# Influenza A virus interference in the mallard duck (*Anas platyrhynchos)* RIG-I pathway

by

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

Doctor of Philosophy

in

Physiology, Cell and Developmental Biology

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## Abstract

Influenza A viruses are a major cause of human and animal disease worldwide. Periodically, avian influenza A viruses from wild waterfowl, like ducks, pass through intermediate agricultural hosts and emerge into the human population as zoonotic diseases with high mortality rates and pandemic potential. Dabbling ducks are the primordial hosts of influenza A viruses and remain an important ecological reservoir. Presumably because of their long co-evolutionary history, ducks are uniquely resistant to influenza disease compared to other birds, animals, and humans. However, relatively little is known about host-pathogen interactions in these hosts that may be contributing to their resistance. Appropriate innate immune signaling, especially through the RIG-I pathway, appears to be crucial for controlling viral replication early and for the survival advantage of ducks infected with highly-pathogenic influenza A viruses. To discover potential unique adaptations between ducks and influenza A viruses, I investigated the interactions of three viral proteins with the duck RIG-I pathway and compared to what is known about these interactions in humans and other hosts.

The non-structural protein 1 (NS1) of influenza A viruses is an important virulence factor that regulates viral replication and controls host cell immune responses. In human cells, NS1 proteins inhibit the induction of innate immune signaling and type-I interferon by preventing the activation of the RIG-I receptor by the ubiquitin ligase TRIM25. It is unclear whether the inhibition of human TRIM25 is a universal function of all influenza A NS1 proteins or is strain-dependent. It is also unclear if NS1 proteins similarly target the TRIM25 and RIG-I pathway of mallard ducks. To answer these questions, I compared the ability of five different NS1 proteins to interact with human and duck TRIM25 and the consequences this had on RIG-I ubiquitination

and signaling in both species. I show that allele A and allele B NS1 proteins from low-pathogenic and highly pathogenic avian influenza viruses efficiently inhibit RIG-I ubiquitination and interferon induction in human cells. In contrast, avian NS1 proteins do not affect duck RIG-I ubiquitination and signaling, despite interacting with duck TRIM25 in co-immunoprecipitations. Avian influenza viruses may lack the ability to dampen RIG-I signaling in ducks, as they do in humans. I also show that a variant NS1 from A/Puerto Rico/8/1934 (H1N1) can enhance TRIM25-mediated human RIG-I ubiquitination, but inhibits interferon by a TRIM25-independent mechanism that involves NS1 residue glycine-184.

PB1-F2 is an accessory protein encoded by most, but not all influenza A virus strains. Several immunomodulatory functions are ascribed to PB1-F2 in mammalian cells, including an interaction with the signal transducer MAVS to inhibit type-I interferon induction. In collaboration with a colleague, I show that PB1-F2 from A/Puerto Rico/8/1934 (H1N1) interacts with duck MAVS and inhibits interferon signaling in avian cells. I also show that PB1-F2 is capable of suppressing TRIM25-mediated RIG-I ubiquitination of both human and duck orthologues. These interactions of PB1-F2 provide an example of the conservation of function across host species.

Finally, the viral polymerase subunit PB2 is also known to traffic to mitochondria and interact with MAVS to suppress interferon in human cells. Mitochondrial localization, but not MAVS interaction specifically, depends on a host-species-specific amino acid polymorphism at position 9 in the PB2 peptide. With a colleague, I show that several avian and one human PB2 proteins all interact with duck MAVS irrespective of the substitution at position 9 or at two other species-specific polymorphism sites close by. However, interferon inhibition efficiencies

differed between the PB2 proteins and suggest the involvement of one or more of the polymorphisms in mitochondrial targeting, as in human cells.

This thesis addresses the understudied subject of host-pathogen interactions of influenza A viruses in their natural host – mallard ducks. The results contribute to our understanding of the mechanisms of host-pathogen co-adaptation and the selective pressures that drive influenza A virus evolution in the reservoir host.

## Preface

Parts of the introduction (Chapter 1) of this thesis, specifically portions of sections 1.1.2, 1.2, and 1.3 were adapted from a review article previously published as Evseev, D., and Magor, K.E. (2019). "Innate Immune Responses to Avian Influenza Viruses in Ducks and Chickens." Vet. Sci. 6(1): pii E5. I researched and wrote that manuscript with editing and input from Dr. Katharine Magor.

A portion of Chapter 3, specifically figure 3.14, has been previously published as 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: 156-165. The experiments in this figure were performed by Adam Caulfield and Matthew Reeves, undergraduate students under my supervision. We worked together to plan, perform, and analyze the experiments.

Parts of Chapter 4 have been previously published as Xiao, Y., Evseev, D., Stevens, C.A., Moghrabi, A., Miranzo-Navarro, D., Fleming-Canepa, X., Tetrault, D.G., and Magor, K.E. (2020). "Influenza PB1-F2 inhibits avian MAVS signaling." Viruses 12(4): 409. The experiments were designed and performed by Dr. Yanna Xiao, Adam Moghrabi, myself, and Chase Stevens, an undergraduate student under my supervision. The manuscript was written by Dr. Yanna Xiao and myself, with editing and input from Dr. Katharine Magor. Dr. Yanna Xiao designed and performed the experiments in figures S1, S2, and most of S3; Adam Moghrabi contributed experiments to figures S2 and S3. Chase Stevens put together figure 4.1 with my help. We worked together to plan, perform, and analyze the experiments. I contributed panel E to figure S3, and the entirety of figures 4.2 – 4.4.

Chapter 5 is the product of a joint effort with a fellow graduate student, David Tetrault (MSc), who contributed panels B and C to figure 5.1, and panel A to figure 5.2.

## Acknowledgements

I would like to thank my supervisor, Dr. Katharine Magor, for her unwavering support, and for providing an excellent environment and all of the tools that I needed to succeed. Thank you, Kathy, for your guidance, encouragement, and knowledge you have shared with me over the past seven years. Thank you for helping me to grow as a scientist and as a person, for always believing that I could do better.

Thank you to my committee members, Dr. Mike Belosevic and Dr. James Stafford, for your time and constructive feedback. Thank you for answering my endless questions and helping me to overcome roadblocks by sharing your ideas and perspectives.

Thank you to my colleagues Dr. Yanna Xiao and David Guy Tetrault for collaborating and combining your creative energies with me; for sharing the stress and the late-night discussions. Thank you to the undergraduate students who worked in the lab and contributed to this thesis – Adam Caulfield, Chase Stevens, Matthew Reeves, Michelle Lee and Leina Saito.

Thank you to all of the members of the lab, without your comradery this would have been an immeasurably difficult journey. Thank you to Dr. Domingo Miranzo-Navarro for mentoring me when I first arrived, and for teaching me the value of being meticulous and not cutting corners. Thank you also for the foundational work that you did that allowed me to pursue this project. Thank you to Ximena Fleming-Canepa for also mentoring me, helping my project to get started, and for being the most supportive friend anybody could ask for. You bring light and joy into the lab, but also discipline. Thank you Graham, Alysson, Karim, Eric, Michelle and Adam for the great friendship, expertise, and assistance.

Thank you to my funding sources. I was financially supported by the Department of Biological Sciences, GSA, FGSR, and QEII scholarships over the course of this degree.

Finally, thank you to my parents, for always supporting me, no matter what, and for being role models of hard work and perseverance. I hope that I make you proud.

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## List of Abbreviations

| | |
|---|---|
| ANP32 | Acidic leucine-rich nuclear phosphoprotein 32 |
| BC500 | A/Duck/British Columbia/500/2005 (H5N2) |
| CA431 | A/Chicken/California/431/2000 (H6N2) |
| CARD | Caspase activation and recruitment domain |
| CCD | Coiled-coil domain |
| CDS | Coding sequence |
| CMV | Cytomegalovirus |
| CNS | Central nervous system |
| CPSF4 | Cleavage and polyadenylation specificity factor 4 |
| Crm1 | Chromosome region maintenance 1 protein homolog |
| d2CARD | Duck RIG-I CARD domains |
| D4AT | A/Duck/D4AT/Thailand/71.1/2004 (H5N1) |
| DI | Defective interfering (particles) |
| DNA | Deoxyribonucleic acid |
| dpi | Days post infection |
| dsRNA | Double-stranded ribonucleic acid |
| ED | Effector domain |
| EID | Egg infectious dose |
| eIF2α | Eukaryotic translation initiation factor 2 alpha |
| eIF4GI | Eukaryotic translation initiation factor 4 gamma 1 |
| h2CARD | Human RIG-I CARD domains |
| HA | Hemagglutinin |

| | |
|---|---|
| HPAI | Highly-pathogenic avian influenza |
| hpi | Hours post infection |
| IAV | Influenza A virus |
| IFN | Interferon |
| IL | Interleukin |
| IRF | Interferon regulatory factor |
| LGP2 | Laboratory of genetics and physiology 2 |
| LPAI | Low-pathogenic avian influenza |
| M1 | Matrix 1 |
| M2 | Matrix 2 |
| MAVS | Mitochondrial antiviral-signaling protein |
| MDA5 | Melanoma differentiation-associated protein 5 |
| mRNA | messenger ribonucleic acid |
| NA | Neuraminidase |
| NEP | Nuclear export protein |
| NLR | NOD-like receptors |
| NLRP3 | The NOD-like receptor family, pyrin domain-containing 3 |
| NLS | Nuclear localization sequence |
| NP | Nucleoprotein |
| NS | Non-structural |
| NS1 | Non-structural protein 1 |
| OAS | 2′-5′-oligoadenylate synthetase |
| PA | Polymerase acidic |

| | |
|---|---|
| PAMP | Pathogen-associated molecular pattern |
| PB1 | Polymerase basic 1 |
| PB1-F2 | Polymerase basic 1 frame 2 |
| PB2 | Polymerase basic 2 |
| PBM | PDZ-binding motif |
| PCR | Polymerase chain reaction |
| pdm09(H1N1) | 2009 pandemic H1N1 |
| PDZ | post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1) |
| PKR | Protein kinase R |
| poly (I:C) | Polyinosinic:polycytidylic acid |
| PR8 | A/Puerto Rico/8/1934 (H1N1) |
| PRR | Pattern recognition receptor |
| qPCR | Quantitative polymerase chain recation |
| RBD | RNA-binding domain |
| RD | Repressor domain |
| RIG-I | Retinoic acid-inducible gene-I |
| RLR | RIG-I-like receptors |
| RNA | Ribonucleic acid |
| RNase L | RNase latent |
| RING | Really interesting new gene domain |
| RNF | RING finger protein |
| RNP | Ribonucleoprotein |

| | |
|---|---|
| SPRY | splA and ryanodine receptor domain |
| TRAF | TNF receptor-associated factor |
| TIR | The Toll/Il-1 receptor domain |
| TLR | Toll-like receptor |
| TM | Transmembrane domain |
| TNF-α | Tumour necrosis factor alpha |
| TRIM25 | Tripartite motif-containing protein 25 |
| VN1203 | A/Vietnam/1203/2004 (H5N1) |
| vRNA | Viral ribonucleic acid |
| vRNP | Viral ribonucleoprotein |
| WT | Wild-type |

# Chapter 1 Introduction

## 1.1 Influenza A virus

Influenza A virus is a member of the *Orthomyxoviridae* family (reviewed in Shaw and Palese, 2013), which includes four *Influenzavirus* genera (*A*,*B*,*C*, and *D*), *Thogotovirus*, *Isavirus*, and *Quaranfilvirus* (King et al., 2011; Su et al., 2017). Of the *Influenzavirus* genera, influenza A viruses are the most clinically significant, have the highest global disease burden, and pose the most serious pandemic threat (Glezen et al., 2013; Hessen, 2009; Monto, 1987; Nolan et al., 1980).

### *1.1.1 Structure and replication*

Influenza A virus (IAV) has a segmented genome consisting of eight single-stranded, negative-sense RNA segments (Palese et al., 1980) that encode up to seventeen proteins. Negative sense means that the genomic RNA segments are complementary to messenger RNA (mRNA) and can serve directly as templates for it (Baltimore, 1971). All IAV strains encode ten proteins: three viral polymerase components PB1, PB2, and PA (Palese et al., 1977); the outer surface proteins hemagglutinin (HA) and neuraminidase (NA) (Palese and Schulman, 1976); nucleoprotein (NP) (Ritchey et al., 1976); the structural matrix protein M1 and the outer surface ion channel M2, expressed from a single genomic segment by alternate splicing and an alternate reading frame (Allen et al., 1980; Lamb and Choppin, 1981; Lamb et al., 1981); the non-structural protein 1 (NS1) and nuclear export protein (NEP), which are also expressed from a single segment (Lamb et al., 1980). Up to seven accessory proteins have been identified that are not universally encoded by all strains. These are PB1-F2 (Chen et al., 2001), PB1-N40 (Wise et

al., 2009), PA-X (Jagger et al., 2012), PA-N155 and PA-N182 (Muramoto et al., 2013), M42 (Wise et al., 2012), and NS3 (Selman et al., 2012).

IAV is an enveloped virus. The virion consists of an outer layer of host-derived lipid membrane and an inner layer of M1 protein. The HA, NA, and M2 proteins stud the outer membrane surface. Inside the virion, the viral RNA (vRNA) segments are coated in nucleoprotein and are attached to the matrix layer (Noton et al., 2007). A trimeric viral polymerase complex is bound to each genomic segment, and all together this is called the viral ribonucleoprotein complex (vRNP) (Compans et al., 1972; Duesberg, 1969). Several nuclear export proteins are also packaged into the virion (Richardson and Akkina, 1991).

Infection begins when viral HA proteins bind glycoprotein receptors with terminal sialic acids on the host cell surface to initiate endocytosis-mediated entry (Matlin et al., 1981; Sieczkarski and Whittaker, 2002; Skehel and Wiley, 2000). Acidification of the endosome triggers a conformational change in HA that stimulates fusion of the viral and endocytic membranes. At the same time, the M2 ion channel directs protons into the virion to induce matrix disassembly and vRNP release – “uncoating” (Pinto and Lamb, 2006; Wharton et al., 1994). The vRNPs then enter host cell nucleus using nuclear localization sequences (NLS) found on all of its protein components (Cros et al., 2005; Jones et al., 1986; Nath and Nayak, 1990; Nieto et al., 1994; O'Neill et al., 1995). The viral polymerase accomplishes both mRNA synthesis and vRNA replication in the host cell nucleus (Beaton and Krug, 1986; Vreede and Brownlee, 2007). Messenger RNA synthesis begins with the snatching of 5' caps from host pre-mRNAs by the PB2 and PA proteins (Dias et al., 2009; Fechter et al., 2003). Negative sense viral genomic RNA is replicated through a complementary RNA intermediate, and depends on the availability of free NP (Beaton and Krug, 1986). New vRNA is encapsidated in nucleoprotein

and interacts with M1 and NEP to exit the nucleus by Crm1-dependent export (Huang et al., 2001; Neumann et al., 2000; Shimizu et al., 2011; Ye et al., 1999). Assembly occurs at the apical surface of polarized epithelial cells, where newly-synthesized HA, NA, and M2 proteins localize (Hughey et al., 1992; Jones et al., 1985; Roth et al., 1983). Budding begins with the accumulation of M1 and vRNP complexes at the plasma membrane (Noton et al., 2007). Virions are released from the cell when NA cleaves sialic acids off of cell surface glycoproteins (Colman et al., 1983; Palese and Compans, 1976; Palese et al., 1974). NA also cleaves sialic acids off of glycoproteins that are incorporated into the virion from the host cell membrane to prevent the clumping of virions (Palese and Compans, 1976). To mediate successful infection of new cells and successful release from infected cells, the HA and NA activities exist in a precise balance that is optimized for individual hosts and viruses (Wagner et al., 2002; Yen et al., 2011).

The HA and NA proteins are abundant on the viral surface and are thus the major antigenic determinants of host humoral immunity. For this reason there is strong selective pressure on the major antigenic sites of HA and NA that helps to generate a large diversity. Currently, 18 antigenically distinct HA subtypes (H1-18) and 11 antigenically distinct NA subtypes (N1-11) are known (CDC, 2019; WHO, 2018). These antigenic subtypes are used to classify viral strains, for example ‘H5N1’.

Influenzaviruses evolve by a complex combination of three mechanisms. The first, “genetic drift,” occurs through the natural accumulation of mutations produced by the relatively error-prone viral RNA polymerase (Parvin et al., 1986). The second mechanism, “genetic shift,” occurs when multiple strains infect the same cells and their segmented genomes are reassorted into new combinations in progeny virions. Unlike the gradual nature of genetic drift, genetic shift can produce large and abrupt changes and is the main driver of the appearance of new pandemic

strains. Members of different *Influenzavirus* genera do not re-assort with each other, demonstrating that they are truly "speciated" (Hause et al., 2014; King et al., 2011). Finally, non-homologous recombination can occur when the viral polymerase switches templates mid-replication and incorporates fragments from other viral RNA segments (Orlich et al., 1994; Pasick et al., 2005) or from host RNA species (Khatchikian et al., 1989). This can also play a significant role in the acquisition of virulence (Abdelwhab et al., 2013).

### *1.1.2 Waterfowl are the natural hosts of influenza A virus*

Influenza A viruses have a common evolutionary ancestor that arose in aquatic birds (reviewed in Webster et al., 1992; Yoon et al., 2014). Phylogenetic analyses of the individual gene segments suggest that all circulating human influenza A viruses have ancestors in the avian lineage (Gammelin et al., 1990; Gorman et al., 1991; Okazaki et al., 1989). The same is true for IAVs in other mammals (Guo et al., 1992; Li et al., 2010). The majority of known influenza A HA and NA subtypes circulate in wild ducks (reviewed in Yoon et al., 2014). H13 and H16 subtypes have not been isolated from ducks, but circulate in gulls (*Laridae* family) (Chambers et al., 1989; Kawaoka et al., 1988). Two HA and two NA subtypes of bat influenza viruses have recently been discovered (Tong et al., 2013), which appear to have been evolving in isolation for a long time (Mehle, 2014).

As the natural host, ducks have co-evolved with influenza A viruses to a state in which a high degree of viral replication is tolerated and damage to the host is limited (Webster et al., 1992). Infection occurs via the fecal-oral route in contaminated water, such as ponds. Ducks may become infected by drinking or eating in contaminated water, by dabbling and preening in it, and by the uptake of water through the cloaca (Wille et al., 2018). Low-pathogenicity avian influenza

viruses (LPAI) typically replicate in duck intestines, where up to $10^{8.7}$ $EID_{50}$ (egg infectious doses) of virus per gram of feces can be shed (Hinshaw et al., 1980; Webster et al., 1978). Mallard ducks (*Anas platyrhynchos*) have a higher prevalence of LPAI infection than other birds in the wild, harbour these viruses without signs of disease, and shed them into bodies of water (Jourdain et al., 2010; Kida et al., 1980; Olsen et al., 2006; Runstadler et al., 2007). The evidence that dabbling and diving ducks of the genus *Anas* are more frequently infected than other ducks and geese implies the importance of these behaviors in the maintenance of IAV in the wild (Munster et al., 2007). Influenza A viruses appear to be stable in cold water, such as ponds, for months (Stallknecht et al., 1990). In their seasonal migrations, ducks circulate influenza viruses along their migration pathways (Hulse-Post et al., 2005; Kilpatrick et al., 2006; Marchenko et al., 2012). Up to 20% of wild ducks at pre-migration staging areas in Canada may be positive for influenza before departure (Hinshaw et al., 1980). The prevalence drops in wintering areas (Stallknecht et al., 1990), but after migration, the arrival of new immunologically-naïve juveniles increases it again. In Sweden and Norway, the seasonal prevalence follows a similar pattern, with prevalence values of 3.4-6.5% reported in the spring (Wallensten et al., 2006), and 13% in autumn (Germundsson et al., 2010). The global patterns of influenza A virus prevalence in wild birds are reviewed in Olsen et al. (2006).

Human influenza pandemics begin with the introduction of new avian genes into mammalian viruses by reassortment (Guan et al., 2010; Smith et al., 2009a). The Asian H2N2 pandemic of 1957 began with the reassortment of a human virus with an avian H2N2 strain, acquiring its HA and NA genes (Schafer et al., 1993). The 1968 H3N2 pandemic was caused by a virus with avian HA and PB1 genes (Kawaoka et al., 1989; Scholtissek et al., 1978). The causative agent of the 2009 H1N1 influenza outbreak was a multiple-reassortant virus that contained avian PB2 and PA

genes in a human and swine background (Smith et al., 2009b). The origins of the infamous 1918 influenza pandemic virus are less clear, but it also appears to have arisen from a swine lineage that had previously been derived from an avian H1N1 virus (Anhlan et al., 2011; Gorman et al., 1991; Guan et al., 2010). The pandemic threat of influenza A viruses owes to the fact that the mammalian and avian pools can mix and successfully reassort in co-infected host 'mixing vessels', like pigs (as reviewed by Shapshak et al., 2011). This can generate viruses with novel antigenicities for which no immunity exists in the population, or viruses with unexpected replicative advantages or virulence characteristics.

Birds are also a source for the spontaneous emergence of highly-pathogenic avian influenza (HPAI) strains that cause severe disease and high death rates in people, but do not necessarily acquire the ability to transmit effectively between them. HPAI strains commonly arise in chickens through mutation of low-pathogenicity strains acquired from ducks. The ability to asymptomatically spread influenza A viruses to domestic poultry has earned ducks the moniker "Trojan horses of influenza" (Kim et al., 2009). By convention, avian influenza viruses are classified as low-pathogenic avian influenza (LPAI) or highly pathogenic (HPAI) based on their pathogenicity in domestic chickens (O.I.E., 2019). LPAI strains with H5 and H7 hemagglutinin subtypes can mutate in gallinaceous poultry to become highly pathogenic by acquiring a multi-basic cleavage site in the HA, a hallmark virulence factor (Pantin-Jackwood et al., 2016; Short et al., 2014; Smith et al., 2015). Influenza hemagglutinin must be cleaved at a specific site by host proteases to make the receptor functional for entry. The amino acid sequence at the HA cleavage site determines how easily it can be cut and by which host proteases. The HAs of LPAI viruses can be cleaved by transmembrane serine proteases and airway trypsin-like proteases, and this limited cleavability restricts infection to certain tissues (Bertram et al., 2010). The HAs of HPAI

viruses with polybasic cleavage sites that contain several consecutive arginine and lysine residues can be activated by a broader repertoire of ubiquitous proteases, such as furin and plasmin, and can thus infect a wider range of tissues and produce more serious diseases (Horimoto and Kawaoka, 1994; Rott, 1992; Stieneke-Gröber et al., 1992). In chickens, multibasic cleavage sites appear to arise by non-homologous recombination of HA genes with other influenza genome segments or with chicken ribosomal RNA (Abdelwhab et al., 2013). In chickens or quail, the H5 or H7 hemagglutinin subtypes also sometimes acquire mutations that make them more similar to those of human seasonal viruses, making the virus more likely to zoonotically infect humans (Matrosovich et al., 1999; Yamada et al., 2012).

Mallards are more resistant to disease caused by influenza A viruses, including highly pathogenic avian influenza viruses, than most other species (Alexander et al., 1986; Cooley et al., 1989; Laudert et al., 1993). However, even mallard ducks may succumb to fatal infection by certain strains of H5 HPAI viruses, particularly some strains belonging to the Eurasian lineage of HPAI H5 viruses tracing back to the Chinese strain A/goose/Guangdong/1/1996 (Chen et al., 2005; Kleyheeg et al., 2017; Njoto et al., 2018; Pantin-Jackwood et al., 2016). Poultry outbreaks and human infections with HPAI H5N1 strains of this lineage began occurring in large numbers in Southeast Asia between 2003 and 2004. Since then, the viruses have been spread to Africa, Europe, and North America, and have become endemic in poultry in China, Indonesia, Vietnam, India, Bangladesh, and Egypt (Food and Agriculture Organization of the United Nations, 2018). More recently, in 2014, a reassortant H5N8 that could kill domestic ducks caused several poultry outbreaks in South Korea (Lee et al., 2014), and arrived on the west coast of North America with migratory waterfowl later that same year (Pasick et al., 2015). It is worth noting, however, that the spread of highly pathogenic influenza strains results from both wild waterfowl migration and

human movement of infected poultry (Kilpatrick et al., 2006), and that the pathogenic strains do not appear to persist for long in wild birds in nature (Kim et al., 2009).

