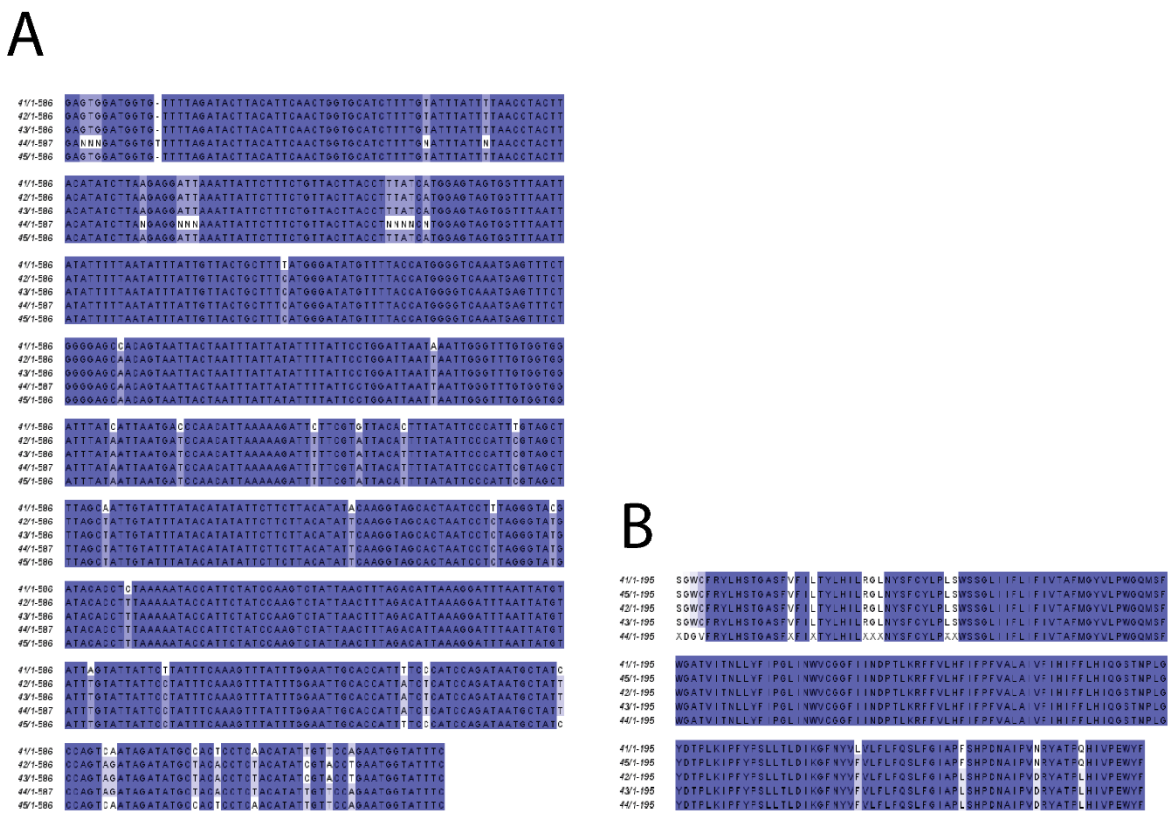


Figure A2.5. Single nucleotide or amino acid changes in *Leucocytozoon simondi cytb* sequences from a 4-week old Pekin duck. Five clones resulting from nested PCR to detect *Leucocytozoon cytb* in liver from sick Muscovy duck were sequenced using Sanger sequencing. The resulting five clones positive for *cytb* were aligned using the MAFFT webserver and visualized using JalView (A) or translated into amino acid (B) and aligned and visualized using MAFFT and JalView.