In summary, ducks are an important natural reservoir host of influenza A viruses. Their co-evolution has produced a unique relationship that allows ducks to act as asymptomatic carriers of LPAI viruses, and to tolerate HPAI virus infections to a greater extent than other species.

### *1.1.3 Host adaptation of influenza A viruses*

Influenza A virus genes accumulate host-specific polymorphisms over time in different hosts that may be adaptive. The PB2, PA, NP, M, and NS genes in particular fall into two distinct phylogenetic branches – an avian lineage and a swine/human lineage (Bean, 1984; Furuse et al., 2009; Gammelin et al., 1990; Gorman et al., 1991; Gorman et al., 1990a; Ito et al., 1991; Okazaki et al., 1989). Large-scale sequence analyses of mutation rates, comparing rates of synonymous versus non-synonymous substitutions, show that viruses in terrestrial poultry, pigs, and humans accumulate amino acid mutations faster than those in wild birds, suggesting that mammalian viruses are adapting more rapidly to these relatively newer hosts (Chen and Holmes, 2006; Furuse et al., 2009; Gorman et al., 1991; Gorman et al., 1990a; Gorman et al., 1990b; Shu et al., 1993; Sugita et al., 1991). The adaptive nature of these mutations is apparent in some cases, such as the alteration of HA binding specificity to accommodate the sialic acid linkages that predominate in human airways. The activities of the HA and NA proteins must be balanced for efficient viral budding and shedding (de Vries et al., 2020). Thus, additional glycolsylation sites in HA, which lower their affinity and render them more human-like tend to occur in tandem with truncations in the NA stalk, which reduces its activity (Matrosovich et al., 1999). In other cases, the function of species-specific polymorphisms is less apparent, as with the negative

selection pressure on amino acid positions 115 and 121 in the viral matrix protein (M1) (Furuse et al., 2009).

The ribonucleoprotein components NP, PA, and PB2 acquire a plurality of species-specific amino acid changes in mammalian hosts (Chen et al., 2006; Finkelstein et al., 2007; Gorman et al., 1991). Many of these polymorphisms fall within domains responsible for interactions between the vRNP components themselves (Chen et al., 2006; Finkelstein et al., 2007). Some enhance polymerase activity in mammalian cells, such as mutations G590S, Q591K/R, and E627K in the polymerase basic protein 2 (PB2) (Hussein et al., 2016; Manz et al., 2016; Mehle and Doudna, 2009; Subbarao et al., 1993). Other substitutions facilitate interaction with host co-factors like acidic leucine-rich nuclear phosphoprotein 32 (ANP32), a nuclear mRNA binding protein that appears to determine host-specificity of the viral polymerase (Hou et al., 2011; Long et al., 2016). Still another substitution affects an accessory function of PB2 – the inhibition of interferon signaling (Graef et al., 2010; Miotto et al., 2008). A subset of PB2 proteins in mammalian cells translocate out of the nucleus and to the mitochondria during infection and suppresses type-I interferon signaling. This activity depends on human-consensus residue asparagine-9 (N9), which replaces the avian-consensus serine-9 (S9). Substituting S9N in an avian H5N1 virus increased its pathogenicity in infected mice (Kim et al., 2010). Viral non-structural protein 1 (NS1) is the most important IAV interferon antagonist and its evolution is treated separately in **section 1.3**.

Complicating the picture, adaptive changes seem to occur at other levels besides protein. For example, two regions in the nucleotide sequence of positive-sense transcripts of the 8$^{th}$ genomic segment (NS) fold into hairpins with species-specific structural differences, the significance of which is still unclear (Vasin et al., 2016). Phylogenetic studies can identify host-

specific signatures faster than they can be characterized and so our understanding of the nature of influenza host-adaptation is far from complete. Furthermore, less attention has been paid to the functions of host-specific polymorphisms in aquatic birds and so we have a shallow understanding of the evolutionary pressures that act on IAV in the reservoir hosts. For this reason, this thesis aims to discover IAV host-pathogen interactions in avian cells.

## 1.2 Innate immune responses to influenza A virus

### *1.2.1 Type-I interferon*

In humans and animals, when death occurs from highly-pathogenic influenza A infection it is often a matter of days, too soon for the full activation of a primary adaptive immune response. Thus, innate immune responses are worth examining when looking for factors that determine influenza disease resistance and susceptibility. The innate immune system is the first line of defense once a pathogen breaches the body's physical barriers. It is an evolutionarily ancient, rapid first-response system that recognizes broad categories of pathogens and initiates non-specific defenses. Innate immune signaling depends on the recognition of non-specific pathogen-associated molecular patterns (PAMPs). Different families of receptors recognize different categories of PAMPs that are broadly shared by pathogens within that category – for example, viral RNA intermediates. Once activated, innate immune signals induce defense systems in the cells surrounding the infection, recruit white blood cells to the site of injury, and direct the mobilization of specific adaptive immune responses.

Type-I interferon (IFN) is a category of related cytokines (signaling molecules) that are produced when the innate immune system detects viral infection. The antiviral effect of interferon in mice was demonstrated with intravenous and intramuscular injections of exogenous

interferon (Finter, 1966, 1967) and with the administration of interferon-neutralizing antiserum (Gresser et al., 1976a; Gresser et al., 1976b). There is a lot of evidence in humans and mice that an early type I interferon response is important for controlling influenza A virus (Haller et al., 1987; Haller et al., 1981; Jørgensen et al., 2018; Koerner et al., 2007; Krishna et al., 2015; Tumpey et al., 2007; Zeng et al., 2007). Pre-treatment of cultured cells with type-I interferon significantly reduces viral replication.

The two most prevalent type-I interferons are interferon-alpha (IFN-α) and interferon-beta (IFN-β). IFN-β is produced by epithelial cells at the initial site of infection and acts in a paracrine fashion to induce an anti-viral state in the surrounding cells. It does this by stimulating the expression of many multifunctional interferon-stimulated genes (ISGs) that modulate host cell metabolism and interact with viral components directly to suppress replication (Alsharifi et al., 2008; Iwasaki and Pillai, 2014; Jiang et al., 2011; Perry et al., 2005; Schoggins et al., 2011; Schultz et al., 2004). Infiltrating leukocytes produce IFN-α (Wyde et al., 1982) and there is a subset of leukocytes called plasmacytoid dendritic cells (pDCs) that secrete large amounts of IFN-α into the circulatory system (Ito et al., 2005; Liu, 2005). Plasmacytoid dendritic cells can detect viral RNA from directly endocytosed viral particles (Diebold et al., 2014), but some evidence suggests that circulating leukocytes that secrete IFN-α may not necessarily act as sentinels of infection but become activated secondarily and serve to amplify the immune response and modulate T-cell activity (Colonna et al., 2004). All type-I interferons signal through the same receptor on target cells.

Type-I interferon production is most important early in a viral infection, as a first checkpoint to delay viral replication and spread while other components of immunity become activated. This was demonstrated by Finter (1966) who found that administration of exogenous interferon before

or simultaneously with arbovirus infection in mice reduced viral replication, but administration seven hours post infection did not. Ducks typically manifest a rapid upregulation of type I interferon in response to HPAI infection, which peaks within the first day. Pekin ducks infected with highly pathogenic H5N1 viruses upregulated *IFNA* and *IFNB* genes early, by 1 day post infection (dpi), in lungs and spleens, by qPCR (Cagle et al., 2012; Saito et al., 2018; Vanderven et al., 2012). The interferon spikes dropped off by the second and third days of infection, but a sustained induction of key ISGs remained (Saito et al., 2018). A transcriptome analysis of H5N1-infected ducks also showed a large and early interferon response underway in the lungs at 1 dpi and the induction of ISGs persistent through 3 dpi (Smith et al., 2015). The ducks also produced interferon in their ilea, though not as rapidly as in their lungs, and ISG induction in both tissues was higher than in similarly infected chickens. The transcriptome comparison showed that the first responses to influenza in chickens and ducks are dramatically different—the genes expressed in the chickens were mostly related to T-cell and B-cell activation, whereas the early genes expressed in ducks were related to pathogen-associated molecular pattern recognition and interferon signaling. Direct comparison was complicated by the need to challenge highly susceptible chickens with a much lower infecting dose than ducks. Kumar et al. (2017) infected domestic ducks with two highly pathogenic IAV strains and found that ISG expression correlates inversely with disease severity. Muscovy ducks, which are more susceptible to influenza disease, have higher pro-inflammatory responses to HPAI viruses compared to Pekin and wild mallard ducks, and have lower IFN-α responses in their spleens (Cagle et al., 2012). Recombinant duck IFN-α was recently shown to inhibit influenza virus replication in duck fibroblasts *in vitro*, and to stimulate the expression of ISGs in the brains and spleens of treated ducks *in vivo* (Gao et al.,

2018). Thus, a rapid type-I interferon response appears to contribute to viral clearance and survival of ducks.

### *1.2.2 Inflammation and immunopathology*

Innate immune signaling is not necessarily always protective, but can also be implicated in influenza pathogenesis (Damjanovic et al., 2012). Immunopathology occurs from aberrant and prolonged pro-inflammatory signaling that leads to tissue damage. Acute inflammation is a broad response to cellular damage or infection in which physical and chemical signals bring an influx of immune cells and fluid from the circulatory system to the site of injury (Ryan and Majno, 1977). Many pro-inflammatory cytokines, such as IL-6 and TNF-α, are released by stressed cells to induce leakiness in the capillaries around the injury and to act as chemoattractants and stimulators to macrophages, neutrophils, and other leukocytes, which can then combat pathogens and clear out debris. However, excessive infiltration of cells and fluid into the submucosal tissues of the airways can destroy tissues, compromise oxygen exchange, and produce hypoxia and respiratory distress syndrome, which have been implicated in severe human H5N1 infections (Peper and Van Campen, 1995; Uyeki, 2009; Ware and Matthay, 2000) and in the pathogenesis of the 1918 "Spanish" influenza (Loo and Gale, 2007). Uncontrolled inflammation in other sensitive tissues, like the central nervous system (CNS), can be equally dangerous (Lucas et al., 2006). Since inflammation is a complex and multifactorial process, many influenza virus studies look at the induction of pro-inflammatory cytokines specifically as a proxy of inflammation in general. An excessive pro-inflammatory response, dubbed "cytokine storm," is suspected to be a major disease mechanism in fulminant highly-pathogenic influenza infections of humans (Cheung et al., 2002; Hagau et al., 2010; Yuen and Wong, 2005), mice (Imai et al., 2008; Sun et al., 2011; Teijaro et al., 2011; Teijaro et al., 2014), and birds, including ducks (Burggraaf et al.,

2014; Wei et al., 2013). In a review of experimental data we found that high levels of pro-inflammatory cytokines tended to correlate with symptom severity in ducks, but that they were less prone to hyper-inflammation in response to influenza than other bird species (Evseev and Magor, 2019).

The relationship between type I interferon and inflammation is complex and involves a lot of context-dependent cross-talk, as reviewed by Trinchieri (2010). In infected animals with inflammatory pathology in the lungs, type-I interferon levels tend to be elevated along with the pro-inflammatory cytokines (Cao et al., 2017; Moulin et al., 2011; Penski et al., 2011; Soubies et al., 2010a). In other situations, type I interferon can oppose pro-inflammatory signaling (Billiau, 2006; Guarda et al., 2011). In the context of influenza infection, type I interferon signaling can promote the secretion of the anti-inflammatory cytokine IL-10 (Arimori et al., 2013) and can oppose neutrophil recruitment (Shahangian et al., 2009). Over all, it seems that a rapid type I interferon response at the site of infection is necessary to limit viral establishment early, but that the innate immune response must not cascade out of control into aberrant activation. In mallard ducks, a well-balanced and temporally limited innate immune response, fine-tuned by a long co-evolutionary history, probably contributes to influenza disease resistance.

### *1.2.3 Pattern recognition receptors*

Innate immune signaling begins when pathogen-associated molecular patterns are recognized by pattern recognition receptors (PRRs). Three classes of PRRs are involved in influenza virus recognition: the membrane-bound Toll-like receptors (TLRs) and two classes of cytoplasmic PRRs – the NOD-like receptors (NLRs) and the RIG-I-like receptors (RLRs).

### *1.2.4 Toll-like receptors*

Three TLRs detect viral RNA species – TLR3, TLR7, and TLR8. TLR3 detects double-stranded RNA in endosomes, but its role in influenza infections is equivocal. Human TLR3 transcription is upregulated *in vitro* in A549 and primary alveolar epithelial cells infected with A/Puerto Rico/8/1934 (H1N1) (PR8), and TLR3 silencing reduces type-I interferon production (Wu et al., 2015). However, in mice infected with A/Scotland/20/1974 (H3N2), upregulation of TLR3 expression in lungs at 3 and 4 dpi was correlated with increased inflammation, and $TLR3^{-/-}$ mice had a survival advantage (Le Goffic et al., 2006). Chicken TLR3 responds to the viral RNA mimic poly (I:C) *in vitro* and was upregulated in lung and brain tissue at 24 hours post infection (hpi) with a highly-pathogenic H5N1 virus (Karpala et al., 2008), and in the lungs at 24 hpi after exposure to A/chicken/Italy/1067/99 (H7N1) (Cornelissen et al., 2012). Muscovy duck TLR3 was cloned and characterized in 2012 (Jiao et al., 2012). It shares 62% amino acid identity with human TLR3, and 87.3% identity with chicken TLR3. The majority of the differences between the duck and chicken TLR3 sequences lie in the cytoplasmic signaling domain, the Toll/Interleukin-1 receptor homology domain (TIR) (Chen et al., 2013).

Toll-like receptors 7 and 8 (TLR7 and TLR8) are endosomal pattern recognition receptors that recognize single stranded viral RNA (Lund et al., 2004). Human TLR7 is highly expressed in the endosomes of plasmacytoid dendritic cells (Ito et al., 2005), which produce the bulk of IFN-α during influenza infection (Feldman et al., 2001; Feldman et al., 1995; Gobl et al., 1988). TLR7 and TLR8 in dendritic cells can respond to viral RNA from ruptured cells or infected cells taken up into endosomes (Iwasaki and Pillai, 2014), but may also function directly in epithelial cells, potentially responding to leaked RNA in the endosome from any defective virions (MacDonald et al., 2008). Intranasal administration of a dual TLR7/TLR8 agonist protected rats

infected with influenza A/Port Chalmers/1/1973 (H3N2) (Hammerbeck et al., 2007). TLR7 signaling also appears necessary for optimal B-cell responses to viral infection (Clingan and Matloubian, 2013). The TLR8 gene is disrupted in ducks, chickens, and other birds, so only TLR7 is functional (MacDonald et al., 2008; Magor et al., 2013; Philbin et al., 2005). Pekin duck TLR7 shares 85% amino acid identity with chicken TLR7 and 66% with human TLR7, and is most highly expressed in the lymphoid tissues of the spleen, bursa, and the lung (MacDonald et al., 2008). In contrast, constitutive expression of chicken TLR7 is low in the lungs (Iqbal et al., 2005). Duck splenocytes respond to human TLR7 agonists by increasing IL-1β, IL-6, and IFN-α mRNA (MacDonald et al., 2008). Chicken splenocytes, similarly stimulated, upregulated IL-1β mRNA, but not IFN-α (Philbin et al., 2005).

### *1.2.5 NOD-like receptors*

The NOD-like receptor family, pyrin domain-containing 3 (NLRP3) inflammasome is a multi-protein complex that responds to danger and damage signals by activating the cysteine protease caspase-1 (Leemans et al., 2011; Tschopp and Schroder, 2010). This leads to the maturation and secretion of pro-inflammatory cytokines IL-1β and IL-18, and to the production of antimicrobial reactive oxygen species (ROS). There is accumulating evidence that the NLRP3 makes important contributions to the innate immune responses to influenza virus in mice (Allen et al., 2009; Thomas et al., 2009) and in humans (Pothlichet et al., 2013) that promote healing. On the other hand, there is also evidence that prolonged NLRP3 activation can contribute to pathology through cytokine storm in humans and non-human primates (Baskin et al., 2009; Tate et al., 2016; Teijaro et al., 2014).

NLRP3 recognizes viral RNA (Allen et al., 2009), but appears to detect other categories of PAMPs as well. Its activation is associated with a range of different pathogens (Kankkunen et

al., 2010; Kanneganti et al., 2006; Martinon et al., 2004) and it also appears able to sense tissue damage directly (Mariathasan et al., 2006; Martinon et al., 2006). The action of the influenza A M2 ion channel in the trans-Golgi network, as it raises pH to prevent the newly-made HA proteins from becoming fusogenic, can activate the NLRP3 inflammasome (Ichinohe et al., 2010). In some cases, inflammasome activation depends on a priming step that requires the prior activation of TLRs (Bauernfeind et al., 2009) or RIG-I (Pothlichet et al., 2013). Once activated, NLRP3 inflammasome action can induce pyroptosis (Fernandes-Alnemri et al., 2007), a cell death mechanism that releases extracellular components into the extracellular space and causes a cascading activation of more inflammatory responses (Bergsbaken et al., 2009).

### *1.2.6 RIG-I-like Receptors*

RIG-I like receptors are a family of three cytoplasmic RNA sensing proteins: LGP2, MDA5 and RIG-I (review by Fan and Jin, 2019). All three members share a DExD/H box RNA helicase domain that recognizes double-stranded RNA species, and a C-terminal repressor or regulatory domain (RD) that undergoes a conformational change when the helicase domain binds to its target. MDA5 and RIG-I, but not LGP2, possess a tandem pair of N-terminal caspase activation and recruitment domains (CARD) that initiate a signaling pathway leading to type I interferon expression, once released from RD repression. In a positive feedback loop, all three RLRs are interferon-inducible in ducks (Barber et al., 2010; Cagle et al., 2011), in fish (Chen et al., 2017), and in humans (Cui et al., 2004; Imaizumi et al., 2002; Imaizumi et al., 2004; Li et al., 2005; Sakaki et al., 2005).

RIG-I is the most important of the RLRs for recognizing influenza viruses and is responsible for the earliest epithelial type-I interferon responses in an influenza infection (Kato et al., 2006; Loo et al., 2008; Opitz et al., 2007; Wu et al., 2012). RIG-I recognizes 5'-

triphosphorylated RNA "panhandle" structures formed by the complementary ends of each influenza genome segment (Liu et al., 2015a; Weber et al., 2013). Short aberrant RNAs (mini viral RNAs) produced by influenza polymerases due to any dysregulation or erroneous activity in the replication process act as strong agonists of RIG-I (te Velthuis et al., 2018). The same is true of RNA species in defective interfering (DI) particles (Baum et al., 2010; Liu et al., 2019; Schlee and Hartmann, 2010), which are virions containing incomplete or faulty genomes that also arise from replication or packaging errors (Lazzarini et al., 1981). Recently, a subpopulation of RIG-I that resides in the nucleus during influenza infection and elicits an interferon response from that compartment has been identified in human cells (Liu et al., 2018). Finally, independent of its IFN signaling function, cytoplasmic RIG-I (but not MDA5) can act as an antiviral effector protein by binding to incoming IAV nucleoprotein and delaying the first cycle of replication (Weber et al., 2015).

In ducks, RIG-I is ubiquitously expressed, especially in mucosal tissues, and the highest constitutive expression in healthy Muscovy ducks is in the trachea (Cheng et al., 2015). Induced by interferon, RIG-I mRNA is highly upregulated in Pekin duck lungs early in HPAI H5N1 infections (Barber et al., 2010; Saito et al., 2018). Transcriptome analysis and qPCR indicate that RIG-I signaling pathway genes play a prominent role in mallard responses to HPAI H5N1 viruses (Fleming-Canepa et al., 2019; Smith et al., 2015). In Muscovy ducks, RIG-I expression increased in brains, spleens, lungs, and bursae in response to infection with LPAI A/Chicken/Shanghai/010/2008 (H9N2) (Cheng et al., 2015). In contrast, RIG-I expression was not significantly induced in Pekin duck intestines in response to LPAI infection (Barber et al., 2010).

Chickens lack the RIG-I gene, and this almost certainly contributes to their extreme susceptibility to influenza disease (Barber et al., 2010; Magor et al., 2013). When chickens and ducks were infected with LPAI H7N1, the chickens highly upregulated MDA5 and TLR7 in their lungs and yet produced higher viral titres, while the ducks upregulated RIG-I and restricted viral replication in their lungs (Costa et al., 2011). Highlighting the importance of RIG-I, a human patient who had a severe infection during the 2009 H1N1 pandemic was found to possess a signaling and RNA-binding-deficient variant of RIG-I (Jørgensen et al., 2018). Correlating with increased pathology, cultured peripheral blood mononuclear cells and macrophages from that patient showed an elevated IL-6 and TNF-α response to infection *in vitro*, compared to controls. Kallfass et al. (2013) showed in mice that epithelial cells are the primary source of IFN-β at the earliest timepoints of influenza infection, if they are not successfully inhibited by viral NS1, which targets the RIG-I pathway. Thus, chickens, without RIG-I, are predicted to be deficient in IFN-β at the first critical epithelial cell barrier. This inference is supported by the fact that chicken splenocytes (likely plasmacytoid dendritic cells and other specialized immune cells) respond with interferon to HPAI A/chicken/Yamaguchi/7/2004 (H5N1) *in vitro*, while chicken fibroblasts do not (Moulin et al., 2011). It seems clear from the fates of HPAI-infected chickens that IFN-α signaling by specialized myeloid cells is not sufficient on its own or occurs too late to limit viral replication in the first cycles of infection.

### *1.2.7 The RIG-I pathway*

The duck RIG-I signaling pathway is summarized in Figure 1.1. RIG-I and MDA5 both signal via a homotypic CARD–CARD interaction with the CARD domain of a signaling adaptor, mitochondrial antiviral-signaling protein (MAVS), which leads to the activation and nuclear translocation of interferon regulatory factors IRF3 and/or IRF7. The mechanism of signal

initiation involves the tandem CARDs of four activated RIG-I or MDA5 proteins nucleating a helical assembly of MAVS CARDs into filamentous structures, a process called "molecular imprinting," in both ducks and humans (Wu et al., 2014). This is a spontaneous process, as demonstrated by the constitutive activity of isolated RIG-I or MAVS CARD domains that are free from auto-repression (Miranzo-Navarro and Magor, 2014; Wei et al., 2014). MAVS oligomers then serve as the assembly platform for downstream signaling intermediates. The interacting surfaces between RIG-I–2CARD :: MAVS–CARD and MAVS–CARD :: MAVS–CARD are not highly conserved between ducks and humans, but T175K/T176E mutations in duck RIG-I permit signaling through human MAVS in HEK293T cells (Wu et al., 2014). Furthermore, wild-type duck RIG-I–2CARD can interact with chicken MAVS in the chicken DF-1 cell line, allowing the RIG-I signaling to be recapitulated (Barber et al., 2013; Barber et al., 2010; Chen et al., 2016; Cheng et al., 2015). Full-length mallard RIG-I transfected into DF-1 cells restores detection of 5'ppp RNA and results in lower titres of LPAI A/Mallard/British Columbia/500/2005 (H5N2) and HPAI A/Vietnam/1203/2004 (H5N1) compared to infected controls (Barber et al., 2010). Induction of IFN-β and ISGs is higher in RIG-I-transfected DF-1 cells, by qPCR, microarray, and transcriptome analyses (Barber et al., 2013; Chen et al., 2016). Muscovy duck RIG-I is 97.4% identical to mallard RIG-I at the amino acid level, and also induces IFN-β signaling, and resistance to LPAI strain A/Chicken/Shanghai/010/2008 (H9N2) in transfected DF-1 cells (Cheng et al., 2015). IRF3 is apparently missing in birds (Barber et al., 2013), so avian MAVS is likely signaling through IRF7. Thus, RLR signaling in ducks mostly resembles the human paradigm.