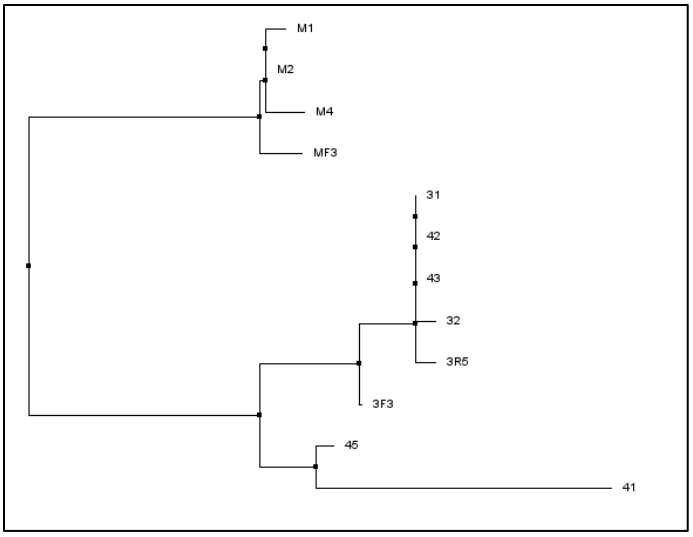


Figure A2.6. *Leucocytozoon cytb* clusters based on the breed of duck *cytb* gene was amplified from. All nucleotide sequences of the *cytb* gene amplified from infected Muscovy and Pekin ducks were aligned using the MAFFT webserver, and a neighbor-joining tree was created using JalView. Cytochrome b sequences originating from the infected Muscovy are designated M#, while *cytb* sequences originating from the 3- and 4-week-old Pekin ducks were designated 3# and 4# (Respectively). The letters "R" or "F" denote that the sequencing was performed using a reverse or forward primer, but not both. The sequence from clone 44 was excluded due to having too many unresolved nucleotides.

A3 MOWOIL mounting media recipe

This protocol is adapted from a protocol obtained from The BioImaging Facility at the University of British Columbia.

Reagents needed:

- Mowiol 4-88 (Sigma #81381).
- DABCO (Sigma # D 2522) (1,4-diazabicyclo[2.2.2]octane or triethylenediamine)
- 0.2 M Tris, pH 8.5
- Glycerol
- Distilled water

To prepare Mowiol:

• Measure out 2.4 g Mowiol and resuspend in 6 mL of glycerol. Add 6 ml of distilled water and stir slowly at room temperature overnight

•Add 12ml of 0.2 M Tris, pH 8.5. Heat the buffer to 50° before next step

• Heat to 50°C while slowly mixing for 15 minutes (or until the Mowiol is dissolved. Can be kept at 50° for up to 1 hour). Clear the solution by centrifugation at 5000 x g for 15 minutes at 4 °C. Decant supernatant (Pellet is invisible. Decant slowly)

• DABCO is added to 2.5 % final concentration and slowly stirred to avoid air bubbles. Invert cylinder to mix. Leave cylinder at 4 °C for 15 mins to allow air bubbles to rise to the top and then aliquot and store at -20 °C

• Aliquot the Mowiol in 1.5 mL epptubes and freeze at -20 °C. Once thawed the Mowiol will remain liquid at 4 °C for a couple of weeks. It can be refrozen after use to prolong the lifespan. To use, add a drop of Mowiol to the slide, coverslip and allow polymerizing in a dry environment for at least 1 hour. Keep the slide dry, dark and at 4°C and your fluorescence should last for months.

A4 TRIM27L Mass spectrometry results

Table A4.1. Mass spectrometric analysis of co-immunoprecipitation using TRIM27L-V5 as bait. Results show TRIM27L and background detection of TRIM27.1 and Vimentin. PSMs value shows relative amounts of proteins found in samples and suggests TRIM27L is associating with other TRIM27L proteins.

Accession	Description	Score	Coverage	# Proteins	# Unique Peptides	# Peptides	# PSMs	# AAs	MW [kDa]	calc. pI
TRIM27-L	TRIM27-L	254.58	57.26	1	24	24	163	489	55.89	7.69
A0A1L1RXL9	Vimentin OS=Gallus gallus GN=VIM PE=3 SV=1 - [A0A1L1RXL9_CHICK]	6.68	5.87	2	2	2	2	392	45.98	4.84

A5 References

Davison, T.F. (2003). The immunologists' debt to the chicken. *British Poultry Science* 44(1)**,** 6-21. doi: 10.1080/0007166031000085364.

García-Posadas, L., Arranz-Valsero, I., López-García, A., Soriano-Romaní, L., and Diebold, Y. (2013). A new human primary epithelial cell culture model to study conjunctival inflammation. *Investigative Ophthalmology & Visual Science* 54(10)**,** 7143-7152. doi: 10.1167/iovs.13-12866.

Herman, C.M., Barrow, J.H., Jr., and Tarshis, I.B. (1975). Leucocytozoonosis in Canada geese at the Seney National Wildlife Refuge. *J Wildl Dis* 11(3)**,** 404-411. doi: 10.7589/0090-3558-11.3.404.

Huth, S., Lausier, J., Rudolph, C., Planck, C., Gersting, S., Welsch, U., et al. (Year). "Characterisation of the Mechanism of Magnetofection", in: *MOLECULAR THERAPY*: ACADEMIC PRESS INC ELSEVIER SCIENCE 525 B ST, STE 1900, SAN DIEGO, CA 92101 …), S372-S372.