### *1.2.8 Ubiquitination regulates RIG-I*

RIG-I signaling is regulated by ubiquitination. Three ubiquitin ligases – tripartite motif-containing protein 25 (TRIM25), RING finger protein 135 (RNF135 or Riplet) and RING finger protein 125 (RNF125) – regulate RIG-I (Arimoto et al., 2007; Gack et al., 2007; Oshiumi et al., 2009; Oshiumi et al., 2010; Rajsbaum et al., 2012). TRIM25 and Riplet are positive regulators, stabilizing activated RIG-I by the addition of lysine(K)63-linked ubiquitin chains (Gack et al., 2007; Oshiumi et al., 2009; Oshiumi et al., 2010). RNF125 mediates K48-linked polyubiquitination, which leads to the degradation of RIG-I (Arimoto et al., 2007).

TRIM25 stabilizes the RIG-I–2CARD :: MAVS–CARD helical structure by furnishing short chains of K63-linked ubiquitin molecules that bind along the outside of the structure (Gack et al., 2007; Wu et al., 2014). Structural studies have shown that these short ubiquitin chains do not necessarily have to be covalently anchored to RIG-I–CARD domains in order to interact and stabilize the assembly (Peisley et al., 2014b; Zeng et al., 2010). A new function of TRIM25 has recently been described in humans—a nuclear subpopulation of human TRIM25 was identified, which bound directly to influenza nucleoprotein and physically prevented RNA strand elongation by the viral polymerase (Meyerson et al., 2017).

In ducks, TRIM25 ubiquitinates RIG-I–2CARD domains and amplifies MAVS signaling (Miranzo-Navarro and Magor, 2014). Two amino acids in the 2CARD—K167 and K193—covalently anchor K63-linked ubiquitin chains, but mutation of both of them does not abolish signal amplification as long as interaction with TRIM25 is preserved. Thus, duck TRIM25 provides both anchored and unanchored K63-linked polyubiquitin chains to potentiate RIG-I signaling. Direct viral restriction by nuclear TRIM25 has yet to be demonstrated in ducks. Rajsbaum et al. (2012) demonstrated that chicken TRIM25 contributes to IFN-β production in

chicken cells during influenza virus infection *in vitro*, but did not identify a mechanism, which remains an open question. In human cells, Gack et al. (2007) showed that only RIG-I, and not MDA5, is ubiquitinated by TRIM25, but perhaps the situation is different in chickens.

Riplet can stabilize RIG-I activation similarly to TRIM25, by ubiquitinating the RIG-I CARD domains with K63-linked polyubiquitin chains (Shi et al., 2017; Gao et al., 2009), but can also ubiquitinate the C-terminal repressor domain (Oshiumi et al., 2009). K63-linked ubiquitination at multiple sites is cooperative and is necessary for maximal type I interferon induction in response to influenza virus infection in mice (Oshiumi et al., 2010; Rajsbaum et al., 2012). Riplet is missing from the chicken genome, and appears to lack exon 1 in Pekin ducks (Magor et al., 2013). Exon 1 encodes the catalytic RING domain, meaning that Pekin duck Riplet would presumably be unable to transfer ubiquitin (Oshiumi et al., 2010).

In summary, TRIM25 is functional and contributes to type I interferon responses in ducks and chickens, though it is still unclear how in the latter. Riplet appears to be missing in chickens and may be non-functional in ducks, and nothing is yet known about RNF125 in either species.

### 1.3 Influenza A NS1 protein

Influenza A virus non-structural protein 1 (NS1) is a virulence factor that opposes host cell innate immune signaling and effector functions in multiple ways, by participating in many protein-protein interactions with host factors (reviewed by Ayllon and García-Sastre, 2015). NS1 proteins are small, consisting of approximately 230 amino acids, but somewhat variable in length between strains. They are composed of two domains joined by a flexible linker region (Bornholdt and Prasad, 2008). The smaller N-terminal domain consists of three alpha-helices between residues 1 and 72, and is termed the RNA-binding domain (RBD) for its first-

discovered function. The C-terminal effector domain (ED) comprises residues 85-207. The very C-terminal tail varies in length from strain to strain, and is structurally disordered (Soubies et al., 2013). Because the NS1 protein is so small and yet participates in so many protein-protein interactions, many interface regions overlap on the NS1 structure (Min et al., 2007).

NS1 proteins are not packaged into the virion, but are expressed only in host cells to facilitate viral replication in several ways. NS1 temporally regulates viral RNA synthesis in host cells (Min et al., 2007). It promotes the translation of viral mRNA by recruiting eukaryotic initiation factor 4GI (eIF4GI) (Aragón et al., 2000; Zhou et al., 2010). Its most important role, however, seems to be the inhibition of type I interferon signaling and effector functions (Hale et al., 2008). This is demonstrated by the fact that recombinant influenza A viruses lacking NS1 replicate normally in IFN-deficient cells and animals, but cannot replicate in normal, IFN-competent organisms (García-Sastre et al., 1998; Kochs et al., 2007; Soubies et al., 2010b; Zhu et al., 2008). In human cells, NS1 proteins oppose interferon induction by blocking the ubiquitination of RIG-I (Gack et al., 2009; Koliopoulos et al., 2018; Rajsbaum et al., 2012), discussed in more detail in **section 1.3.3**. NS1 can interact directly with, and block the action of the host antiviral effector protein kinase R (PKR), which blocks viral mRNA translation by phosphorylating eukaryotic translation initiation factor eIF2α (Li et al., 2006; Min et al., 2007). NS1 prevents the activation of another interferon-stimulated gene, RNase L, by binding and sequestering any free double stranded RNA (dsRNA) generated during viral replication. RNase L is a ribonuclease that destroys all RNA in the cell when activated by 2′-5′-oligoadenylate synthetase (OAS). OAS detects the presence of (viral) double-stranded RNA, but with a weak affinity that can be outcompeted by NS1 (Krug, 2014; Min and Krug, 2006). Another important function of many NS1 proteins is broad suppression of host protein expression (Das et al., 2008;

Hale et al., 2010b; Khaperskyy and McCormick, 2015; Kuo and Krug, 2009; Nemeroff et al., 1998; Noah et al., 2003), discussed in **section 1.3.2**.

### *1.3.1 NS1 protein phylogeny and host adaptation*

Apart from the surface antigens HA and NA, NS1 is the most polymorphic protein of influenza A viruses (Chen and Holmes, 2006; Kumar et al., 2006; Obenauer et al., 2006). IAV NS1 proteins fall into two phylogenetic clades that diverged very early in IAV evolution, called allele A and allele B (Treanor et al., 1989; Webster et al., 1992; Xu et al., 2014). Between members of a single allele, whether A or B, there is approximately 90% amino acid sequence conservation; between alleles, NS1 proteins share approximately 72% identity (Treanor et al., 1989). With rare exceptions, all known mammalian strain NS1 proteins fall into allele A, while avian strains can possess either allele A or allele B NS1 proteins (Guo et al., 1992; Ludwig et al., 1991; Xu et al., 2014; Zohari et al., 2008). Within each allele can be found species-specific, and geographically isolated lineages (Xu et al., 2014), but allele A and allele B strains can also be found co-circulating in wild birds in the same geographic regions at the same time, though allele B appears more rare (Zohari et al., 2008).

The observation that B-allele NS segments are virtually restricted to birds initially led to the hypothesis that the NS1 or the NEP proteins belonging to that allele are less well-adapted to replication in mammalian hosts. This was supported by the fact that recombinant influenza A/Udorn/1972 (H3N2) bearing a B-allele NS1 gene was attenuated in the respiratory tracts of squirrel monkeys compared to the wild-type virus (Treanor et al., 1989). Similarly, an allele A NS1 from A/Mink/Sweden/1984 (H10N4) was more potent at inhibiting an interferon-stimulated response element reporter system than B-allele NS1 from A/Chicken/Germany/N/1949 (H10N7) in human A549 cells (Zohari et al., 2010). On the other hand, Turnbull et al. (2016) found that

swapping NS alleles in the context of human H1N1 and H3N2 viruses did not attenuate them in various mammalian cell lines or in mice, and did not reduce their ability to inhibit interferon signaling. Furthermore, replacing the endogenous NS segment (allele A NS1) in A/FPV/Rostock/1934 (H7N1) with the NS segment from A/Goose/Guangdong/1/1996 (H5N1) (allele B NS1) improved replication efficiency in human and murine cells, and pathogenicity in live mice (Ma et al., 2010).

Phylogenetically, the NS segments of all currently circulating human and swine viruses derive from a single lineage that traces back to the 1918 pandemic H1N1 strain. Since then, human and swine NS1 proteins have been evolving separately from the avian lineages, and have accumulated species-specific substitutions in both protein domains and in the inter-domain linker region (analyzed systematically by Vasin et al., 2017). The dimerization interface of the N-terminal RNA-binding domain of NS1 depends on six amino acids (Liu et al., 1997; Wang et al., 1999) that are universally conserved in all IAV strains. RNA binding depends, in part, on residues R35, R38, and K41, which also form a nuclear localization sequence (NLS) essential for interaction of NS1 with importin α (Greenspan et al., 1988; Melén et al., 2007). R35 and R38 are universally conserved, as is K41, with the exception of the human H3N2 lineage, which acquired a K41R mutation in 1974 (Vasin et al., 2017). An R44K substitution arose in the seasonal human H1N1 NS1 lineage in 1935 and also independently in the classical swine lineage in 1971, which was maintained in the triple-reassortant swine and subsequent pdm09 viruses. However, in the seasonal H2N2 viruses that arose out of the seasonal H1N1 lineage in 1957, and the H3N2 lineage that replaced it in 1968, this amino acid reverted to the original R44. The functional consequences of this polymorphism are unknown, but in combination with mutations V18A and S195P it yields an attenuated, temperature-sensitive virus (Garaigorta et al., 2005). Several other

substitutions exist in the NS1 RBD that only occur in the human lineage – R21Q, F22V, A23V, A60V, and I81 – which fall outside of known interaction interfaces and whose functional consequences are unknown. A serine at position 42 (S42) has been linked to pathogenicity in mice (Jiao et al., 2008), but this residue is conserved in the majority of human, swine, and avian strains in the NCBI influenza database.

Another polymorphism in the NS1 RBD that may be associated with host adaptation is located at position 55. According to the sequences in the NCBI influenza database, 68% of avian NS1 proteins (22986 sequences on January 9, 2020) encode a glutamic acid at this position (E55). The next most common residue is an arginine (R55), and a small fraction of the sequences encode a lysine (K55). On the other hand, approximately 63% of human and swine influenza NS1 proteins (44326 sequences on January 9, 2020) encode K55, with E55 being the next most prevalent, and a small fraction of sequences encoding Q55 and, even more rarely, R55. Li et al. (2018) examined growth kinetics and interferon induction of three different H5N1 viruses in human A549 cells and macrophages. They found that having a glutamic acid (E) instead of a lysine (K) at position 55 in NS1 enhanced viral replication and interferon suppression. The A/Puerto Rico/8/1934 (H1N1) strain (PR8), which has been propagated in laboratories around the world since the 1940s, exists as several variants, some of which encode K55 in the NS1 protein, and some of which encode E55. Several groups have investigated the contribution of this polymorphism to viral replication and virulence. Liedmann et al. (2014) compared two PR8 variants with different passage histories that had different pathogenicities in mice. The two variants differed by multiple substitutions across 8 viral proteins, including K55E in NS1. The PR8 variant encoding NS1-K55 was lethal to mice and induced less interferon, but the authors found that swapping the NS gene did not alter the viral phenotype. Instead, the changes in

pathogenicity and immune induction were attributed to changes in the PB1 and PA proteins. On the other hand, in their investigation of several PR8-based recombinant vaccine viruses, Murakami et al. (2008) found that a glutamic acid at position 55 in PR8 NS1 enhanced interferon inhibition and growth kinetics. It is possible that viruses with NS1 proteins encoding E55 may be more virulent in humans and thus less likely to be maintained in the population. However, more systematic research is necessary to establish if that is the case, and to investigate the consequences of these substitutions in avian hosts.

A flexible 12-aa linker (residues 73-84) joins the N-terminal RBD to the C-terminal effector domain. Its flexibility allows the domains to move relatively independently and to adopt several different conformations relative to each other (Carrillo et al., 2014). A five-amino-acid truncation in this region arose in avian H5N1 viruses that reassorted with the progeny of the highly pathogenic A/Goose/Guangdong/1/1996 (H5N1) and became prevalent in HPAI H5N1 strains circulating in the first decade of the 2000s (Guan et al., 2004; Li et al., 2004; Zhou et al., 2006). The presence of this truncation increases H5N1 strain virulence in chickens and mice (Long et al., 2008).

The effector domain of NS1 is responsible for many important protein-protein interactions in host cells. The region between residues 81 and 113 mediates the interaction with eIF4GI that promotes viral mRNA translation (Aragón et al., 2000; Zhou et al., 2010). Several lineage-specific substitutions have occurred in this region, including T91A, which was replaced by A91S in pdm09 strains, and L91I, which became I95T in seasonal H3N2 strains since 1975, and I95V in seasonal H1N1 strains since 2001 (Vasin et al., 2017). Avian H5N1 strains between 1957 and 2001 acquired a glutamic acid at position 92 (E92) that enhanced pathogenicity in experimentally-infected pigs (Seo et al., 2002). However, all other known human, swine, avian

and equine influenza A viruses have an aspartic acid at this position (D92), and the D92E polymorphism has since disappeared from circulating viruses (Jiao et al., 2008; Li et al., 2004). Tandem glutamic acids at positions 96 and 97 (E96, E97) have been identified as critical for several protein-protein interactions (Gack et al., 2009; Hale et al., 2010a). These residues are universally conserved, and appear to be involved more so in the maintenance of NS1 structural stability than directly in protein-protein contacts (Hale et al., 2010a; Koliopoulos et al., 2018).

Several conserved residues of NS1 are sites of covalent modifications. A proline-to-tyrosine substitution (P215T) in the human NS1 lineage (Finkelstein et al., 2007) has been linked to phosphorylation. In human cells, NS1 proteins are phosphorylated at T215, and loss of phosphorylation by mutating T215A reduces viral propagation independently of interferon suppression (Hale et al., 2009). Another covalent modification occurs at two lysine residues close to the C-terminus, K217 and K219, which serve as substrate for the conjugation of small ubiquitin-related modifier 1 (SUMO1) in human cells (Xu et al., 2011). Mutating these residues to glutamic acids attenuates viruses in cell culture, but the mechanism is unclear (Xu et al., 2011). Both lysines are highly-conserved in avian NS1 sequences, but K217 is present in only 50% of the human and swine NS1 sequences available on NCBI.

One of the most striking examples of NS1 species-specific adaptation occurs at the very C-terminal end of the effector domain, in the structurally disordered tail between residues 208 and 230. The length of most avian and mammalian NS1 proteins is 230 amino acids, but the length of this tail varies. Spontaneous truncations occur in both avian and mammalian isolates. In the late 1940s, circulating human seasonal H1N1 strains acquired a seven-amino-acid extension at the C-terminus, which was maintained in the progeny H2N2 and H3N2 lineages until it was lost in the 1980s (Hale et al., 2008). Amino acids 227-230 in avian NS1 isolates contain a PDZ-

binding motif. PDZ is a structurally conserved protein domain that appears in many diverse proteins. Its name represents the three proteins first discovered to contain it – post synaptic density protein (PSD95), Drosophila disc large tumor suppressor (Dlg1), and zonula occludens-1 protein (zo-1). PDZ domains recognize and bind short peptide motifs to facilitate protein-protein interactions, similarly to the well-known Src-homology (SH2 and SH3) domains (reviewed by Fanning and Anderson, 1996). PDZ domains are found in a variety of signaling proteins, tyrosine phosphatases, at neuronal synapses, and in association with the cytoskeleton where they participate in motor trafficking of protein complexes. They are divided into several families that bind to the carboxyl-terminus of various proteins bearing an appropriate sequence of amino acids (Nourry et al., 2003). One such consensus sequence bound by a family of closely-related PDZ domains is an (S/T)xV motif.

Large-scale sequencing studies have revealed a PDZ-binding motif (PBM) in the last four amino acids of a majority of avian influenza A virus NS1 proteins (Obenauer et al., 2006; Thomas et al., 2011). The majority of circulating avian influenza A viruses have NS1 proteins with a C-terminal consensus sequence ESxV. The majority of circulating human strains in the past decades share the C-terminal consensus sequence RSxV. The human RSxV motif has a lower affinity than ESxV for mammalian PDZ domain-containing proteins (Golebiewski et al., 2011; Liu et al., 2010). NS1 proteins with an 'ESEV' PBM specifically associate with the PDZ proteins Scribble, Dlg1, MAGI-1, MAGI-2, and MAGI-3, while 'RSKV' does not. Infection with a virus that expresses a NS1 protein with the ESEV PBM results in co-localization of NS1, Scribble, and Dlg1 within perinuclear puncta. Association with Scribble prevents apoptosis and increases viral replication compared to an 'ESEA' point-mutant virus in HeLa cells (Liu et al., 2010). Infection of polarized MDCK cells with the 'ESEV' virus additionally results in

functional disruption of cellular tight junctions (Golebiewski et al., 2011). The consequences of this species-specific difference for viral fitness and pathogenicity are unclear, however. The introduction of 'ESEV' sequence into the swine strain A/WSN/1933 (H1N1) increased virulence in mice in an interferon-independent manner (Jackson et al., 2008). Replacing 'ESEV' with the human 'RSKV' in A/Turkey/Italy/977/1999 (H7N1) had different effects in human, mouse, and duck cells *in vitro*. 'RSKV' virus replicated more efficiently in human A549 cells and in duck embryonic fibroblasts, whereas in mouse 3T3 cells 'ESEV' virus replicated to higher titres (Soubies et al., 2010a; Volmer et al., 2011). In live mice, the same 'ESEV' virus was more virulent and lethal and induced orders of magnitude more type-I interferon in the lungs, but in ducks neither virus was pathogenic, with 'RSKV' inducing slightly higher ISG transcription in the lungs, by qPCR. Removing the 'ESEV' motif from the NS1 of LPAI A/Turkey/Italy/977/1999 (H7N1) by truncation slightly increased the histopathology in infected chicken lungs without increased replication, and no pathology or replication differences were seen in ducks (Soubies et al., 2013). In the context of highly-pathogenic H5N1, the addition of either the avian 'ESEV' or the human 'RSKV' motifs to the naturally-truncated NS1 of A/Vietnam/1203/2004 (H5N1) did not affect its virulence or replication efficiency in mice and chickens (Zielecki et al., 2010).

More species-specific polymorphisms exist in NS1 than described here, but phylogenetic analyses to identify them have so far outpaced the experimental investigations of their significance. Evidence for host-adaptations in two specific functions of NS1 most relevant to this thesis is discussed in the sections below.

### *1.3.2 NS1 inhibition of CPSF4*

NS1 broadly and non-specifically blocks host protein expression in infected cells by interacting with a component of host mRNA processing machinery, cleavage and polyadenylation specificity factor 4 (CPSF4, 30 kDa subunit, also called CPSF30) (Das et al., 2008; Hale et al., 2010b; Kuo and Krug, 2009; Nemeroff et al., 1998; Noah et al., 2003) (Fig 1.2). By sequestering CPSF4, NS1 prevents the proper 3'-end processing of host pre-mRNA species and their export from the nucleus. This theoretically benefits the virus in two ways – by liberating more cellular resources for viral protein synthesis and by repressing innate immune signaling and effector functions because mRNA is not being translated (Khaperskyy et al., 2014).

A crystal structure of NS1 in complex with a CPSF4 fragment is available (Das et al., 2008) and is congruent with NS1 mutagenesis studies (Hale et al., 2010b; Steidle et al., 2010). NS1 proteins bind the second and third zinc fingers of CPSF4, which are perfectly conserved at the amino acid level between humans, pigs, chickens, and ducks. The interaction occurs through two separate interfaces involving NS1 residues F103 and M106 at one and K108, D125, GGLEWND183-189 at the other (Das et al., 2008; Hale et al., 2010b). Mutation of either of these interfaces abolishes the interaction. Examining their conservation among avian strains versus human and swine strains available on the NCBI influenza database revealed an interesting inverse pattern. Residues 183-188 are universally conserved. In human and swine NS1 peptide sequences available on NCBI (44326 sequences on January 9, 2020) residues F103 and M106 are conserved in 98% of proteins, but residues 108, 125, and 189 are poorly conserved. K108 is conserved in only 54% of human and swine sequences, D189 in 55%, and a D125E substitution occurs in 77% - a majority of human and swine isolates. The NS1 protein of A(H1N1)pdm09, which is of human origin, does not bind CPSF4 because of substitutions at these three poorly

conserved residues (Hale et al., 2010b). In avian NS1 sequences (22986 sequences on January 9, 2020) the pattern is the opposite. Residues 108, 125, and 189 are highly conserved, while residue F103 is conserved in only 69% of sequences, and residue M106 in 86%. The NS1 proteins of some avian viruses that infect humans, like A/Hong Kong/156/1997 (H5N1) and A/Shanghai/patient1/2013 (H7N9), do not interact with CPSF4 because of tandem substitutions at positions 103 and 106 (Dankar et al., 2013; Liu et al., 2013). It appears that CPSF4 binding is not strictly conserved in viruses of either host pool, but that different selective pressures act on different residues that make up the two interacting interfaces.

A/Puerto Rico/8/1934 (H1N1) NS1 also lacks the ability to interact with CPSF4 due to paired substitutions at positions 103 and 106 (Steidle et al., 2010). This is unusual for human strains, but PR8 has been a prototypical laboratory strain for generations, and had been propagated in mice for a long time before the advent of sequencing (Bouvier, 2015). Dankar et al. (2011) showed that loss of function mutations at positions 103 and 106 were spontaneously selected when a human H3N2 virus was serially passaged in mice.

The intuitive hypothesis is that losing the ability to bind CPSF4 would attenuate the virus (Noah et al., 2003), but the reality appears more complicated. Spesock et al. (2011) found that restoring CPSF4 binding by mutating residues 103 and 106 in recombinant A/Hong Kong/483/1997 (H5N1) increased its virulence in mice by enhancing systemic spread. On the other hand, restoring CPSF4 binding in A(H1N1)pdm09 NS1 attenuated the virus in mice and in ferrets (Hale et al., 2010b). Similarly, Dankar and colleagues reported increased lethality in mice when CPSF4 binding was abolished in an H3N2 background (Dankar et al., 2013; Dankar et al., 2011). Examining equine H3N8 virus evolution since its introduction from birds in 1963, Chauché et al. (2018) found that the original avian NS1 protein was a competent CPSF4 binder,

but progeny of that lineage in horses acquired an E186K mutation that abolished this interaction, but enhanced the viruses' ability to control JAK/STAT signaling in equine cells. This seems similar to the spontaneous selection of CPSF4 non-interacting mutants in mice (Dankar et al., 2011). Thus, there is some evidence that influenza A viruses evolve away from the ability to interfere with CPSF4 in mammalian hosts.

### *1.3.3 NS1 interference in the RIG-I pathway*

There are three ways in which NS1 potentially inhibits the signaling of the human RIG-I pathway: by sequestering double-stranded RNA (dsRNA), by interacting directly with RIG-I, or by blocking TRIM25-mediated ubiquitination of RIG-I CARD domains. Early studies suggested that dsRNA binding was a key mechanism of innate immune suppression by NS1 (Lu et al., 1995; Ludwig et al., 2002). However, it appears that the affinity of NS1 for the dsRNA backbone is relatively weak and insufficient to competitively inhibit human RIG-I (Krug, 2014; Min and Krug, 2006). There is some evidence for high-affinity co-operative binding of NS1 RBDs to certain virus-specific RNA sequences (Marc et al., 2013), but there are also studies suggesting that very little free dsRNA actually accumulates within influenza A-infected cells (Pichlmair et al., 2006; Weber et al., 2006; Wisskirchen et al., 2011).

NS1 can interact with RIG-I CARD domains directly, in a strain-specific manner (Dankar et al., 2013; Jureka et al., 2015; Mibayashi et al., 2007). This interaction appears to involve residues in both the N-terminal RNA-binding domain (Jureka et al., 2015) and residues 103 and 106 in the C-terminal effector domain (Dankar et al., 2013). Crude purifications from cells overexpressing recombinant proteins showed that NS1 could be found in insoluble fractions containing RIG-I and MAVS (Mibayashi et al., 2007). However, a more recent study by the same group, using bimolecular fluorescence complementarity and super-resolution microscopy

showed that, in fixed cells, NS1 localizes to cytoplasmic foci that contain TRIM25 and RIG-I, but exclude MAVS, and that NS1 interacts to a greater extent with TRIM25 than with RIG-I in those cells (Sánchez-Aparicio et al., 2017).

NS1 proteins bind human TRIM25 to prevent K63-linked ubiquitination of RIG-I CARD domains and thus destabilize the RIG-I/MAVS interaction (Gack et al., 2009; Koliopoulos et al., 2018; Woo et al., 2019) (Fig 1.2). This interaction depends on four critical residues in the NS1 protein – arginine 38 (R38), lysine 41 (K41), and two glutamic acids at positions 96 and 97 (E96, E97) (Gack et al., 2009; Rajsbaum et al., 2012). As described above, residue R38 is universally conserved in virtually all known IAV isolates, and K41 is conserved in all lineages except the human H3N2, which acquired a K41R mutation whose functional significance is unknown (Vasin et al., 2017). Residues E96 and E97 are universally conserved and make internal polar contacts within the NS1 protein (Koliopoulos et al., 2018). There are currently two model mechanisms to explain how NS1 binding blocks TRIM25 enzymatic activity. The first model proposes that NS1 prevents TRIM25 dimerization (Gack et al., 2009). Antiparallel homo-dimerization is necessary for TRIM25 proteins to be able to covalently attach K63-linked ubiquitin chains to target molecules (Sanchez et al., 2016; Sanchez et al., 2014) Gack *et al.* (2009) proposed that NS1 binding to the TRIM25 coiled-coil domain prevents dimerization. The second model arises from a recent crystal structure of NS1 in complex with TRIM25, showing it binding to already-formed dimers (Koliopoulos et al., 2018). The Koliopoulos *et al.* model proposes that NS1 binds to TRIM25 dimers and sterically inhibits ubiquitin ligation to the target protein by occupying the space where ubiquitin ligation should happen.

An earlier investigation of several different NS1 proteins from mammalian and avian influenza A strains found host species-specific and strain-specific differences in their interactions

with human, mouse, and chicken TRIM25 (Rajsbaum et al., 2012). In that study, all the tested NS1 proteins bound to human TRIM25, but only avian virus NS1 bound strongly to chicken TRIM25. Chickens are missing the RIG-I gene, but possess the related MDA5 receptor and many other functional components of the RIG-I signalling pathway (Barber et al., 2010; Magor et al., 2013). In murine cells, NS1 proteins did not bind mouse TRIM25 and were unable to inhibit mouse RIG-I-CARD ubiquitination (Rajsbaum et al., 2012). In terms of interactions with the RIG-I pathway in ducks, all we know so far is that NS1 from strain A/Duck/Guangdong/212/2004 (H5N1) blocks Muscovy duck MDA5 signaling (Wei et al., 2014). It is not known whether this is achieved by blocking the ubiquitination of MDA5 CARD domains, or in another manner. Thus, there is evidence of host-specific adaptations in NS1 function that I aimed to explore further in this thesis.

### 1.4 Experimental aims and results summary

Mallard ducks, the natural hosts of influenza A viruses, have co-evolved a relationship that permits tolerance of infection and resistance to disease. A well-adapted innate immune response is likely an important element of that relationship. LPAI viruses replicate in duck intestines and do not elicit much immunity. In response to HPAI infections, ducks respond with a rapid and potent type-I interferon response that is also brief, but maintains a prolonged upregulation of ISGs. Aberrant and prolonged inflammation is rare. The observation that the lack of RIG-I in chickens makes them extremely susceptible to influenza A viruses, and that reconstituting with duck RIG-I ameliorates this phenotype, has focused our lab on the duck RIG-I signaling pathway. Little is known about host-pathogen interactions of influenza A virus with the duck RIG-I pathway, which is the subject of this thesis.

The primary objective of this thesis work, outlined in **chapter 3**, was to investigate whether NS1 proteins from different IAV strains interfere with the duck RIG-I pathway at the level of TRIM25 and K63-linked ubiquitination, as they do in human cells. I compared four NS1 proteins from avian influenza virus strains of high and low pathogenicity, and one mammalian strain, in their abilities to interact with human and duck TRIM25. I found that all of the NS1 proteins bound efficiently to duck TRIM25, but were unable to prevent duck RIG-I-CARD ubiquitination. In contrast, I saw strain-specific differences in the ability of avian NS1 proteins to interact with human TRIM25 *in vitro*, but they all potently inhibited human RIG-I CARD ubiquitination and signaling.

**Chapter 4** outlines a collaboration with Dr. Yanna Xiao to study the effects of the influenza accessory protein PB1-F2 on duck MAVS signaling. We found that PR8 PB1-F2 co-localizes with duck MAVS in avian cells and inhibits interferon induction. To our surprise we found that PR8 PB1-F2 was able to inhibit TRIM25-mediated K63-linked ubiquitination of both human and duck RIG-I CARD domains through a mechanism that is still under investigation.

**Chapter 5** describes collaboration with David Tetrault to investigate the anti-interferon activity of the IAV polymerase subunit PB2. Knowing that PB2 localizes to mitochondria in human cells and interacts with MAVS to inhibit interferon, we sought to determine if this also occurs in avian cells with duck MAVS. I show preliminary evidence suggesting that this does appear to be the case, and that strain-specific differences may influence the localization of PB2 proteins and the magnitude of interferon inhibition.

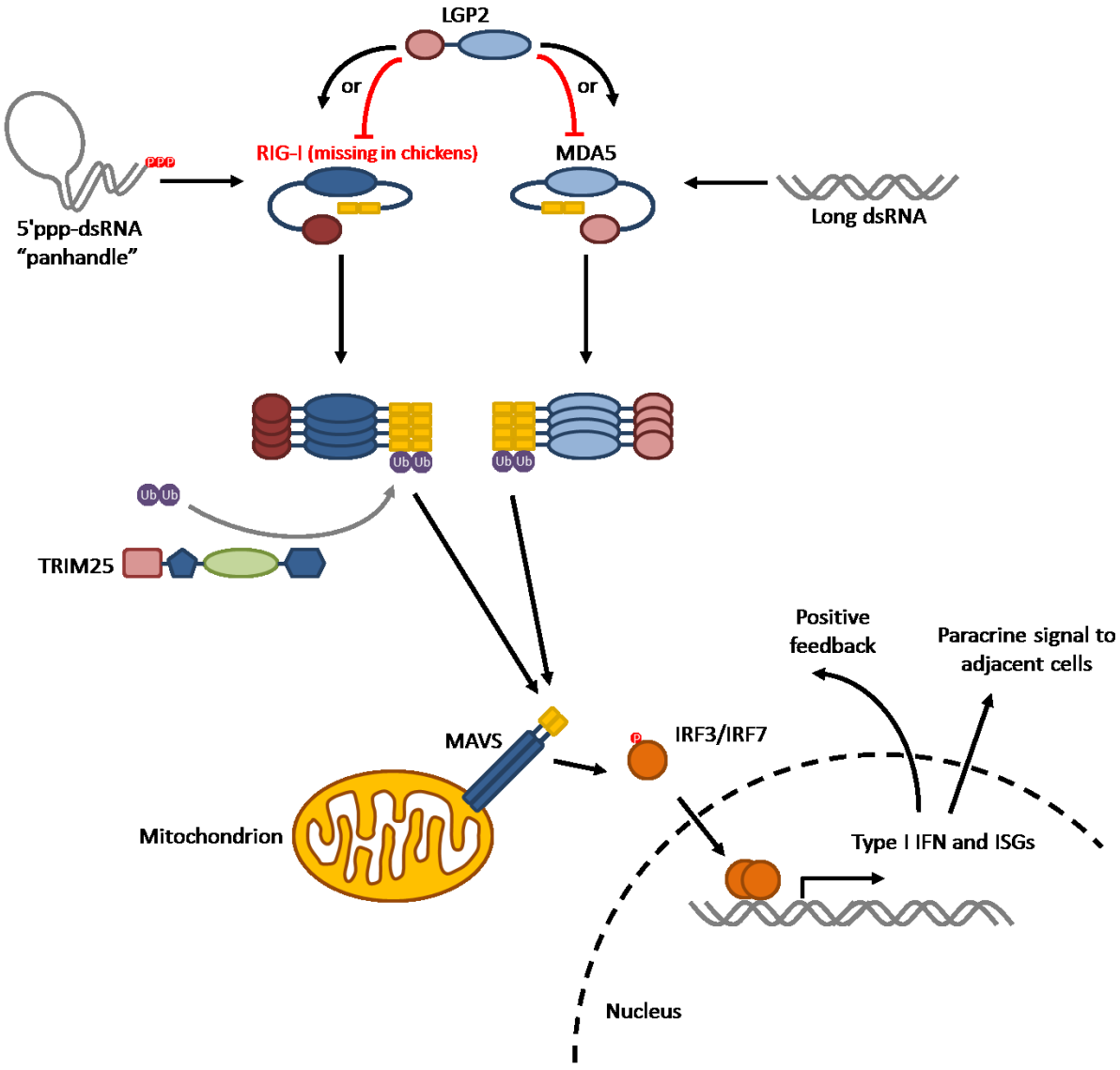



**Figure 1.1. The RIG-I signaling pathway.**

The RLR family consists of three cytoplasmic receptors: RIG-I, MDA5, and LGP2. RIG-I and MDA5 detect viral RNA species and initiate interferon signaling. LGP2 does not possess a signaling domain and may act as a positive or negative regulator of the other two. RIG-I recognizes 5'-triphosphorylated RNA "panhandle" structures formed by the complementary ends of each influenza genome segment. MDA5 recognizes long double-stranded RNA. Ligand recognition leads to oligomerization of the receptors and releases the N-terminal caspase activation and recruitment domains (CARD) domains (shown as yellow rectangles) from repression by the C-terminal regulatory domain. Short K63-linked polyubiquitin chains synthesized by tripartite motif-containing protein 25 (TRIM25) stabilize the RIG-I CARD domain oligomers, which interact with similar CARD domains on mitochondrial MAVS. Activation of MAVS proteins leads them to multimerize in turn, and to serve as an assembly platform for a kinase cascade, which terminates in the phosphorylation of the transcription

factors IRF3 and IRF7. Phosphorylated IRF dimerizes and is translocated to the nucleus where it initiates transcription of type I and type III interferon and interferon-stimulated genes. The expressed interferon is secreted to induce an antiviral state in the neighbouring cells and can also amplify RLR signaling in a positive feedback loop, since the pathway components are themselves interferon-inducible.

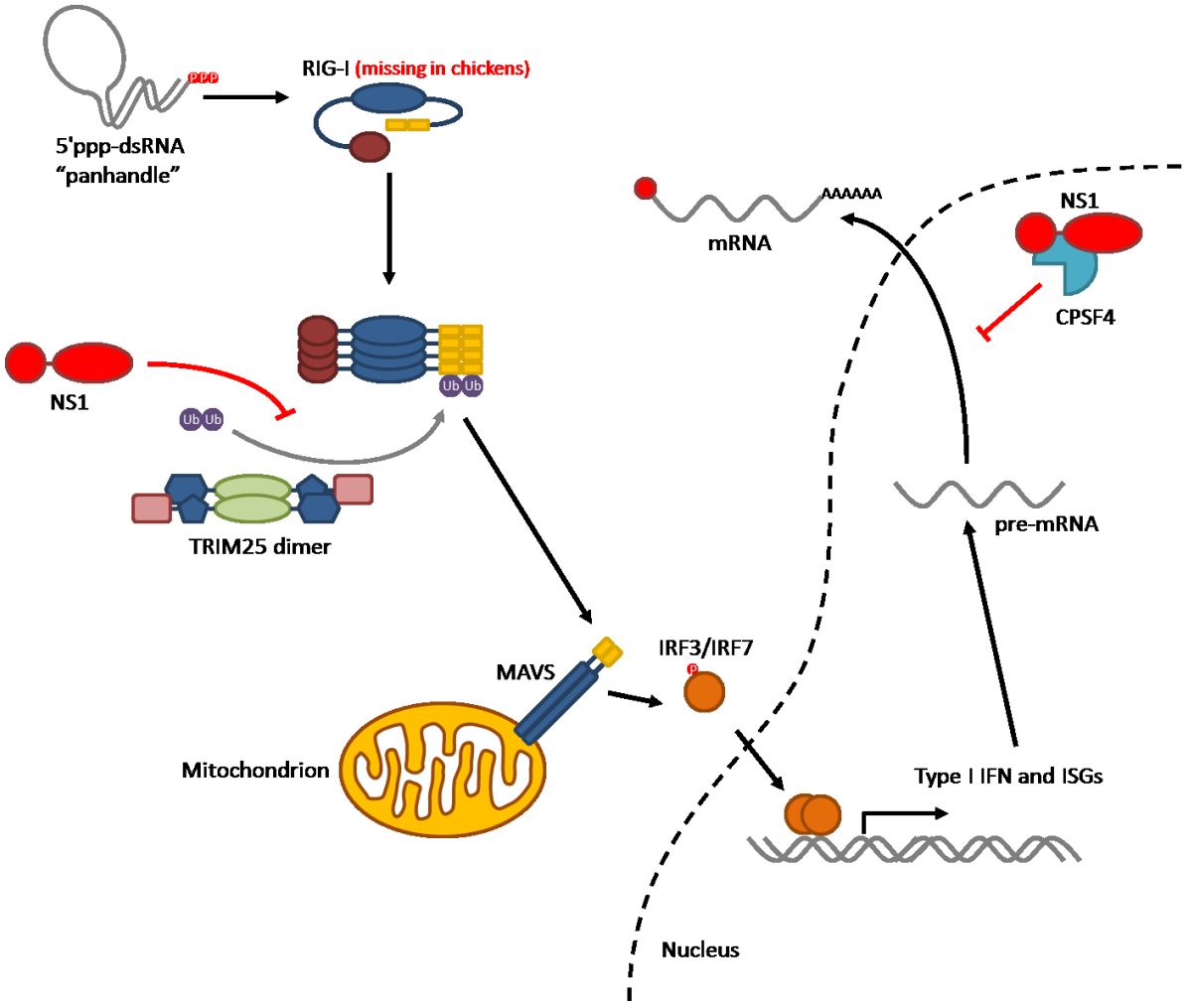



**Figure 1.2. Two mechanisms of interferon inhibition by IAV NS1 in human cells.** Influenza A virus NS1 proteins target human TRIM25 to block K63-linked ubiquitination of activated RIG-I CARD domains. In the nucleus, NS1 binds to CPSF4 and prevents the host mRNA polyadenylation and nuclear export.

# Chapter 2 Materials and Methods

## 2.1 Cell culture and transfection.

HEK293T cells, DF-1 cells, A549 cells, and HeLa cells were maintained in Dulbecco's modified Eagle's medium (DMEM) plus 10% FBS. DF-1 cells are a chicken embryonic fibroblast cell line derived from an East Lansing line (EV-0) embryo (Schaefer-Klein et al., 1998). For recombinant protein expression, cells were seeded overnight in 6-well plates ($8\times10^5$ cells/well) and 24 h later transfected with 1-2 μg/well of each DNA construct using Lipofectamine 2000™ reagent (Invitrogen) (DNA:lipofectamine ratio 1:2.5).

## 2.2 Plasmids and constructs.

pcDNA3.1/Hygro(+) (Invitrogen) served as the backbone plasmid for all the constructs used in this work. Phusion® High-Fidelity DNA polymerase (NEB) was used in all PCR and site-directed mutagenesis reactions. Avian NS1 protein coding sequences were amplified from cDNA of mallard duck lung samples collected at 1 day post infection with the appropriate viruses (A/Duck/British Columbia/500/2005(H5N2); A/Duck/D4AT/71.1/2004(H5N1); A/Vietnam/1203/2004(H5N1)) and cDNA from DF-1 cells infected with A/Chicken/California/431/2000(H6N2). Primers used for amplification can be found in table 2.1. The CDS of PR8 NS1 (A/Puerto Rico/8/1934 (H1N1)) was kindly provided by Dr. Yan Zhou. Avian NS1 proteins were amplified using primers that incorporated an N-terminal single Flag tag (DYKDDDDK) and BamHI and NotI restriction enzyme sites (Fig 2.1A). PR8 NS1 was amplified and Flag-tagged in the same manner, but using NheI and XhoI sites (Fig 2.1A). PCR

products were first cloned into pCR2.1-TOPO (Invitrogen) and sequenced in the forward and reverse directions using BigDye Terminator v3.1 (Applied Biosystems), then subcloned into the pcDNA3.1/Hygro(+) expression vector and sequenced again (Table 2.2). Human GST-RIG-I-CARD (GST-h2CARD) and human TRIM25-V5 constructs were kindly provided by Dr. Michaela U. Gack. Duck GST-RIG-I-CARD (GST-d2CARD) and TRIM25-V5 constructs were cloned previously (Miranzo-Navarro and Magor, 2014). GST-tagged coiled-coil domains (CCD) of human and duck TRIM25 were constructed by amplifying the CCDs from human and duck TRIM25-V5 constructs (nucleotide residues 538-1347 for human, 565-1401 for duck) and cloning them into the pcDNA3.1-GST vector described previously (Miranzo-Navarro and Magor, 2014). Primers used for amplification can be found in table 2.1. pcDNA3.1-HAUB (Addgene plasmid 18712) (Kamitani et al., 1997) was used to detect ubiquitination of RIG-I CARD domains. PR8 PB1-F2 was amplified from the cDNA of infected DF-1 cells and cloned into pcDNA3.1/Hygro (+) between BamHI and NotI restriction sites with an N-terminal Flag tag, by Dr. Yanna Xiao. V5-tagged duck MAVS (V5-MAVS) and viral PB2 proteins were cloned previously (Tetrault, 2015). All plasmids were transformed into *Escherichia coli* DH5α. Individual clones of each transformation were used for plasmid isolation using the Qiagen Miniprep Kit (Qiagen). High-concentration and high-purity plasmid preps for transfection were generated using the Qiagen Midiprep Kit (Qiagen).

**2.3 Site-directed mutagenesis.**

Site directed mutagenesis was performed with a one-step PCR method based on the QuickChange protocol. Forward and reverse primers were designed for increased QuickChange efficiency (Zheng et al., 2004) (Table 2.3). NS1 mutants (G184R and R38A) and RIG-I CARD domain mutants (h2CARD-D122A and d2CARD-A120D) were generated by PCR amplification

of the entire expression vector with partially-overlapping mutagenesis primers and Phusion® High-Fidelity DNA polymerase (NEB). A DpnI digests were performed to degrade the methylated template DNA, PCR products were transformed into *E. coli* DH5α, screened and sequenced (Table 2.2).

**2.4 Immunoprecipitation, GST pulldown, and immunoblotting.**

Immunoprecipitations and GST pulldowns were performed as previously described (Miranzo-Navarro and Magor, 2014). Briefly, cells in a 6-well plate were transfected with DNA constructs, one well per sample. 24 h later, cells were lysed in 1000 µL of lysis buffer (50 mM TRIS pH 7.2, 150 mM NaCl, 1% [vol/vol] Triton X-100, protease inhibitor cocktail [Roche]), followed by two 10-second cycles of sonication. Whole cell lysate (WCL) samples were collected post-sonication, and the remainder of the lysates were centrifuged at 14,000 rpm for 15 min. For GST-pulldown assays, clarified supernatants were mixed with 50 µL of glutathione Sepharose 4B resin (GE Healthcare) pre-equilibrated with lysis buffer, and the binding reaction mix was incubated for 3 to 4 h at 4°C. The GST-pulldown was washed four times with ice-cold lysis buffer and then the beads were boiled in Laemmli sample buffer for 10 min. Immunoprecipitations with anti-V5 agarose were performed analogously, using anti-V5 Agarose Affinity Gel (A7345; Sigma-Aldrich). For immunoblotting, proteins were separated by SDS–polyacrylamide gel electrophoresis (SDS–PAGE) and transferred to a nitrocellulose membrane. Proteins were detected with anti-V5 monoclonal (1:5000) (R96025; Fisher), anti-Flag-M2 monoclonal (1:5000) (F3165; Sigma-Aldrich), anti-HA-HRP monoclonal (1:5000) (A01296; GenScript), and anti-GST polyclonal (1:5000) (G7781; Sigma-Aldrich) antibodies and were

visualized by chemiluminescence using Pierce® ECL Western blotting substrate (Thermo Scientific) and a ChemiDoc imager (Bio-Rad).

**2.5 Confocal microscopy and co-localization analysis.**

DF-1, HeLa, or HEK293T cells were seeded onto coverslips in 6-well plates at $8\times10^5$ cells/well and transfected with 1 μg of each DNA construct. 24 h after transfection, cells were fixed in 1% PFA overnight at 4°C. Fixed cells were permeabilized by a 10-minute incubation in PBS + 0.2% [vol/vol] Triton X-100 and blocked in 4% BSA for 1 h. The subcellular distributions of the recombinant proteins were determined by staining with rabbit anti-V5 conjugated to Dylight650 (ab117489; Abcam) and mouse anti-Flag-M2 monoclonal (F3165; Sigma-Aldrich) followed by a secondary goat anti-mouse antibody conjugated to Alexa Fluor 488 (A-11001; Thermo Scientific). The cell nuclei were stained with Hoechst 33342 (Life Technologies). Images were taken on a Leica TCS SP5 confocal microscope. Co-localization analysis by Pearson's coefficient was completed using ImageJ (Schindelin et al., 2012).

**2.6 Dual luciferase reporter assays and interferon beta protein quantification.**

The human IFN-β promoter luciferase reporter plasmid (pLuc125) was generously provided by Dr. Yan Zhou. The chicken IFN-β promoter luciferase reporter plasmid (pGL3-chIFNβ) was derived from the chicken IFN2 gene as previously described (Barber et al., 2010; Childs et al., 2007; Sick et al., 1998). Luciferase activity was measured using the Dual-Luciferase Reporter Assay System (Promega) according to the manufacturer's instructions. Briefly, DF-1 or HEK293T cells were seeded in 24-well plates at 2×105 cells per well. 24 h later

they were transfected with fixed amounts of the appropriate IFN-β reporter plasmid (150 ng) and the synthetic *Renilla* luciferase internal control construct phRTK (10 ng). Cells also received 5 ng of GST-RIG-I-CARD construct, 150 ng of TRIM25-V5 construct, and 500 ng of Flag-NS1 construct, as indicated. Variable amounts of pcDNA3.1/Hygro(+) backbone vector were added to keep the amount of transfected DNA constant. 24 h after transfection, cells were lysed with 100 uL/well passive lysis buffer for 15 min. at room temperature. 20 µL of cell lysate was transferred to a 1.5 ml Eppendorf tube containing 100 µL Luciferase Assay Reagent II and firefly luciferase activity was measured using the GloMax 20/20 Luminometer (Promega). Then, 100 µL Stop & Glo® reagent was added to the lysate mix, and *Renilla* luciferase activity was measured.

For quantification of secreted human IFN-β, HEK293T cells were transfected with 500 ng of GST-RIG-I-CARD construct, 1 µg of TRIM25-V5 construct, and 2 µg of Flag-NS1 construct, as indicated. Culture supernatants were collected 24 h-post transfection, cleared by centrifugation at 18,000 x g for 5 min. at 4°C, and sent to Eve Technologies for Luminex bead-based quantification (Human IFN beta Array 1-plex).

**2.7 Virus infections in cell culture.**

Wild-type A/Puerta Rico/8/1934(H1N1) (PR8) was propagated in 10-day-old chicken eggs. Recombinant del-NS1 PR8 (ΔNS1 rPR8) was propagated in MDCK cells stably expressing PR8 NS1. The titre of all stocks was determined by plaque assay on MDCK cells or by hemagglutination of chicken erythrocytes. 24 hours after plating or transfection HEK293T and A549 cells were infected at a multiplicity of infection of 2 (MOI=2) for 11 hours before lysis.