Ishiguro, K., Watanabe, O., Nakamura, M., Yamamura, T., Matsushita, M., Goto, H., et al. (2017). Combinational use of lipid-based reagents for efficient transfection of primary fibroblasts and hepatoblasts. *Biotechniques* 63(1)**,** 37-39. doi: 10.2144/000114569.

Jordan, I., Vos, A., Beilfuß, S., Neubert, A., Breul, S., and Sandig, V. (2009). An avian cell line designed for production of highly attenuated viruses. *Vaccine* 27(5)**,** 748-756. doi: https://doi.org/10.1016/j.vaccine.2008.11.066.

Khan, R.A., and Fallis, A.M. (1968). Comparison of infections with *Leucocytozoon simondi* in black ducks (*Anas rubripes*), mallards (*Anas platyrhynchos)* and white Pekins (*Anas bochas*). *Can J Zool* 46(4)**,** 773-780. doi: 10.1139/z68-107.

Loye, J.E., and Zuk, M. (1991). "Bird-parasite interactions: ecology, evolution and behaviour," in *Bird-parasite interactions: ecology, evolution and behaviour*. Oxford University Press; Ornithology Series, 2).

Nooroong, P., Watthanadirek, A., Minsakorn, S., Poolsawat, N., Junsiri, W., Srionrod, N., et al. (2022). Molecular genetic diversity and bioinformatic analysis of *Leucocytozoon sabrazesi* based on the mitochondrial genes *cytb*, *coxI* and *coxIII* and co-infection of *Plasmodium spp*. *Parasite* 29**,** 22. doi: 10.1051/parasite/2022022.

Pal, K., and Grover, P.L. (1983). A simple method for the removal of contaminating fibroblasts from cultures of rat mammary epithelial cells. *Cell Biol Int Rep* 7(10)**,** 779-783. doi: 10.1016/0309-1651(83)90181-9.

Pantin-Jackwood, M., Swayne, D.E., Smith, D., and Shepherd, E. (2013). Effect of species, breed and route of virus inoculation on the pathogenicity of H5N1 highly pathogenic influenza (HPAI) viruses in domestic ducks. *Vet Res* 44(1)**,** 62. doi: 10.1186/1297-9716-44-62.

Pérez-Rodríguez, A., de la Puente, J., Onrubia, A., and Pérez-Tris, J. (2013). Molecular characterization of haemosporidian parasites from kites of the genus Milvus (Aves: *Accipitridae*). *International Journal for Parasitology* 43(5)**,** 381-387. doi: https://doi.org/10.1016/j.ijpara.2012.12.007.

Plank, C., Zelphati, O., and Mykhaylyk, O. (2011). Magnetically enhanced nucleic acid delivery. Ten years of magnetofection-progress and prospects. *Adv Drug Deliv Rev* 63(14-15)**,** 1300-1331. doi: 10.1016/j.addr.2011.08.002.

Vainauska, D., Kozireva, S., Karpovs, A., Čistjakovs, M., and Bariševs, M. (2012). A novel approach for nucleic acid delivery into cancer cells. *Medicina (Kaunas)* 48(6)**,** 324-329.

Valkiūnas, G. (2005). *Avian Malaria Parasites and Other Haemosporidia.* Boca Raton: CRC Press.

Wang, W., Said, A., Wang, B., Qu, G., Xu, Q., Liu, B., et al. (2018). Establishment and evaluation of the goose embryo epithelial (GEE) cell line as a new model for propagation of avian viruses. *PLOS ONE* 13(3)**,** e0193876. doi: 10.1371/journal.pone.0193876.

Wang, W., Said, A., Wang, Y., Fu, Q., Xiao, Y., Lv, S., et al. (2016). Establishment and characterization of duck embryo epithelial (DEE) cell line and its use as a new approach toward DHAV-1 propagation and vaccine development. *Virus Res* 213**,** 260-268. doi: 10.1016/j.virusres.2015.12.021.

Waterhouse, A.M., Procter, J.B., Martin, D.M.A., Clamp, M., and Barton, G.J. (2009). Jalview Version 2—a multiple sequence alignment editor and analysis workbench. *Bioinformatics* 25(9)**,** 1189-1191. doi: 10.1093/bioinformatics/btp033.

Wettere, A.J.V. (2020). *Leucocytozoonosis in Poultry* [Online]. https://www.merckvetmanual.com/: Merck Veterinary Manual Available: https://www.merckvetmanual.com/poultry/bloodborne-organisms/leucocytozoonosis-in-poultry [Accessed July 16 2022].