For infections, cells were cultured in DMEM supplemented with 0.3% BSA and L-(tosylamido-2-phenyl) ethyl chloromethyl ketone-treated trypsin (Worthington Biochemical) (0.2 μg/mL).

**2.8 Recombinant virus plasmid rescue.**

Eight ambisense plasmids (pHW) containing each genome segment of influenza A/Puerto Rico/8/1934 (H1N1) between PolI and PolII promoters (Fig 2.2) was kindly provided by Dr. Yan Zhou, and sequenced (Table 2.4). An NS-split plasmid containing full coding sequences of PR8 NS1 and PR8 NEP separated by an autocatalytic cleavage site was kindly provided by Dr. Adolfo García-Sastre, and sequenced (Table 2.4). In this vector, the splice acceptor site in the NS1 CDS is silently mutated and the complete CDS of the NEP gene is included downstream of a porcine teschovirus-1 2A site (Nogales et al., 2016; Perez et al., 2013) (Fig 2.2). NS1 coding sequences from other viral strains were cloned into this vector using a Gibson Assembly (New England Biolabs) approach from three fragments. The vector backbone was generated by a restriction digest of the purified parent vector at SacI and XhoI restriction sites. A fragment containing the 2A site and the adjacent PR8 NEP CDS was amplified by PCR from the parent vector by Michelle Lee, an undergraduate student under my supervision (Table 2.1). NS1 CDS fragments for wild-type avian, G184R-mutant PR8, and R38A-mutant PR8, with the splice acceptor sites silently mutated, were synthesized by Integrated DNA Technologies. Identity of assembled vectors was confirmed by sequencing using internal primers (Table 2.4).

Virus generation from plasmids was performed as previously described (Martínez-Sobrido and García-Sastre, 2010). Briefly, an almost confluent co-culture of Madin Darby canine kidney (MDCK) cells and human embryonic kidney (HEK293T) cells was transfected

simultaneously with seven ambisense plasmids (pHW) containing seven of the eight genome segments of influenza A/PR/8/34 between PolI and PolII promoters (kindly provided by Dr. Yan Zhou) and one NS-split plasmid containing full coding sequences for NS1 and PR8 NEP separated by an autocatalytic cleavage site (kindly provided by Dr. Adolfo García-Sastre (Nogales et al., 2016; Perez et al., 2013)). After 72 hours the cell culture supernatant was collected, centrifuged to remove cellular debris, and used to infect fresh monolayers of MDCK cells for plaque assay.

**2.9 Plaque assays**

MDCK cells were cultured in 1X Minimal Essential Media (MEM) supplemented with 10% FBS, sodium bicarbonate, PSF (Streptomycin, Penicillin, Amphotericin B), MEM vitamins, and L-glutamine (Gibco). Infection media for MDCK cells was the same as growth media, but with 0.3% BSA in place of 10% FBS, and with addition of 1 μg/mL of L- (tosylamido-2-phenyl) ethyl chloromethyl ketone–treated trypsin (Worthington Biochemical). Supernatant from infected HEK293T cells were collected 24 hours post infection, and serially diluted in infection media. Monolayers of MDCK cells grown in 6-well plates were infected with serially diluted supernatants. After one hour, supernatants were removed and cells were washed with 1X PBS. Cells were then overlaid with infection media containing 0.9% agar. After 72 hours agar was removed and cells were stained with 0.1% crystal violet solution.

**2.10 Viral RNA extraction**

Recombinant virus rescue supernatants were used to infect fresh monolayers of HEK293T cells as previously described. 24 hours after infection, cell culture supernatant was

collected and centrifuged for 5 minutes at 13000 rpm at 4°C to remove cellular debris. 250 µL of each cleared cell culture supernatant was transferred to a new tube and mixed with 750 µL of TRIzol reagent (Invitrogen) for extraction of total RNA. cDNA was synthesized using SuperscriptIII (Invitrogen) reverse transcriptase and a universal influenza A genome primer targeting the conserved sequences at the termini of influenza A genome fragments (Hoffmann et al., 2001). The NS1 coding sequences were amplified out of the viral cDNA using Phusion® High-Fidelity DNA polymerase (NEB), verified on agarose gels, and sequenced to confirm identity (primers in Table 2.4).

**Table 2.1. Primers for amplifying and cloning recombinant proteins.**

| Protein | Primer | Primer Sequence (5'→3') |
|---|---|---|
| NS1 PR8 | Forward | GCTAGCGCCACCATGGATCCAAACACTGTGTCAAGCTTTCAGGTAG |
| | Reverse | CTCGAGTCATCAAACTTCTGACCTAATTGTTCCCGCCATTTCTCGTTTCT |
| | Forward Flag | GCTAGCGCCACCATGGATTATAAAGATGATGATGATAAAGGAATGGATCC AAACACTGTGTCAAGCTTTCAGGTAG |
| NS1 BC500 | Forward | GGATCCGCCACCATGGATTCCAACACGATAACCTCGTTTCAGGTAG |
| | Reverse | GCGGCCGCTCATCAAACTTCTGACTCAACTCTTCTCGCCATGTAGCGTT |
| | Forward Flag | GGATCCGCCACCATGGATTATAAAGATGATGATGATAAAGGAATGGATTC CAACACGATAACCTCGTTTCAGGTAG |
| NS1 CA431 | Forward | GGATCCGCCACCATGGACTCCAACACGATAACCTCGTTTCAGGTAG |
| | Reverse | GCGGCCGCTTATCAAACTTCTGACTCAGCTCTTCTCGCCATGTAGCGTTTCT |
| | Forward Flag | GGATCCGCCACCATGGATTATAAAGATGATGATGATAAAGGAATGGACTC CAACACGATAACCTCGTTTCAGGTAG |
| NS1 D4AT | Forward | GGATCCGCCACCATGGATTCCAACACTGTGTCAAGCTTTCAGGTAG |
| | Reverse | GCGGCCGCTCATCAAACTTCTGACTCAATTGTTCTCGCCATTTTCC |
| | Forward Flag | GGATCCGCCACCATGGATTATAAAGATGATGATGATAAAGGAATGGATTC CAACACTGTGTCAAGCTTTCAGGTAG |
| NS1 VN1203 | Forward | GGATCCGCCACCATGGATTCCAACACTGTGTCAAGCTTTCAGGTAG |
| | Reverse | GCGGCCGCTTATTACCGTTTCTGATTTGGAGGGAGTGGAAGTCTCC |
| | Forward Flag | GGATCCGCCACCATGGATTATAAAGATGATGATGATAAAGGAATGGATTC CAACACTGTGTCAAGCTTTCAGGTAG |
| hTRIM25 CCD | Forward | GGATCCCTGGTGGAGCATAAGACCTGCTCTCC |
| | Reverse | ATCGATTTATTATCTGGACTTGGCCAGGAAGGTCTCC |
| dTRIM25 CCD | Forward | GGATCCCTCCTCAGCCACAAGCTGTGTAACGC |
| | Reverse | ATCGATTTATTATCTGTCTTTCTGAAGAAAGCTGCTAATAAGATCTTTTGTA TTAGCCGC |
| 2A-NEP | Forward | GCGACCAACTTTAGCCTGCTG |
| | Reverse | GGGAAAAAGATCTGCTAGCTCGAGC |

**Table 2.2. Primers for sequencing expression constructs.**

| Construct | Primer | Primer Sequence (5'→3') |
|---|---|---|
| pCR2.1-TOPO | M13F(-20) | CAGGAAACAGCTATGAC |
| (vector) | M13R | GTAAAACGA CGGCCAG |
| pcDNA3.1/Hygro(+) | T7-pgem | TAATACGACTCACTATAGGG |
| (vector) | BGHR | TAGAAGGCACAGTCGAGG |
| hTRIM25-V5 | Internal F1 | CAGGACCACCCGCTGCAGCC |
| | Internal F2 | AGATTGAACAGAGCCTGACC |
| | Internal F3 | GCTTCTGTGGCTGAGATGCC |
| | Internal R1 | GTGCCAGGCAGAGATCTTGG |
| | Internal R2 | GTGGACGCTGGGTCATGCTC |
| | Internal R3 | CAGGTCGGCGCTGGCCTGGC |
| dTRIM25-V5 | Internal F1 | GAGACAGCTGCCAGAAAGCGAG |
| | Internal F2 | GGGAGCACCAAGCACAGCAGCA |
| | Internal F3 | GCGCTGACCCGAGCCGTGTCC |
| | Internal R1 | TGCCATCTCAATCTGGTCTCTG |
| | Internal R2 | GGTGAAACGCTGAGGGTGGTGG |
| | Internal R3 | TCCGCCACCGCCTCATCTTCC |
| GST-h2CARD | Internal F1 | GGGCTGGCAAGCCACGTTTGGTG |
| GST-d2CARD | Internal F1 | GGGCTGGCAAGCCACGTTTGGTG |
| Flag-hRIG-I | Internal F1 | GATGTTGAAACAGAAGATCTTGAGG |
| | Internal F2 | GATGCGTCAGTGATAGCAACAGTC |
| | Internal F3 | GCATTCAAAGCCAGTGGAGATC |
| | Internal R1 | CTCACTAAGATTCTGGCATTCTGG |
| | Internal R2 | CAACTTGCTCCAGTTCCTCCAG |
| | Internal R3 | GTAATGGCATTCCTCTATCACTC |
| Flag-dRIG-I | Internal F1 | CCCATCAAAACACCTGGCTTTATGT |
| | Internal F2 | ACTGGAAGAGCTTGTCTGCATCCTG |
| | Internal F3 | GATGATGCGTACCGCTATAACCCA |
| | Internal R1 | CTACCTTGACATACAGGCCATATCC |
| | Internal R2 | GTGAAATGACAGATGCCTCTGAG |
| | Internal R3 | CACTGAGATTATCATCAGGTTCTGC |

**Table 2.3. Primers for mutagenesis.**

| Protein | Mutation | Primer | Primer Sequence (5'→3') |
|---|---|---|---|
| NS1 PR8 | R38A | Forward | GGCGATGCCCCATTCCTTGATCGGCTTCGCGCAGATCAGAAATCCCTA AGAGGAAGGGGCAGTACTCTCGGTCTGGACATCAAGA |
| | | Reverse | CCAGACCGAGAGTACTGCCCCTTCCTCTTAGGGATTTCTGATCTGCGC GAAGCCGATCAAGGAATGGGGCATCGCCTAGTTCTT |
| NS1 PR8 | G184R | Forward | GTCAAAAATGCAGTTGGAGTCCTCATCGGACGACTTGAATGGAATGAT AACACAGTTCGAGTCTCTGAAACTCTACAGAGATTCG |
| | | Reverse | GTAGAGTTTCAGAGACTCGAACTGTGTTATCATTCCATTCAAGTCGTC CGATGAGGACTCCAACTGCATTTTTGACATCCTCAG |
| NS1 BC500 | G184R | Forward | GTCAAAAATGCAATTGGAATCCTCATCGGTCGACTTGAATGGAATGAT AACTCAATTCGAGCGTCTGAAAATATACAGAGATTCG |
| | | Reverse | GTATATTTTCAGACGCTCGAATTGAGTTATCATTCCATTCAAGTCGACC GATGAGGATTCCAATTGCATTTTTGACATCCTCTG |
| NS1 CA431 | G184R | Forward | GTCAAAAATGCAATTGGAATCCTCATCGGTCGACTTGAATGGAATGAT AACTCAATTCGAGCGTCTGAAAATATACAGAGATTCG |
| | | Reverse | GTATATTTTCAGACGCTCGAATTGAGTTATCATTCCATTCAAGTCGACC GATGAGGATTCCAATTGCATTTTTGACATCCTCTG |
| NS1 D4AT | G184R | Forward | GTCAAAAATGCAATTGGCGTCCTCATCGGACGACTTGAATGGAATGAT AACACAGTTCGAGTCACTGAAACTATACAGAGATTCG |
| | | Reverse | GTATAGTTTCAGTGACTCGAACTGTGTTATCATTCCATTCAAGTCGTCC GATGAGGACGCCAATTGCATTTTTGACATCCTCAC |
| NS1 VN1203 | G184R | Forward | GTCAAAAATGCAATTGGCGTCCTCATCGGACGACTTGAATGGAATGAT AACACAGTTCGAGTCACTGAAACTATACAGAGATTCG |
| | | Reverse | GTATAGTTTCAGTGACTCGAACTGTGTTATCATTCCATTCAAGTCGTCC GATGAGGACGCCAATTGCATTTTTGACATCCTCAC |
| h2CARD | D122A | Forward | AACCAGAATTATCCCAACCGCTATCATTTCTGATCTGTCTGAATGTTTA ATTAATCAGG |
| | | Reverse | CAGACAGATCAGAAATGATAGCGGTTGGGATAATTCTGGTTTTAAATT CTGG |
| d2CARD | A120D | Forward | GTTAGAAGTCGACCCAGTAGATCTCATTCCCTATATAAGCACATGCCT GATAGAC |
| | | Reverse | GTGCTTATATAGGGAATGAGATCTACTGGGTCGACTTCTAACATTGTT GCCTCTATCCG |

**Table 2.4. Primers for sequencing A/Puerto Rico/8/1934 (H1N1) recombinant viral rescue plasmids.**

| Construct | Primer | Primer Sequence (5'→3') |
|---|---|---|
| pHW191 | Internal F1 | AGCGAAAGCAGGTCAATTATATTC |
| (genome segment 1) | Internal F2 | GGATACTAACATCGGAATCGC |
| | Internal F3 | AGAACAAGCGGATCATCAGTC |
| | Internal R1 | AGTAGAAACAAGGTCGTTTTTAAACTATTCG |
| | Internal R2 | CTGCATTCTACTTTGCTTTGGTGG |
| | Internal R3 | CGTGCTGGAGTACTCATC |
| pHW192 | Internal F1 | AGCGAAAGCAGGCAAACC |
| (genome segment 2) | Internal F2 | CAGAGAAAGAGACGGGTGAG |
| | Internal F3 | GAGAGCAAGAGTATGAAACTTAGAACTC |
| | Internal R1 | AGTAGGAACAAGGCATTTTTTCATG |
| | Internal R2 | TTCAGTGGGTTGCATAAACG |
| | Internal R3 | GAATTCAAATGTACCTGTTCTGTTTATG |
| pHW193 | Internal F1 | AGCGAAAGCAGGTACTGATC |
| (genome segment 3) | Internal F2 | GGCAGACTACACTCTCGATG |
| | Internal F3 | CTTCTGTCATGGAAGCAAGTACTG |
| | Internal R1 | AAGGTACTTTTTTGGACAGTATGG |
| | Internal R2 | TCTCAAGAACACAGTACTTCTCCC |
| pHW194 | Internal F1 | AAATGAAGGCAAACCTACTGG |
| (genome segment 4) | Internal F2 | CGGAGAAGGAGGGCTC |
| | Internal R1 | AGTAGAAACAAGGGTGTTTTTCC |
| | Internal R2 | CTCGATAACAGTGTTCACCTTG |
| pHW195 | Internal F1 | AGCAAAAGCAGGGTAGATAATCAC |
| (genome segment 5) | Internal F2 | GGAATGGATCCCAGGATGTG |
| | Internal R1 | AGTAGAAACAAGGGTATTTTTCTTTAATTGTCG |
| | Internal R2 | CAGAATGGCATGCCATCCAC |
| pHW196 | Internal F1 | AGCGAAAGCAGGGGTTTAAAATG |
| (genome segment 6) | Internal F2 | GGCTAACAATCGGAATTTCAGG |
| | Internal R1 | AGTAGAAACAAGGAGTTTTTTGAACAG |
| pHW197 | Internal F1 | AGCGAAAGCAGGTAGATATTGAAAG |
| (genome segment 7) | Internal R1 | AGTAGAAACAAGGTAGTTTTTTACTCCAG |
| pHW198 | Internal F1 | AGCAAAAGCAGGGTGACAAAG |
| (genome segment 8) | Internal R1 | AGTAGAAACAAGGGTGTTTTTTATTATTAAATAAGCTG |
| pDZ-NS1-2A-NEP | Forward | TCTGGCGTGTGACCGGCGGCT |
| (NS split plasmid) | Reverse | TTGCCCTCCCATATGTCCTTCCGAG |
| | Internal F B/C/D/V | GAGGATGTCAAAAATGCAATTGG |
| | Internal F PR8 | GAGGATGTCAAAAATGCAGTTGG |
| | Internal R1 | GTTATCATTCCATTCAAGTCC |

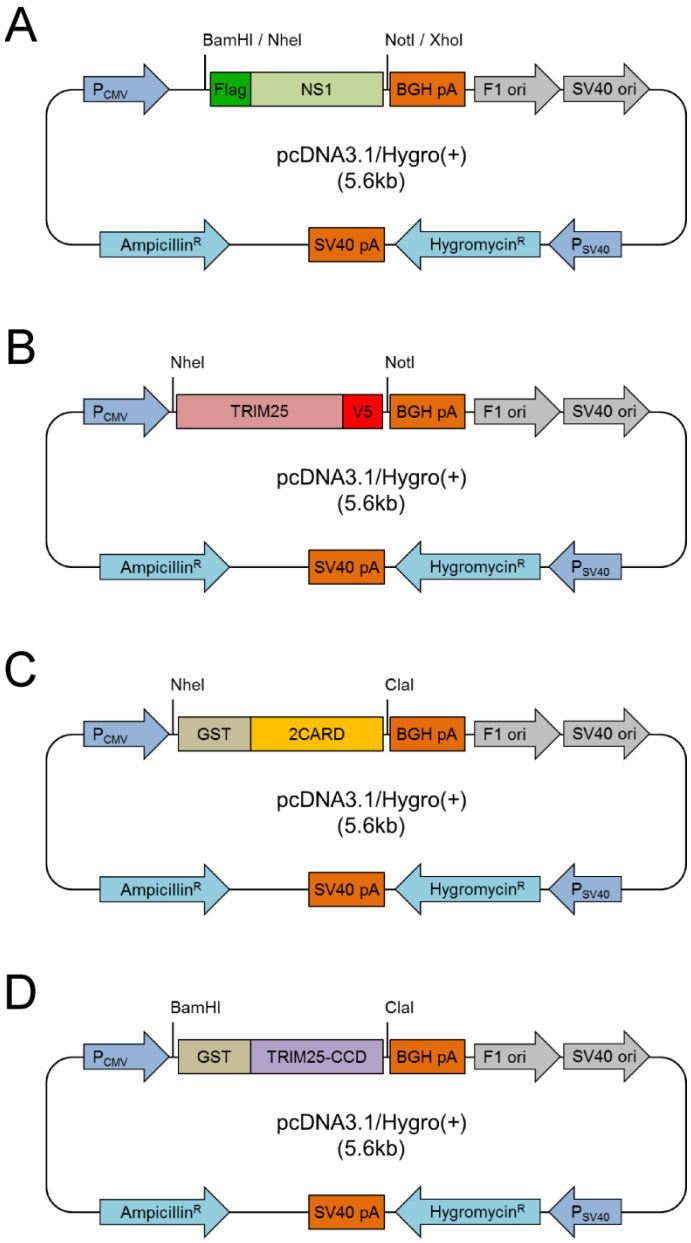



**Figure 2.1. Maps of expression constructs in the pcDNA3.1/Hygro(+) vector backbone.** (A) Flag-tagged NS1 (Flag-NS1) (B) V5-tagged TRIM25 (TRIM25-V5) (C) GST-tagged RIG-I CARD domains (GST-2CARD) (C) GST-tagged coiled coil domain of TRIM 25 (GST-TRIM25-CCD).

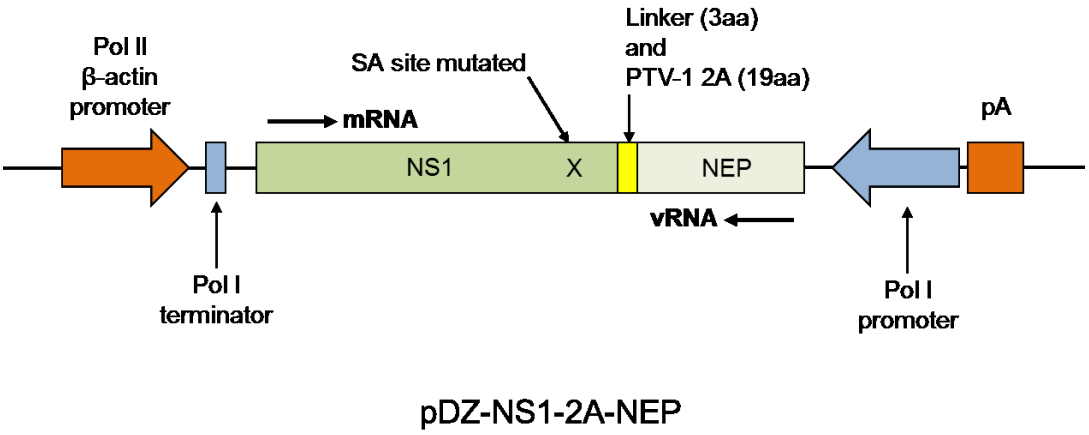



**Figure 2.2. Schematic of the pDZ-NS1-2A-NEP plasmid that separates the two protein coding sequences in the 8th (NS) genomic segment of PR8 virus.**
All of the viral rescue plasmids used in this study (pHW and pDZ) contain genomic segments of PR8 virus between Pol II and Pol I promoters to allow the generation of mRNA and negative-sense vRNA copies in transfected eukaryotic cells. The non-coding ends of the influenza A genomic segments are included, but not shown here for clarity. The pDZ-NS1-2A-NEP plasmid contains the full coding sequence of PR8 NS1 with the splice acceptor site silently mutated. The full coding sequence of the NEP protein is directly downstream, separated from NS1 by a 3-amino-acid linker and a 19-amino-acid porcine teschovirus-1 2A site that catalyzes its own proteolytic cleavage. NS1 and NEP proteins are expressed as a polycistron and then cleaved apart. *This figure is adapted from (Perez et al., 2013).*

# Chapter 3 Avian influenza NS1 proteins inhibit human, but not duck, RIG-I-CARD ubiquitination and interferon signaling.

## 3.1 Introduction

The influenza A virus non-structural protein 1 (NS1) is a major virulence factor that facilitates viral replication and controls host cell immune responses. It is a small, multifunctional protein that is expressed only in host cells and participates in multiple protein-protein interactions to regulate viral RNA synthesis (Min et al., 2007), control host protein expression (Nemeroff et al., 1998), and inhibit innate immune signaling (Ayllon and García-Sastre, 2015; Hale et al., 2008). Without NS1, influenza A viruses do not replicate efficiently in immunologically competent hosts (García-Sastre et al., 1998; Kochs et al., 2007; Soubies et al., 2010b; Zhu et al., 2008). However, NS1 is also the most polymorphic gene of influenza A viruses, after the hypervariable surface antigens, in mammalian and in avian hosts (Obenauer et al., 2006). This means that not all NS1 proteins participate in the entire repertoire of interactions and functions that have been experimentally described. Two of the most well-studied functions of NS1 proteins are an interaction with cleavage and polyadenylation specificity factor 4 (CPSF4) and interference in the RIG-I signaling pathway. By binding to and sequestering human CPSF4, NS1 inhibits host mRNA maturation and achieves a broad and non-specific shutoff of host protein synthesis (Das et al., 2008; Hale et al., 2010b; Kuo and Krug, 2009; Nemeroff et al., 1998; Noah et al., 2003). By interfering with the retinoic acid-inducible gene I (RIG-I) signaling pathway, a key component in the early detection of influenza virus infection, NS1 proteins block the induction of type I interferons (IFN-α and IFN-β) and the ensuing antiviral response (Gack et al., 2009; Koliopoulos et al., 2018).

In humans, RIG-I is a key pattern recognition receptor for influenza virus detection (Kato et al., 2006; Liu et al., 2015a; Loo et al., 2008; Opitz et al., 2007; Weber-Gerlach and Weber, 2016). It detects viral 5'-triphosphate RNA species and signals through the mitochondrial antiviral signaling (MAVS) protein to induce nuclear translocation of interferon response factors (IRF3 and IRF7) and the transcription of type I interferons and antiviral interferon-stimulated genes (Weber, 2015; Weber et al., 2013; Wu et al., 2014). RIG-I interacts with MAVS through a homotypic interaction between mutual caspase activation and recruitment (CARD) domains, an interaction that is stabilized by K63-linked polyubiquitination of the RIG-I CARD domains by tripartite motif-containing protein 25 (TRIM25) (Sanchez et al., 2016; Wu et al., 2014). NS1 binds human TRIM25 to prevent this ubiquitination and thus destabilize the RIG-I/MAVS CARD interaction (Gack et al., 2009; Koliopoulos et al., 2018)

TRIM25 is comprised of four protein domains (Fig 3.1). The N-terminal RING-type zinc finger domain is responsible for E3 ubiquitin transferase activity – it synthesizes K63-linked ubiquitin chains and attaches them to a target (Sanchez et al., 2016). The C-terminal B30.2/SPRY domain recruits the RIG-I CARD domains for ubiquitination (D'Cruz et al., 2013; Gack et al., 2008; Gack et al., 2007). In the interior of the protein lie the B boxes domain and the coiled-coil domain. The B boxes have a potential role in protein-protein interactions that are not defined with respect to RIG-I ubiquitination (Sanchez et al., 2016). The coiled-coil domain is a long alpha-helix responsible for antiparallel homo-dimerization of TRIM25 proteins (Sanchez et al., 2014), which is necessary for their enzymatic activity (Sanchez et al., 2016). In human cells, NS1 binds to the coiled-coil domain of TRIM25 to prevent RIG-I-CARD ubiquitination (Gack et al., 2009; Koliopoulos et al., 2018). An investigation of several NS1 proteins from representative human, swine, and avian influenza A virus strains found host species-specific and strain-specific

differences in their interactions with human, mouse, and chicken TRIM25 (Rajsbaum et al., 2012). In that study, all of the tested NS1 proteins bound to human TRIM25, but only the representative avian virus NS1 bound strongly to chicken TRIM25. Chickens are missing the RIG-I gene, but possess the related MDA5 receptor and many other functional components of the RIG-I signalling pathway (Barber et al., 2010; Magor et al., 2013). In murine cells, NS1 proteins did not bind mouse TRIM25 and were unable to inhibit mouse RIG-I-CARD ubiquitination (Rajsbaum et al., 2012). NS1 proteins did bind mouse Riplet, another E3 ubiquitin ligase that helps to fully activate RIG-I by ubiquitinating at its other end, the C-terminal repressor domain (Oshiumi et al., 2009; Oshiumi et al., 2010; Rajsbaum et al., 2012). Riplet functions analogously in human cells and is also targeted by NS1, but this is beyond the scope of this study.

Waterfowl are the primordial hosts of influenza viruses (Webster et al., 1992) and today dabbling ducks are the main ecological reservoir of low-pathogenic avian influenza viruses (Yoon et al., 2014). Mallards are more resistant to disease caused by influenza viruses than most other afflicted species, including, in many cases, disease caused by highly pathogenic avian influenza viruses (Alexander et al., 1986; Cooley et al., 1989; Laudert et al., 1993). The co-evolution of mallard ducks with influenza viruses has likely produced unique host–pathogen interactions that allow ducks to tolerate disease and act as asymptomatic carriers of almost all known strain types. It is possible that this co-evolution has affected duck RIG-I pathway regulation and its inhibition by influenza A virus NS1.

We recently cloned duck TRIM25 and duck RIG-I and characterized the induction of the MAVS signaling pathway (Miranzo-Navarro and Magor, 2014). Full-length human and duck TRIM25 proteins share 49% amino acid identity (Fig 3.2). Despite this low conservation, we

found that duck RIG-I signaling functions analogously to the human mechanism (Wu et al., 2014), with RIG-I signaling through MAVS, stabilized by TRIM25-mediated polyubiquitination of the RIG-I CARD domains. Unlike human RIG-I CARD domains, which are ubiquitinated at a single lysine residue, duck RIG-I CARD domains can be ubiquitinated at either of two lysines, and can still be activated by unanchored ubiquitin chains synthesized by TRIM25 when these lysines are mutated (Miranzo-Navarro and Magor, 2014). The coiled-coil domain of human TRIM25, which is bound by NS1 (Gack et al., 2009; Koliopoulos et al., 2018), shares only 37% amino acid identity with the homologous region of duck TRIM25 (Fig 3.2). To answer the question of whether NS1 proteins bind duck TRIM25 to inhibit interferon induction in the natural host, I compared the abilities of several different avian influenza NS1 proteins and one mammalian NS1 protein to interact with human and duck TRIM25, and compared the consequences on RIG-I ubiquitination and signaling in both species. I found that NS1 proteins associated with both human and duck TRIM25 *in vitro*, but could only block ubiquitination of and interferon induction by human RIG-I CARD domains, while duck RIG-I CARD ubiquitination and interferon induction remained unaffected. I also show that the major NS1 binding pocket for host CPSF4, centered on NS1 residue glycine-184, may also affect interferon regulation through a separate, unknown mechanism.

## 3.2 Results

### *3.2.1 The NS1 proteins used in this study and their sequence features.*

I cloned five influenza A virus NS1 proteins for use in this study (Fig 3.3A). Two NS1 proteins were cloned from highly pathogenic avian influenza (HPAI) viruses – A/Duck/D4AT/Thailand/71.1/2004 (H5N1) abbreviated D4AT, and A/Vietnam/1203/2004

(H5N1) abbreviated VN1203. These two HPAI H5N1 viruses are closely phylogenetically related (both belong to the A/goose/Guangdong/1/1996 lineage, HA antigenic clade 1) (Kim et al., 2008; WHO/OIE/FAO H5N1 Evolution Working Group, 2012), but whereas D4AT was isolated from a duck (Songserm et al., 2006), VN1203 was isolated from a fatal human case (Govorkova et al., 2005). Both of these NS1 proteins belong to allele A. In experimental infections, D4AT was more lethal in ducks (Sturm-Ramirez et al., 2005) and induced more interferon (Saito et al., 2018), compared to VN1203. We were interested to see if differences between these NS1 proteins could be correlated with virulence and interferon induction, with respect to the duck RIG-I pathway. Two NS1 proteins were also cloned from low-pathogenic avian influenza (LPAI) viruses – A/Duck/British Columbia/500/2005 (H5N2) abbreviated BC500, and A/Chicken/California/431/2000 (H6N2) abbreviated CA431. Both of these NS1 proteins belong to allele B. We also cloned the NS1 protein from a well-studied, mouse-adapted laboratory strain A/Puerto Rico/8/1934 (H1N1), abbreviated PR8. PR8 NS1 belongs to allele A.

NS1 proteins are relatively small (~230 amino acids) and possess a two-domain structure – an N-terminal RNA-binding domain (RBD) (residues 1-72) and a C-terminal effector domain (ED) (residues 85-207) joined by a flexible linker region (Fig 3.3A). The linker region varies in length between different strains; the two H5N1 NS1 proteins I used contain a 5-amino-acid truncation in that region (Fig 3.3B), which may affect the structural conformations that can be adopted (Bornholdt and Prasad, 2008; Carrillo et al., 2014) and is associated with enhanced virulence (Long et al., 2008). The extreme C-terminal end of NS1 is also highly variable in sequence and in length, and possesses species-specific signatures. The majority of circulating avian influenza A viruses sampled between 1976 and 2004 contain NS1 proteins that are 230 amino acids long, with the last four amino acids forming the consensus sequence ESxV

(Obenauer et al., 2006; Thomas et al., 2011); three of the avian NS1 proteins used in this study belong to this group (Fig 3.3B). The majority of low-pathogenic human strains sampled in that timeframe share the C-terminal consensus sequence RSxV (Obenauer et al., 2006; Thomas et al., 2011), which is borne by PR8 NS1 (Fig 3.3B). The C-terminal tail of VN1203 NS1 is truncated by ten amino acids, which is the most striking difference between it and the highly-related D4AT NS1. Overall, the two LPAI NS1 proteins (BC500 and CA431, allele B) share 96.5% amino acid sequence identity and the two HPAI NS1 proteins (allele A) share 97.7% amino acid sequence identity. PR8 NS1, belonging to allele A, is more similar to the HPAI NS1 proteins (89.8% identity with VN1203) than to the LPAI (67.4% identity with BC500). The interaction of NS1 proteins with human TRIM25 involves both the RNA-binding and effector domains (Gack et al., 2009; Rajsbaum et al., 2012). Four critical residues for this interaction have been identified, R38 and K41 in the RNA-binding domain, and E96 and E97 in the effector domain (Gack et al., 2009). These residues are conserved in all of the NS1 proteins that I cloned (Fig 3.3B).

### *3.2.2 Recombinant NS1 expression and the glycine-184-to-arginine (G184R) mutation.*

For protein interaction studies, I amplified the avian NS1 protein coding sequences out of infected duck mRNA and cloned them into an overexpression vector. However, the wild-type avian NS1 proteins were expressed only to a very low level in transfected cells (Fig 3.4), even under the control of a highly active CMV promoter, due to their interaction with host cleavage and polyadenylation specificity factor 4 (CPSF4). NS1 proteins that bind and sequester host CPSF4, broadly and non-specifically repress host mRNA maturation and nuclear export in those cells (Nemeroff et al., 1998). When NS1 is expressed heterologously, without viral polymerase, nascent NS1 proteins repress their own continued expression by host machinery and are virtually undetectable by Western blot (Chauché et al., 2018). The four avian NS1 proteins used in this

study possess the amino acids required to interact with human CPSF4 (Das et al., 2008), while wild-type PR8 NS1 does not (Steidle et al., 2010). Thus, of the wild-type NS1 proteins, only PR8 could be expressed to a high level in HEK293T cells (Fig 3.4A). NS1 binds the second and third zinc fingers of CPSF4 (Das et al., 2008), which are perfectly conserved at the amino acid level between humans, chickens, and ducks. Thus, in the DF-1 chicken cell line also, wild-type avian NS1 proteins could only be expressed to a low level (Fig 3.4B). A glycine-184-to-arginine (G184R) point mutation rescued heterologous protein expression in both cell types (Fig 3.4A and B). G184 is a surface-accessible residue that sits at the center of a small binding pocket on NS1 into which CPSF4 docks. Mutating G184 abrogates the interaction with CPSF4, but does not affect the ability of PR8 NS1 to bind TRIM25 and inhibit interferon induction in HEK293T cells, according to a previous study using intentionally mutated recombinant viruses (Steidle et al., 2010). I included a G184R-mutant PR8 NS1 as a control, to confirm that this mutation would not affect interactions with TRIM25. I also cloned a loss-of-function mutant of PR8 NS1, substituting an alanine in place of arginine-38 (R38A), to abolish TRIM25 interaction (Gack et al., 2009) and serve as a negative control (Fig 3.4C).

### *3.2.3 NS1 proteins co-precipitate with human and duck TRIM25 proteins, with strain-specific differences.*

To validate my controls, I co-expressed the PR8 NS1 variants with hTRIM25-V5 in HEK293T cells for anti-V5 co-immunoprecipitation. As expected, both wild-type and G184R-mutant PR8 NS1 proteins co-precipitated with hTRIM25, while the R38A mutant did not (Fig 3.5A). I compared the abilities of the avian NS1 proteins to interact with human and duck TRIM25 by similar anti-V5 co-precipitations with hTRIM25-V5 in HEK293T cells (Fig 3.5B) and with dTRIM25-V5 in DF-1 cells (Fig 3.5C). Of the four avian NS1 proteins, three co-

precipitated with human TRIM25: BC500, D4AT, and VN1203 NS1 proteins were pulled down, while CA431 was not, consistently (Fig 3.5B). In contrast, all of the NS1 proteins tested, except the negative control mutant R38A, co-precipitated readily with dTRIM25 (Fig 3.5C). Thus, both avian and mammalian-origin NS1 proteins associate with duck TRIM25, and demonstrate apparent strain-specific differences in the ability to associate with human TRIM25.

### *3.2.4 NS1 proteins co-localize with human and duck TRIM25 in the cytoplasm of human and avian cells.*

To compare the interactions of the NS1 proteins with human and duck TRIM25, I began by examining their subcellular distributions and co-localization (Figs 3.5 and 3.6). To demonstrate that NS1 proteins co-localized with TRIM25 to the same subcellular compartment, I performed confocal microscopy on Flag-tagged NS1 proteins (Flag-NS1) and V5-tagged human TRIM25 (hTRIM25-V5) co-expressed in HEK293T cells (Fig 3.6A), and Flag-NS1 and V5-tagged duck TRIM25 (dTRIM25-V5) co-expressed in DF-1 cells (Fig 3.6B). In both cell types, TRIM25 proteins localized to the cytoplasm while Flag-NS1 proteins were always diffusely distributed throughout both the nuclei and the cytoplasm (Fig 3.6A and B). Pearson's co-localization coefficient analysis in one plane showed a low degree of quantifiable co-localization between human TRIM25 and all of the tested NS1 proteins in the cytoplasm of HEK293T cells – mean R values between 0.0 and 0.5 (Fig 3.6C). The R value for co-localization of hTRIM25-V5 with D4AT-G184R NS1 was lower than the rest, reflecting an overall lower abundance of that NS1 in the cytoplasm that was visually apparent. In DF-1 cells, NS1 proteins also co-localized with dTRIM25-V5 in the cytoplasm. Pearson's co-localization analysis produced mean R values above 0.5 in all cases, with no statistical differences between any of the groups (Fig 3.6D). When the NS1 proteins and the two TRIM25 orthologues were expressed separately in cells, their

subcellular distributions looked similar to what I saw in co-expressing cells (Fig 3.7A and B). To control for the potential influences of cell type and the Flag tag, I performed similar microscopy with untagged PR8 NS1 proteins labeled with anti-NS1 antibodies and hTRIM25-V5 in HeLa cells and observed similar intracellular distributions (Fig 3.7C).

### *3.2.5 NS1 proteins do not inhibit TRIM25 dimerization through its coiled-coil domain.*

To test the functional consequences of the NS1 association with TRIM25, I investigated whether NS1 would prevent the association of TRIM25 with RIG-I-CARD domains and whether NS1 would prevent homo-dimerization of the TRIM25 coiled-coil domain (CCD). According to the two currently available model mechanisms for NS1 TRIM25 interference, NS1 should either inhibit CCD dimerization to block enzymatic activity (Gack et al., 2009), or should bind to TRIM25 dimers and inhibit activity by steric interference (Koliopoulos et al., 2018). In both cases, NS1 should not affect the association of TRIM25 with RIG-I CARD domains through its C-terminal B30.2/SPRY domain (Gack et al., 2009; Koliopoulos et al., 2018).

To test if NS1 proteins inhibit the association between TRIM25 and the RIG-I CARD domains, I performed GST pulldowns from cells co-expressing a GST-tagged RIG-I CARD domains fragment and TRIM25, in the absence and presence of NS1 proteins. None of the NS1 proteins prevented association between human RIG-I CARD domains (GST-h2CARD) and hTRIM25 in HEK293T cells (Fig 3.8A), or between duck RIG-I CARD domains (GST-d2CARD) and dTRIM25 in DF-1 cells (Fig 3.8B). To determine if NS1 proteins interfere with TRIM25 homo-dimerization via the coiled-coil domains, I cloned GST-tagged CCD fragments of human and duck TRIM25. The CCD constructs were used to co-precipitate full-length TRIM25 by GST pulldown, in the absence and presence of NS1 proteins (Fig 3.9A-C). None of

the NS1 proteins prevented the association of human TRIM25 with its cognate CCD, when co-expressed in HEK293T cells (Figs 3.8A and B). Similarly, none of the NS1 proteins prevented the association of duck TRIM25 with its cognate CCD in DF-1 cells (Fig 3.9C). These results fit with the model proposing that NS1 proteins bind to already-dimerized TRIM25 proteins (Koliopoulos et al., 2018).

### *3.2.6 Avian NS1 proteins inhibit TRIM25-mediated ubiquitination of human RIG-I CARD domains, but do not affect duck RIG-I-CARD ubiquitination; PR8 NS1 appears to stimulate human RIG-I-CARD ubiquitination.*

To investigate the effect of the NS1 proteins on TRIM25-mediated ubiquitination of RIG-I in both species, I co-expressed HA-tagged ubiquitin in cells with RIG-I CARD domains and TRIM25, in the presence and absence of NS1 (Figs 3.9 and 3.10). Co-expression of TRIM25 with RIG-I CARD domains increased the amount of ubiquitination compared to CARD domains alone, for both the human and duck orthologues (Fig 3.10A and B). In HEK293T cells, the PR8 NS1 variants increased the proportion of ubiquitinated human RIG-I CARD domains whereas all four avian NS1 proteins decreased the proportion of ubiquitinated RIG-I-CARD (Fig 3.10A). On the other hand, in DF-1 cells, NS1 co-expression did not significantly affect the level of ubiquitination of duck RIG-I CARD domains by dTRIM25, for all variants and strains of origin (Fig 3.10B). Thus, NS1 proteins from four avian influenza virus strains inhibit human TRIM25-mediated RIG-I ubiquitination, but not duck TRIM25-mediated RIG-I ubiquitination. Figure 3.11 shows more clearly that, in HEK293T cells, PR8 NS1 variants appear to stimulate RIG-I-CARD ubiquitination (Fig 3.11A) while the avian NS1 proteins potently suppress it (Fig 3.11B).

### *3.2.7 Avian NS1 proteins inhibit interferon induction by human RIG-I CARD domains in HEK293T cells; Wild-type PR8 NS1 inhibits interferon by a separate, TRIM25-independent mechanism.*

To examine the effect of NS1 proteins on type-I interferon induction by the human RIG-I pathway, I performed dual luciferase reporter assays measuring interferon-beta (IFN-β) promoter activity (Fig 3.12A-C). I stimulated the human IFN-β promoter in HEK293T cells by co-expressing hTRIM25 with the constitutively active human RIG-I CARD domain fragment. When using small quantities of the h2CARD construct, the addition of hTRIM25 significantly elevates its IFN-β promoter stimulating activity, presumably through ubiquitination. Reflecting the ubiquitination patterns I observed, co-transfected avian NS1 proteins (BC500, CA431, D4AT, VN1203) significantly reduced hIFN-β promoter activity compared to the empty vector control and to the G184R and R38A PR8 NS1 variants (Fig 3.12A). However, I also found that wild-type PR8 NS1, but not PR8-G184R, suppressed IFN-β promoter activity in this system (Fig 3.12B) even though both of these NS1 proteins did not inhibit human RIG-I-CARD ubiquitination *in vitro*. The G184R mutation did not interfere with the ability of the avian NS1 proteins to inhibit TRIM25-mediated RIG-I ubiquitination, which suggests that interferon suppression by WT PR8 occurs through a distinct mechanism. Inhibition of hIFN-β promoter activity by wild-type avian NS1 proteins, possessing also a functional CPSF4 interaction, was more potent than inhibition by WT PR8 NS1 (Fig 3.12C).

To corroborate the luciferase reporter results, I quantified secreted IFN-β protein in the supernatants of co-transfected HEK293T cells (Fig 3.12D). As in the hIFN-β promoter reporter assays, avian NS1 proteins significantly reduced the amount of secreted interferon. It was unclear whether wild-type PR8 NS1 inhibited secreted interferon - the mean concentration value

is lower than the control and the other PR8 NS1 variants, but the difference is not statistically significant. In order to produce an appreciable amount of interferon-beta secretion for this analysis, I had to stimulate the cells with much larger quantities of transfected DNA than in the luciferase assays, which, in turn, produced high variability.

### *3.2.8 NS1 proteins do not inhibit interferon induction by duck RIG-I in DF-1 cells; Wild-type PR8 NS1 inhibits interferon by a separate, RIG-I-independent mechanism.*

To test the effects of the NS1 proteins on interferon-beta induction by duck RIG-I, I performed dual luciferase assays measuring chicken IFN-β promoter induction in DF-1 cells co-expressing duck RIG-I CARD domains and dTRIM25 (Fig 3.13). The four avian NS1 proteins did not inhibit chIFN-β promoter activity (Fig 3.13A), just as they did not inhibit d2CARD ubiquitination. The two PR8 mutants also did not inhibit interferon induction, but a slight inhibition by WT PR8 NS1 was recorded. To examine this further, I compared the effect of the PR8 NS1 variants on interferon induction downstream of d2CARD, and downstream of duck MAVS, also constitutively active when overexpressed (Fig 3.13B). Wild-type PR8 NS1 inhibited interferon downstream of both proteins, suggesting a RIG-I-independent mechanism of inhibition that is affected by the G184R mutation, similar to what I saw in human cells. I performed a co-immunoprecipitation to determine if inhibition downstream of MAVS was due to a unique interaction by wild-type PR8 NS1 with duck MAVS itself (Fig 3.13C). I found that trace amounts of all the NS1 proteins, except the R38A mutant, were pulled down in complexes containing duck MAVS (dMAVS-V5) Thus, interferon inhibition by wild-type PR8 NS1 was not due to a specific interaction with duck MAVS.

In an effort to find an additional way of quantifying interferon signaling in DF-1 cells, I investigated nuclear translocation of IRF7 proteins. Overexpressed duck IRF7 (not shown) and chicken IRF7 translocated to the nucleus of cells stimulated with duck RIG-I CARD domains (Fig 3.14), but the system did not prove sensitive enough to assay NS1-mediated inhibition.

### *3.2.9 Recombinant virus infection studies.*

To complement recombinant protein work, I wanted to investigate RIG-I ubiquitination and interferon signaling in cells infected with recombinant viruses bearing different NS1 genes in identical genetic backgrounds. Using wild-type PR8 virus and a del-NS1 mutant version lacking the NS1 gene, I wanted to investigate the effect of PR8 NS1 on endogenous human RIG-I ubiquitination in HEK293T cells, as in Gack et al. (2009). I found, however, that I was unable to immunoprecipitate any detectable amount of endogenous RIG-I from HEK293T cells, and found a statement in the literature to the same effect (Linehan et al., 2018). To determine which cell type would be more appropriate for me to use, I stimulated three human cells lines with poly (I:C) and found that A549 cells had the highest inducible endogenous RIG-I expression (Fig 3.15A). Upon infecting A549 cells, I found that I could immunoprecipitate RIG-I, but not in sufficient quantities to produce a visible and clean ubiquitin blot (Fig 3.15B). I tried compensating for this by transfecting the cells with recombinant HA-tagged ubiquitin, but found that this did not solve the problem (Fig 3.15C). I also tried blotting the whole-cell lysates for human IFN-β, but observed what may be non-specific binding (Fig 3.15C). There is a band at approximately 22 kDa, the expected size of IFN-β, but the band intensity is equivalent in the infected and in the mock-infected samples. To overcome protein quantity problems I have decided to look at ubiquitination of overexpressed full-length RIG-I in cells infected with WT

and del-NS1 PR8 viruses. To date, I have made V5-tagged full-length RIG-I expression vectors, for both human and duck orthologues, and have verified protein expression.

I will also generate a set of recombinant PR8 viruses encoding the wild-type versions of each avian NS1 used in this study, along with the G184R and R38A mutants of PR8 NS1. Recombinant influenza A viruses can be generated in cell culture from plasmid starting material, using a rescue method devised by Palese and García-Sastre (Martínez-Sobrido and García-Sastre, 2010; Schickli et al., 2001). PR8 strain genomic segment rescue plasmids were kindly provided to me by Dr. Yan Zhou and Dr. Adolfo García-Sastre, and their sequences were confirmed. I have generated seven rescue plasmids encoding each different NS1 CDS within the PR8 genomic segment 8. In all cases, the PR8 NEP protein coding sequence is preserved by silently mutating the splice acceptor site in the variant NS1 and including the complete PR8 NEP CDS downstream of the NS1 gene (Nogales et al., 2016; Perez et al., 2013). In a test rescue, I generated two functional infectious viruses – a wild-type PR8 virus and a PR8 virus encoding BC500 NS1. Infectivity was verified by plaque assay on MDCK cells, using dilutions of the viral rescue supernatants (Fig 3.16). Mock rescue supernatant produced no cell death or evidence of infection. With both viral rescue supernatants, most of the MDCK cells died even at the 1000-fold dilution. No further dilutions were done because I had not expected the viral rescue to be so efficient. Viral RNA was extracted from centrifuged supernatants of secondarily infected MDCK cells (to reduce the possibility of rescue plasmid contamination) and the NS1 genes were sequenced and their identities confirmed. No viral RNA could be obtained from cells infected with the mock rescue sample. These rescued viruses still need to be plaque purified.

## 3.3 Discussion

A lot of research has been done on the molecular interactions of influenza A NS1 proteins in mammalian cells, but little work has been done in mallard ducks, an important ecological host species with unique disease dynamics (Evseev and Magor, 2019). Here I show that NS1 proteins from two LPAI strains and two HPAI strains associate with duck TRIM25, but this does not reduce the ubiquitination of duck RIG-I CARD domains and their induction of interferon, as it does in human cells (Fig 3.17). I also show the existence of potential strain-specific differences with respect to human TRIM25 binding and ubiquitination inhibition. I provide support for a model of TRIM25 binding by NS1 that does not block TRIM25 dimerization or its association with RIG-I CARD domains, in both species. Finally, I provide evidence for the existence of a separate interferon suppression mechanism that functions downstream of RIG-I and MAVS in both species, which is affected by the residue at position 184 in NS1.

### *3.3.1 Avian influenza NS1 proteins inhibit human, but not duck, RIG-I-CARD ubiquitination and interferon signaling.*

I mutated the four avian NS1 proteins at glycine-184 to abrogate their interaction with cleavage and polyadenylation specificity factor 4 (CPSF4), to be able to express them heterologously (Das et al., 2008). I chose to mutate the single glycine-184 to an arginine (G184R) because this was sufficient to increase expression, and because it was previously demonstrated to have no effect on the interaction of PR8 NS1 with human TRIM25 and on the induction of interferon in HEK293T cells and in mice (Steidle et al., 2010). Wild-type PR8 NS1 possesses two rare substitutions at amino acid positions 103 and 106 that prevent it from interacting with CPSF4. Using both wild-type and G184R-mutant PR8 NS1 proteins as controls,

I confirmed that the mutation does not affect association with TRIM25. A five-amino-acid mutation to increase NS1 expression, at positions 184-188, has also previously been described (Kumar et al., 2012; Noah et al., 2003).

I tested the interaction of NS1 proteins with human and duck TRIM25 using co-immunoprecipitation. The PR8 NS1 variants behaved as expected with respect to human TRIM25 - WT and G184R-mutant interacting comparably, and R38A-mutant losing the interaction. The same pattern held with duck TRIM25. Among the four avian NS1 proteins, there were consistent differences in the amounts pulled down with human TRIM25, despite the universal conservation of four amino acid residues that have been identified as critical for this interaction – R38, K41, E96 and E97 (Gack et al., 2009). This suggests that additional amino acids contribute to its stability. In fact, a crystal structure of PR8 NS1 bound to hTRIM25 from Koliopoulos et al. (2018) showed that NS1 residues E96 and E97 do not make contact with TRIM25 itself, but stabilize the NS1 effector domain through internal polar interactions, whereas NS1 residue L95 fits into a hydrophobic pocket on TRIM25 and makes several important non-polar contacts. All of the NS1 proteins in my set encode either a leucine (L) or an isoleucine (I) at this position, except CA431, which does not co-precipitate readily with hTRIM25 and encodes a smaller valine (V) residue at position 95. It will be interesting to mutate that valine to a leucine or an isoleucine to see if that enhances the interaction of CA431 NS1 with human TRIM25. Strain-specific and host-species-specific differences in the ability of NS1 proteins to interact with TRIM25 have been documented before (Rajsbaum et al., 2012), but in that study NS1 proteins from representative avian, human, and swine influenza A strains bound comparably to human TRIM25 (and all encoded either a leucine or an isoleucine at position 95). In my comparison of

four avian strains it appears that polymorphisms in NS1 result in strain-specific differences with respect to the human TRIM25 interaction, at least *in vitro*.

In a comparison of avian, porcine, and human influenza A NS1 proteins, Rajsbaum et al. (2012) found that only the avian NS1 bound strongly to chicken TRIM25. In my set, PR8 and all four avian NS1 proteins bound duck TRIM25 efficiently in DF-1 cells. The uniform interaction of the four avian NS1 proteins with duck TRIM25, in contrast to the strain-specific differences observed in the human TRIM25 co-precipitation, suggest a potential species-specific difference in the binding mechanics to the TRIM25 orthologues. The R38A NS1 mutation that abolishes human TRIM25 binding also abolished binding of PR8 NS1 to duck TRIM25. R38 is an important residue for multiple NS1 functions, including multimerization (Bornholdt and Prasad, 2008) and dsRNA binding (Min and Krug, 2006). It is conserved in over 99% of NS1 sequences in the NCBI influenza database, and mutating results in severe attenuation (Donelan et al., 2003; Steidle et al., 2010).

Certain limitations of the co-immunoprecipitation technique must be kept in mind when interpreting this data. Co-precipitation of proteins from a cell lysate does not necessarily imply direct interaction, but can also result from common association of the bait and prey with a larger protein complex. In the case of NS1 and TRIM25, pre-existing functional research and X-ray crystallography suggest that a direct protein-protein interaction does occur. However, it is also important to remember that the system itself is somewhat artificial because the molar ratios of the overexpressed proteins probably do not reflect their actual biological proportions in an infected cell. Furthermore, in doing these experiments, I did not systematically test different buffers of varying stringency. To overcome these limitations I incorporated a good negative control, the loss-of-function mutant NS1 R38A. Within the parameters set by the positive and

negative controls, I assume that co-immunoprecipitation experiments provide an approximation of biological reality.

I examined the subcellular distributions of co-expressed TRIM25 and NS1 proteins and observed some co-localization in the cytoplasmic compartment, with both human and duck TRIM25 orthologues. In HEK293T and in DF-1 cells, NS1 was distributed throughout the nuclei and cytoplasm, both in the presence and in the absence of TRIM25. Previous studies using recombinant NS1 proteins showed higher proportions of cytoplasmic versus nuclear NS1 in the presence of human TRIM25 compared to NS1 alone (Gack et al., 2009; Rajsbaum et al., 2012), but I observed a consistent distribution of NS1 to both cellular compartments independent of TRIM25 co-expression. Over all, the degree of co-localization between the NS1 proteins and either orthologue of TRIM25 does not appear to correlate with the co-immunoprecipitations of NS1 with TRIM25. These observations differ from previous studies (Gack et al., 2009; Rajsbaum et al., 2012), but are in line with data demonstrating that inability to bind CPSF4 affects the subcellular distribution of NS1 (Han et al., 2010). Han et al. compared chimeric GFP-NS1 proteins and showed that wild-type PR8 NS1, which is naturally unable to interact with CPSF4, was distributed throughout the nuclei and cytoplasm of HEK293T cells, while wild-type NS1 from A/Sydney/5/1997(H3N2), a competent CPSF4 binder, was exclusively nuclear (Han et al., 2010). Mutating A/Sydney/5/1997 NS1 to abolish CPSF interaction, either by mutating residues 103 and 106 to resemble PR8, or by mutating residues 184-188, induced these NS1 proteins to localize diffusely to the nucleus and cytoplasm, similar to WT PR8 NS1 (Han et al., 2010). A more recent study of NS1 subcellular localization, employing super-resolution microscopy, found that a fraction of PR8 NS1 co-localizes with hTRIM25 and RIG-I-containing puncta in the cytoplasm of HeLa cells (Sánchez-Aparicio et al., 2017). The co-localization

experiments presented here also have limitations that should be considered in interpretation. Fluorescent signal overlap, as quantified by Pearson's correlation coefficient, does not necessarily imply protein-protein interactions. Furthermore, signal overlap between two diffusely-cytoplasmic proteins may be incidental, as appears to be the case with NS1-R38A. These experiments do show us, at minimum, that TRIM25 and NS1 proteins localize to the same cellular compartment where they could theoretically interact.

There are currently two different models in the literature that describe how NS1 blocks TRIM25-mediated ubiquitination. According to the first model, NS1 binds to the central coiled-coil domain of TRIM25 and prevents the homo-dimerization that precedes catalytic activity (Gack et al., 2009; Sánchez-Aparicio et al., 2017; Sanchez et al., 2016; Sanchez et al., 2014). When I co-precipitated full-length TRIM25 proteins with recombinant CCD-only fragments, with and without co-expressed NS1, I found that none of the NS1 proteins prevented the association between TRIM25 and its corresponding CCD, for both human and duck orthologues. This did not agree with the established model, but soon after, Koliopoulos et al. (2018) proposed an alternative mechanism based on a co-crystallization of NS1 with TRIM25. According to their crystal structure, NS1 binds to the CCDs of already-dimerized TRIM25 proteins and sterically prevents the close association of the ubiquitin-bearing RING domain with the B30.2/SPRY domain, which recruits RIG-I-CARD – this prevents covalent ubiquitin ligation to the target. Thus, the model proposed by Koliopoulos et al. (2018), in which NS1 does not block TRIM25 homo-dimerization but binds to TRIM25 dimers, corroborates my results. According to both models, NS1 should not interfere with the interaction between the TRIM25 B30.2/SPRY domain and RIG-I CARD domains, and I confirmed this with both human and duck orthologues.

Next, I investigated how the different NS1 proteins would inhibit TRIM25-mediated ubiquitination of human RIG-I CARD domains, and whether they would inhibit duck RIG-I-CARD ubiquitination, using a similar method to those employed by Gack et al. (2009), Rajsbaum et al. (2012), and Koliopoulos et al. (2018). I was surprised to find that the PR8 NS1 variants, wild-type and both mutants, appeared to increase human RIG-I-CARD ubiquitination rather than decrease it. In all previous work with PR8 NS1, researchers reported inhibition of TRMI25-mediated ubiquitination, but the details were not unequivocal. Gack et al. (2009) found that wild-type PR8 NS1 inhibited RIG-I-CARD ubiquitination, but that mutating the critical residues for TRIM25 interaction in NS1 (R38A+K41A or E96A+E97A) abrogated this inhibition. In contrast, Koliopoulos et al. (2018) observed ubiquitination inhibition by wild-type PR8 NS1 and also by mutant NS1 proteins where the same critical residues were mutated to alanine, in a similar experimental system. These discrepancies demonstrate either the technical limitations of these assays or that the inhibition of human RIG-I ubiquitination is a complex and context-dependent process. The single difference between the PR8 NS1 variant that I used and the one used in the above cited studies is an amino acid polymorphism at position 55. This naturally occurring polymorphism in PR8 strain variants is discussed in Chapter 1 (section 1.3.1). My PR8 NS1 variant encodes a lysine at position 55 (K55) while the variant used in Gack et al. (2009) and Koliopoulos et al. (2018) encodes a glutamic acid (E55). Two studies have found that the presence of E55 in NS1 enhances interferon inhibition and viral replication in human and mammalian cells (Li et al., 2018; Murakami et al., 2008), and it is notable that E55 is the most prevalent residue in avian influenza A isolates while K55 is the most common in human and swine isolates. It is possible that this polymorphism contributes to the ability of NS1 proteins to suppress RIG-I-CARD ubiquitination, which would be consistent with my results and the

observation that E55-encoding NS1 proteins inhibit interferon more effectively. I am currently working on experiments to test this hypothesis. The other notable difference between my ubiquitination experiments and the ones cited above is that I overexpressed HA-tagged ubiquitin in the cells whereas Gack et al. and Koliopoulos et al. blotted for endogenous ubiquitin. It is possible that the addition of exogenous ubiquitin contributed somehow to the difference that I saw in my results. However, my results also clearly show that various avian influenza NS1 proteins are able to potently inhibit human RIG-I-CARD ubiquitination, despite variable association with hTRIM25 in co-immunoprecipitations.

The ubiquitination levels of human RIG-I-CARD that I saw in the GST pull down experiments correlated with interferon-β promoter induction and interferon-β protein secretion downstream of RIG-I. WT PR8 NS1 was an exception, since it appeared to enhance human RIG-I-CARD ubiquitination, but to decrease interferon signaling and this is discussed in more detail below, in section 3.3.2. The suppression of ubiquitination by the four avian NS1 proteins translated into inhibition of IFN-β promoter activity and IFN-β protein secretion in HEK293T cells stimulated with TRIM25 and RIG-I CARD domains. Thus, in human cells, avian influenza NS1 proteins interact with human TRIM25 to block RIG-I-CARD ubiquitination and signaling. These findings agree with a recent paper demonstrating that avian NS1 proteins are effective at suppressing innate immunity in human cells (Monteagudo et al., 2019).

It was striking that none of the tested NS1 proteins decreased the ubiquitination of duck RIG-I CARD domains even though all of them bound to duck TRIM25. Correspondingly, I saw no inhibition of chicken IFN-β promoter activity in dRIG-I-CARD-stimulated DF-1 cells (excepting PR8 WT NS1, discussed below). DF-1 chicken cells lack endogenous RIG-I, but

possess the related MDA5 receptor and the downstream components of the signaling pathway, beginning with MAVS (Barber et al., 2010). Duck RIG-I-CARD domains interact with chicken MAVS to induce interferon, recapitulating RIG-I signaling (Barber et al., 2013; Chen et al., 2016; Cheng et al., 2015; Miranzo-Navarro and Magor, 2014; Wu et al., 2014). In DF-1 cells, avian influenza NS1 proteins interacted with duck TRIM25, but did not block duck RIG-I-CARD ubiquitination and signaling. Human and duck TRIM25 proteins share 49% amino acid identity overall, and the human TRIM25 CCD shares only 37% identity with the CCD of duck TRIM25. Of the 9 residues of human TRIM25 that make important contacts with PR8 NS1 according the Koliopoulos et al. (2018) crystal structure, only three are strictly conserved in duck TRIM25 (Fig 3.18). The fact that all of the NS1 proteins bound dTRIM25 efficiently, without the sequence-dependent differences that I saw with human TRIM25, suggests that different amino acid contacts are made. The fact that ubiquitination is not inhibited suggests that binding might occur in a region that does not sterically inhibit ubiquitin transfer, like it may with human TRIM25 (Koliopoulos et al., 2018). To discover where on duck TRIM25 do NS1 proteins bind, we could test a series of fragments of dTRIM25 for NS1 binding by co-precipitation, or perform X-ray crystallography. It is noteworthy that both human (Zeng et al., 2010) and duck (Miranzo-Navarro and Magor, 2014) RIG-I-CARD tetramers can also be stabilized by unanchored K63-linked ubiquitin chains, a redundancy that may partially compensate for viral inhibition. In fact, according to the Koliopoulos et al. (2018) model, TRIM25 dimers bound by NS1 do not lose their ability to synthesize unanchored ubiquitin chains, and this likely explains some of the residual signaling I saw in my human interferon assays.

### *3.3.2 RIG-I-independent mechanism of interferon inhibition that requires NS1 residue G184.*

Surprisingly, wild-type PR8 NS1, but not PR8-G184R, inhibited IFN-β promoter activity in both HEK293T and DF-1 cells even though, in my experiments, both NS1 proteins did not block TRIM25-mediated ubiquitination of either RIG-I-CARD orthologue. Secreted human interferon quantification also suggested potential inhibition by PR8-WT, but the effect was not statistically significant. NS1 is a multifunctional regulator of host cell dynamics and interferes with innate immune signaling in multiple ways and in multiple signaling pathways (Ayllon and García-Sastre, 2015; Hale et al., 2008). The interaction of most NS1 proteins with host CPSF4 to non-specifically block pre-mRNA maturation is a powerful mechanism that shuts down immune signaling and host protein production generally (Das et al., 2008; Hale et al., 2010b). The wild-type versions of the avian NS1 proteins, competent in CPSF4 binding, inhibited human IFN-β promoter activity more potently than wild-type PR8 NS1. Steidle et al. (Steidle et al., 2010) studied the effect of several different amino acid mutations in PR8 NS1, which is naturally unable to interact with CPSF4 due to two rare substitutions - F103S and M106I. In their report, Steidle and colleagues showed that introducing the G184R mutation into PR8 NS1 did not affect its *in vitro* association with human TRIM25, nor the ability of recombinant viruses bearing this mutation to inhibit interferon production in HEK293T cells and in reporter-mice (Steidle et al., 2010). However, they also showed that recombinant viruses bearing the G184R mutant NS1 proteins replicated more slowly than their wild-type counterparts in mice, despite growing at equivalent rates in MDCK and A549 cells. The authors concluded that the G184R mutation affects viral fitness by a mechanism unrelated to interferon production. My experiments suggest that the G184R mutation may actually impact some aspect of interferon inhibition by NS1, which functions separately from TRIM25 binding and RIG-I ubiquitination. The fact that WT

PR8 NS1 also inhibited chicken IFN-β promoter activity downstream of duck RIG-I and duck MAVS suggests that this is a RIG-I-independent mechanism that is conserved in chickens. I showed that this was not due to a specific interaction with duck MAVS. Kuo et al. (2010) have previously reported that NS1 proteins from fifteen human influenza strains differed in their ability to block IRF3 activation and IFN-β transcription in human cells irrespectively of TRIM25 binding. They found that TRIM25 binding did not always correlate with IRF3 suppression, but that the ability to block IRF3 activation coincided with glutamic acid (E) at position 196 in NS1. The authors concluded that E196 likely participates in an important protein-protein interaction. One such interaction could be with host TRAF3 (Qian et al., 2017), which NS1 targets in human cells to reduce IRF3 activation downstream of MAVS. Human TRAF3 interacts with the C-terminal end of NS1, between residues 126 and 225 (Qian et al., 2017). All of the NS1 proteins used in this study conserve E196. On currently-available NS1 crystal structures, E196 is a surface-exposed amino acid proximal to G184. It is possible that the protein-protein interaction that occurs at E196 to suppress IRF3 activation also involves G184, and that the G184R mutation disrupts this interaction as well as the interaction with host CPSF4. This hypothesis could be tested with the help of additional mutagenesis and protein mass spectrometry. The first step would be to mutate E196 in WT PR8 NS1 to see if that also abolishes interferon inhibition under these conditions. Wild-type and mutant NS1 proteins could be co-precipitated with human and duck TRAF3 to find out if this interaction may be responsible for the inhibition. Alternatively, mass spectrometry could be used to find other interacting partners from immunoprecipitated whole-cell lysates.

It is unclear if this inhibitory function that depends on the C-terminus of PR8 NS1 would be shared by the avian NS1 proteins in this study, if they were not also mutated at residue G184.

The C-terminal region of NS1 proteins is highly variable. In their investigation of NS1 and TRAF3, Qian et al. (2017) found that NS1 proteins from PR8 and from an H5N1 avian strain bound human TRAF3, while NS1 proteins from avian H7N9 and H9N2 strains did not. Due to the plethora of protein-protein interactions in which NS1 despite its small size, it is very difficult to isolate specific functions with mutagenesis, without unintentionally affecting others.

### *3.3.3 Recombinant virus infection studies and future directions*

I have begun recombinant virus infection studies to corroborate my recombinant protein results in a more biologically-relevant context. I will examine RIG-I ubiquitination and interferon signaling in cells infected with recombinant viruses expressing different NS1 proteins. Using wild-type and del-NS1 PR8 viruses I can further investigate the effects of PR8 NS1 on human RIG-I-CARD ubiquitination. Using recombinant PR8 viruses expressing different NS1 proteins, I can investigate strain-specific NS1 differences and compare functions, like the inhibition of RIG-I ubiquitination, in human and avian cells. This work has not been completed yet because I had an unexpected number of difficulties with recombinant protein experiments.

It is important to remember that the RIG-I pathway is only one of many innate antiviral detection mechanisms, and that interference in this pathway is only one of many functions of influenza A NS1. Furthermore, NS1-host protein-protein interactions are only one level in a hierarchy of variables that determine viral fitness and pathogenicity. For example, Ma et al. (2010) showed that two recombinant viruses containing different NS1 proteins in an identical background had different replication efficiencies and virulence in mice. The two NS1 proteins had equivalent PKR and CPSF4 binding affinities *in vitro*, but in infected mice, one of the NS1 proteins was expressed to a higher level and resulted in faster and stronger PKR and IFN-β

inhibition (Ma et al., 2010). Thus, in the future, these experiments should be expanded to *in vivo* models. It would be interesting to investigate the importance of RIG-I signaling in juvenile ducks using wild-type and RIG-I-knock-out animals infected with recombinant viruses encoding different NS1 proteins. Conversely, our lab is also working on knocking duck RIG-I into chicken embryos, which will add another fascinating platform for the study of antiviral innate immune signaling.

### *3.3.4 Conclusion*

In this chapter I presented evidence that avian influenza A NS1 proteins may lack the ability to downregulate duck RIG-I ubiquitination and signaling, as they do in human cells. Comparing NS1 proteins from several viral strains, I showed that RIG-I antagonism in the human host is a complex and multifactorial process with potential strain-dependent differences, the full scope of which we do not yet fully understand. Clearly, avian NS1 proteins from both A and B alleles are able, in principle, to potently inhibit human TRIM25-mediated RIG-I ubiquitination. The same avian influenza NS1 proteins interact with duck TRIM25, but do not interfere with duck RIG-I ubiquitination and signaling. The function of PR8 NS1 remains unclear, but highlights the functional plasticity of NS1 proteins and the existence of multiple mechanisms by which they interfere with host innate immune signaling, across multiple pathways and host species.

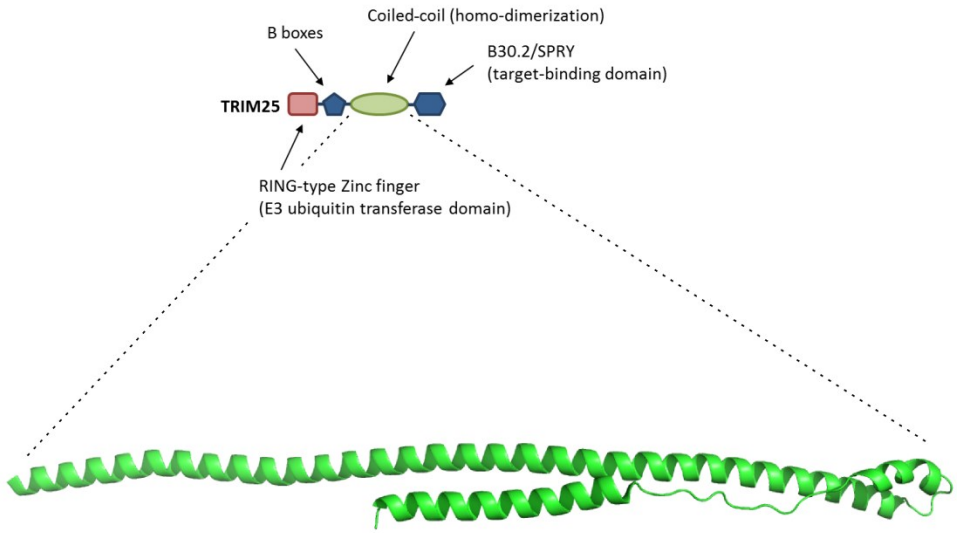



**Figure 3.1. TRIM25 domain architecture.**
TRIM25 proteins comprise four protein domains. The N-terminal RING-type zinc finger domain is responsible for enzymatic E3 ubiquitin transferase activity. Two tandem B box motifs may participate in protein-protein interactions and higher-order multimerization. The central coiled-coil domain (CCD) is a long alpha-helix responsible for antiparallel homo-dimerization of TRIM25 proteins, which is necessary for enzymatic activity. The C-terminal B30.2/SPRY domain recruits RIG-I CARD domains for ubiquitination. Ribbon diagram of CCD structure is adapted from Sanchez et al. (2014).

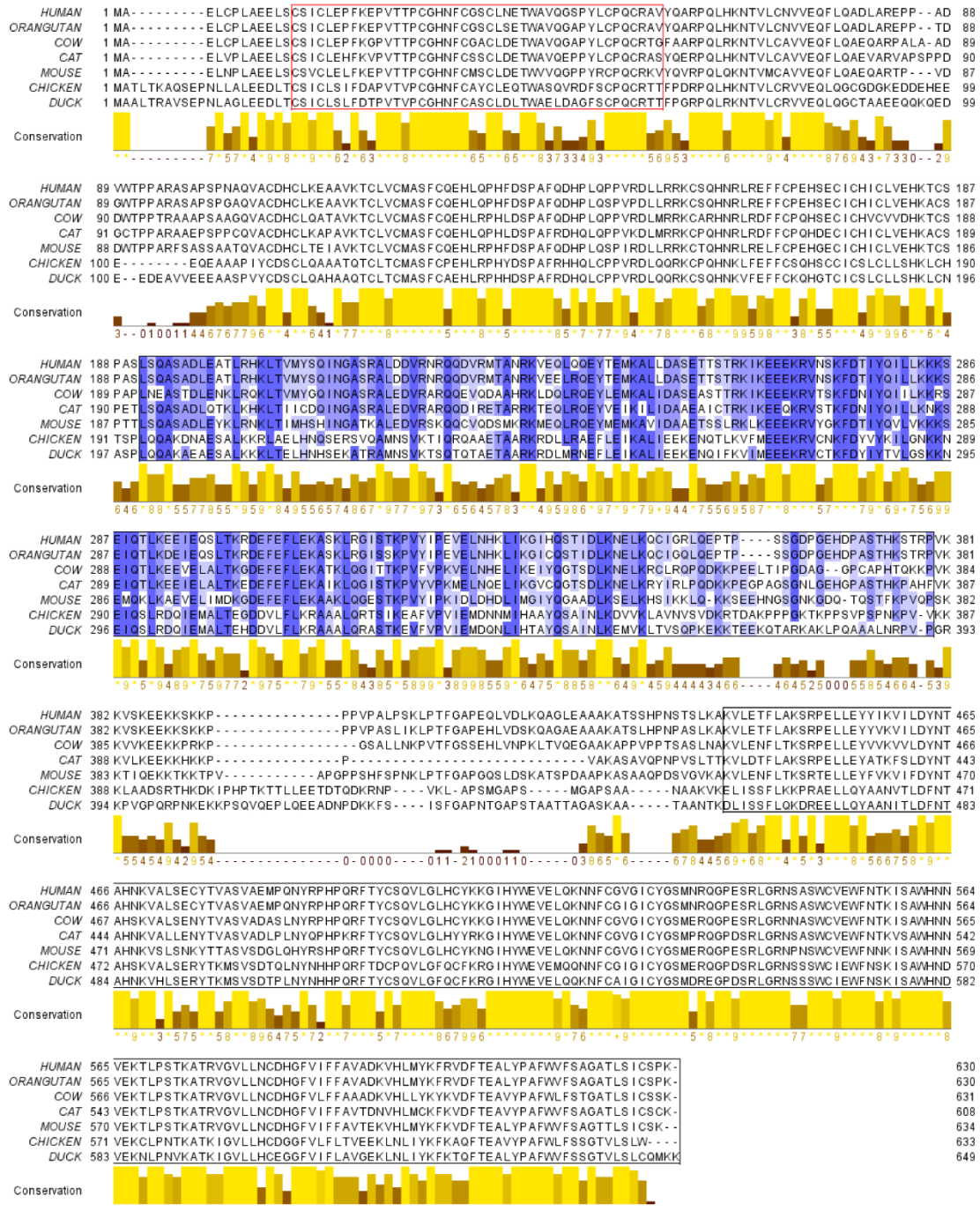



**Figure 3.2. TRIM25 multiple sequence alignment.**
Peptide alignment of TRIM25 proteins from humans (GenBank accession: NM_005082.4), orangutans (KX000923.1), cattle (NM_001100336.1), cats (NM_001290251.1), mice (NM_009546.2), chickens (KM879874.1), and mallard ducks (KF483852.1). Aligned in Jalview using MAFFT L-INS-i preset. RING domain is boxed in red, coiled coil domain is coloured by conservation, B30.2/SPRY domain is boxed in black.

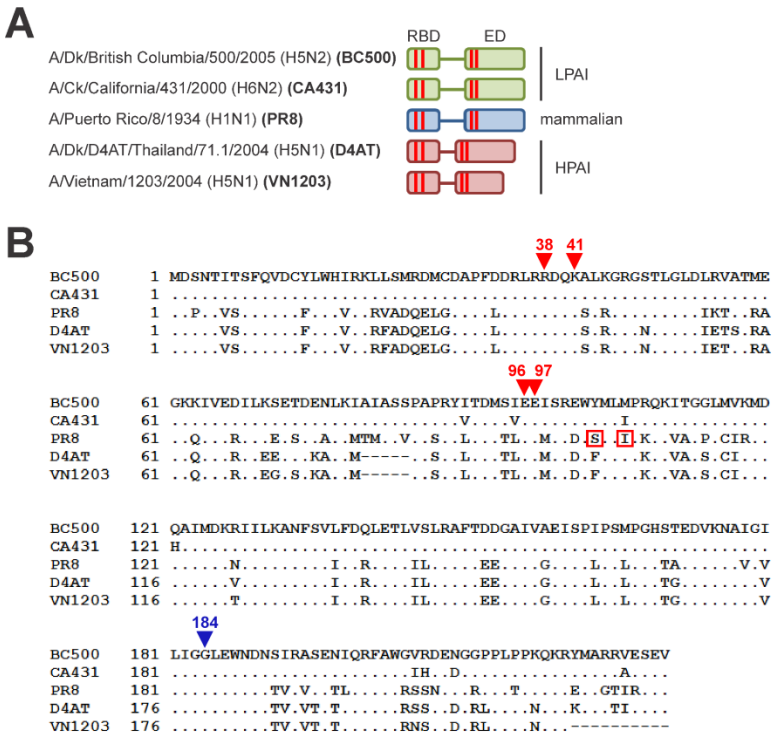



**Figure 3.3. NS1 proteins used in this study and their sequence features.**
(A) NS1 proteins represented schematically beside their corresponding virus strain names. Strain name abbreviations are shown in brackets in bold. Red bands on the NS1 proteins represent conserved critical residues for human TRIM25 interaction. LPAI, low-pathogenicity avian influenza; HPAI, highly pathogenic avian influenza. (B) NS1 peptide sequences were aligned using MAFFT. Red arrows indicate conserved critical residues for human TRIM25 interaction. Blue arrow indicates conserved residue glycine-184. Red boxes highlight PR8 NS1 residues S103 and I106, which prevent CPSF4 interaction.

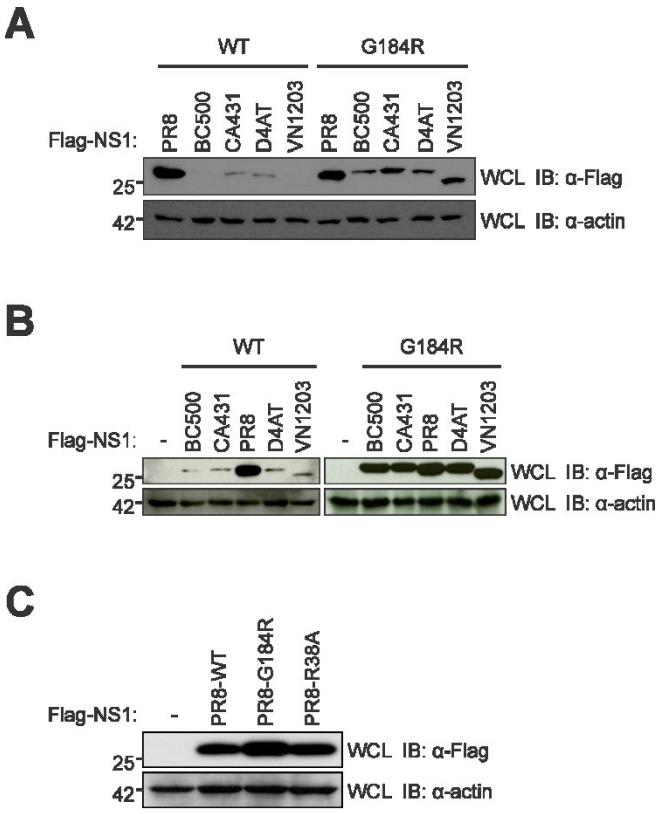



**Figure 3.4. Glycine-184-to-arginine (G184R) mutation rescues recombinant avian NS1 protein expression.**

(A) HEK293T cells were transfected with the indicated Flag-tagged NS1 proteins (Flag-NS1), either wild-type (WT) or G184R mutants. Whole cell lysates (WCL) were immunoblotted (IB) with anti-Flag and anti-actin antibodies. (B and C) DF-1 cells were transfected with the indicated Flag-tagged NS1 proteins (Flag-NS1). Whole cell lysates (WCL) were immunoblotted as in (A). Each experiment was repeated at least three times.

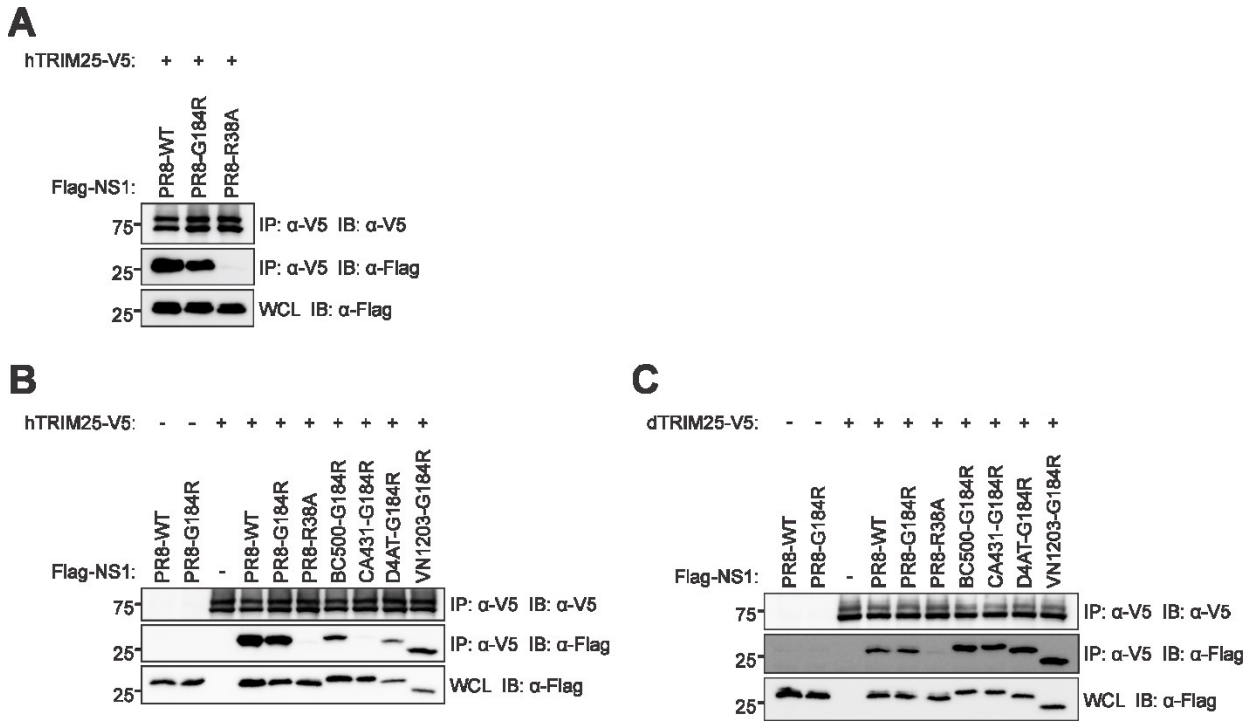



**Figure 3.5. NS1 proteins interact with human and duck TRIM25.**
(A and B) HEK293T cells were transfected with V5-tagged human TRIM25 (hTRIM25-V5) together with the indicated Flag-tagged NS1 proteins (Flag-NS1). Clarified whole cell lysates (WCL) were subjected to anti-V5 immunoprecipitation (IP), followed by immunoblotting (IB) with anti-V5 and anti-Flag antibodies. (C) DF-1 cells were transfected with V5-tagged duck TRIM25 (dTRIM25-V5) together with the indicated Flag-tagged NS1 proteins (Flag-NS1). Co-precipitation performed as in (A). Each experiment was repeated at least three times.

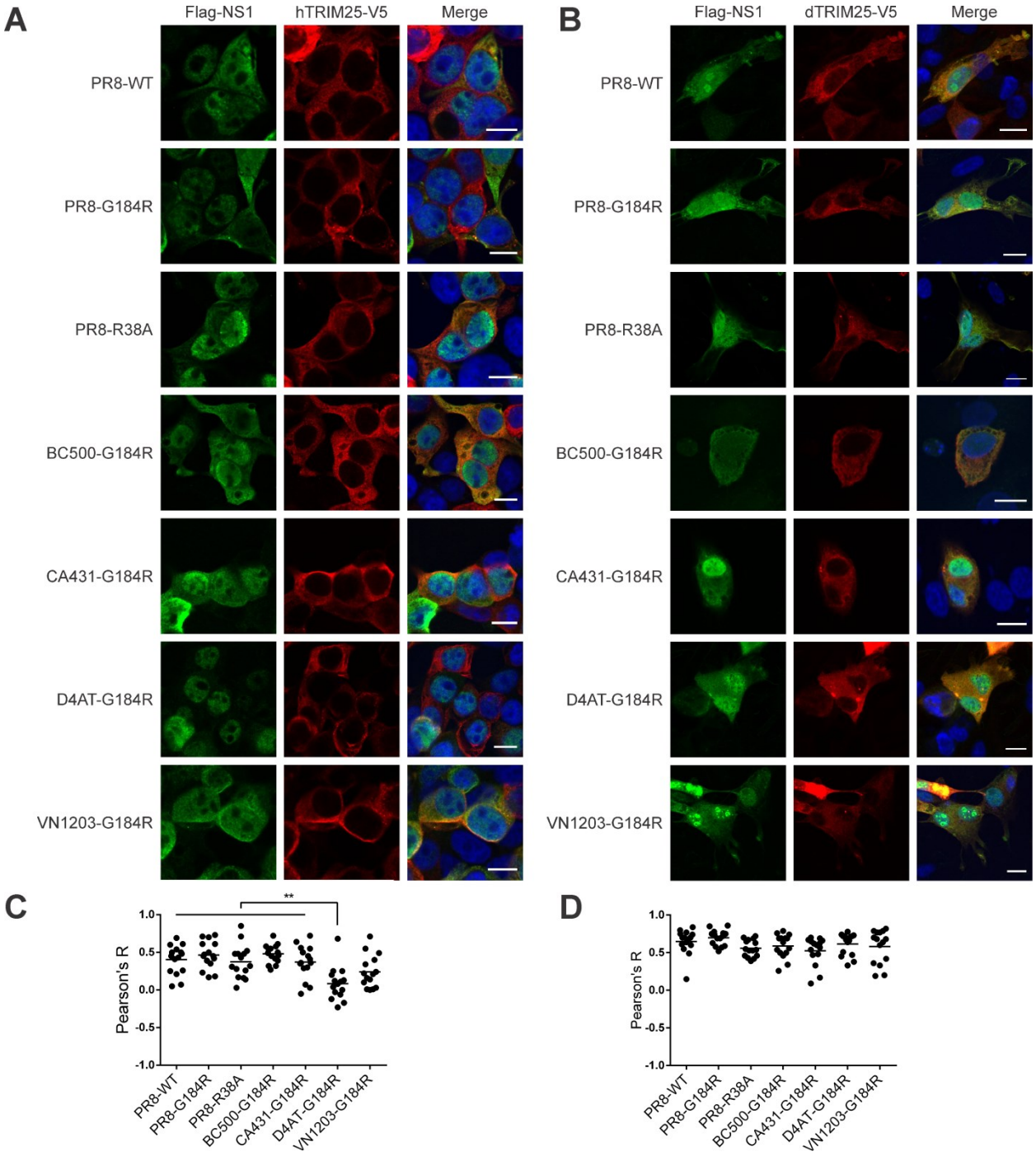



**Figure 3.6. NS1 proteins co-localize with human and duck TRIM25 in the cytoplasm of cells.**

(A) HEK293T cells overexpressing hTRIM25-V5 and the Flag-NS1 proteins indicated on the left border were fixed, stained, and imaged using confocal microscopy. Panels show staining with Hoechst 33324 (nuclei-blue), anti-Flag antibodies (green), anti-V5 antibodies (red), and a merged image. (B) DF-1 cells overexpressing dTRIM25-V5 and the Flag-NS1 proteins indicated

on the left border were fixed, stained, and imaged using confocal microscopy as in (A). Scale bars show 10 μm. (C and D) Co-localization of each indicated Flag-NS1 with hTRIM25-V5 (C) or with dTRIM25-V5 (D) was quantified using Pearson's correlation coefficient (Pearson's R). Bars show mean values from 15 analyzed cells taken from three different slides. Means were compared using a one-way ANOVA with a Tukey's multiple comparisons post-hoc test (**, $P \leq 0.01$).

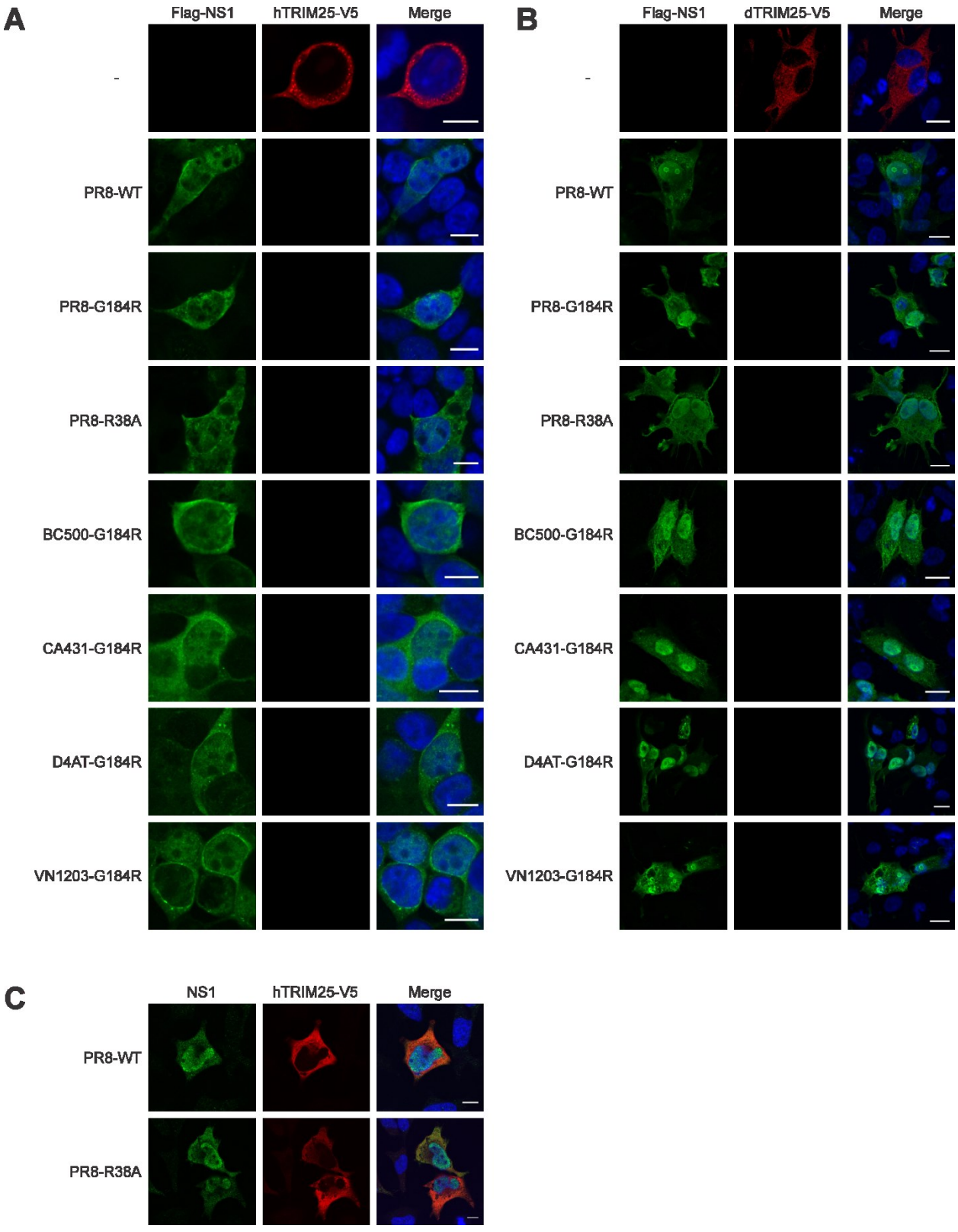

A
Flag-NS1
hTRIM25-V5
Merge
-
PR8-WT
PR8-G184R
PR8-R38A
BC500-G184R
CA431-G184R
D4AT-G184R
VN1203-G184R
B
Flag-NS1
dTRIM25-V5
Merge
-
PR8-WT
PR8-G184R
PR8-R38A
BC500-G184R
CA431-G184R
D4AT-G184R
VN1203-G184R
C
NS1
hTRIM25-V5
Merge
PR8-WT
PR8-R38A

**Figure 3.7. NS1 and TRIM25 intracellular distributions.**
(A) HEK293T cells overexpressing hTRIM25-V5 or the Flag-NS1 proteins indicated on the left border were fixed, stained, and imaged using confocal microscopy. Panels show staining with Hoechst 33324 (nuclei-blue), anti-Flag antibodies (green), anti-V5 antibodies (red), and a merged image. (B) DF-1 cells overexpressing dTRIM25-V5 or the Flag-NS1 proteins indicated on the left border were fixed, stained, and imaged as in (A). (C) HeLa cells overexpressing hTRIM25-V5 and the untagged NS1 proteins indicated on the left border were fixed, stained, and imaged using confocal microscopy. Panels show staining with Hoechst 33324 (nuclei-blue), anti-NS1 antibodies (green), anti-V5 antibodies (red), and a merged image. Scale bars show 10 μm.

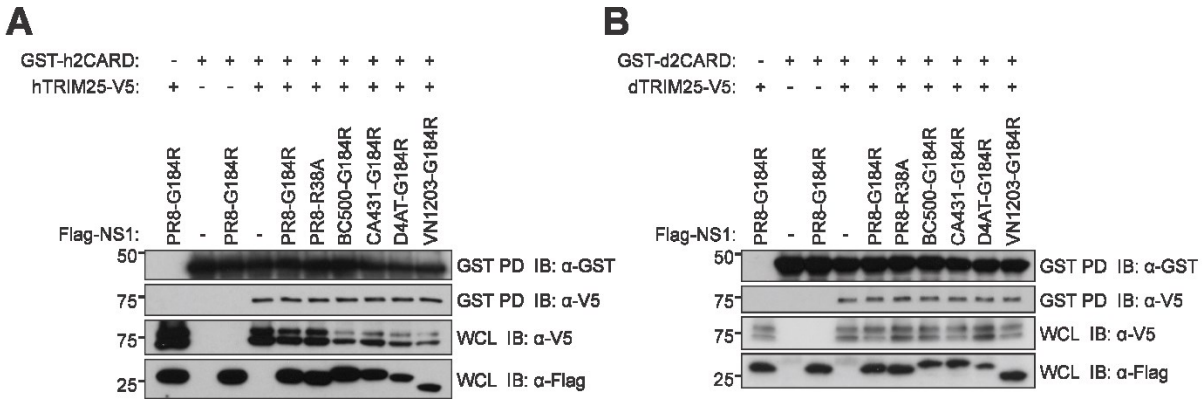



**Figure 3.8. NS1 proteins do not prevent the association between TRIM25 and RIG-I CARD domains.**

(A) HEK293T cells were transfected with GST-tagged human RIG-I CARD domains (GST-h2CARD) and V5-tagged human TRIM25 (hTRIM25-V5) together with the indicated Flag-tagged NS1 proteins (Flag-NS1). Clarified whole cell lysates (WCL) were subjected to GST pulldown (GST PD), followed by immunoblotting (IB) with anti-GST, anti-V5, and anti-Flag antibodies. (B) DF-1 cells were transfected with GST-tagged duck RIG-I CARD domains (GST-d2CARD) and V5-tagged duck TRIM25 (dTRIM25-V5) together with the indicated Flag-tagged NS1 proteins (Flag-NS1). GST pulldown and immunoblotting were performed as in (A). Each experiment was repeated at least three times.

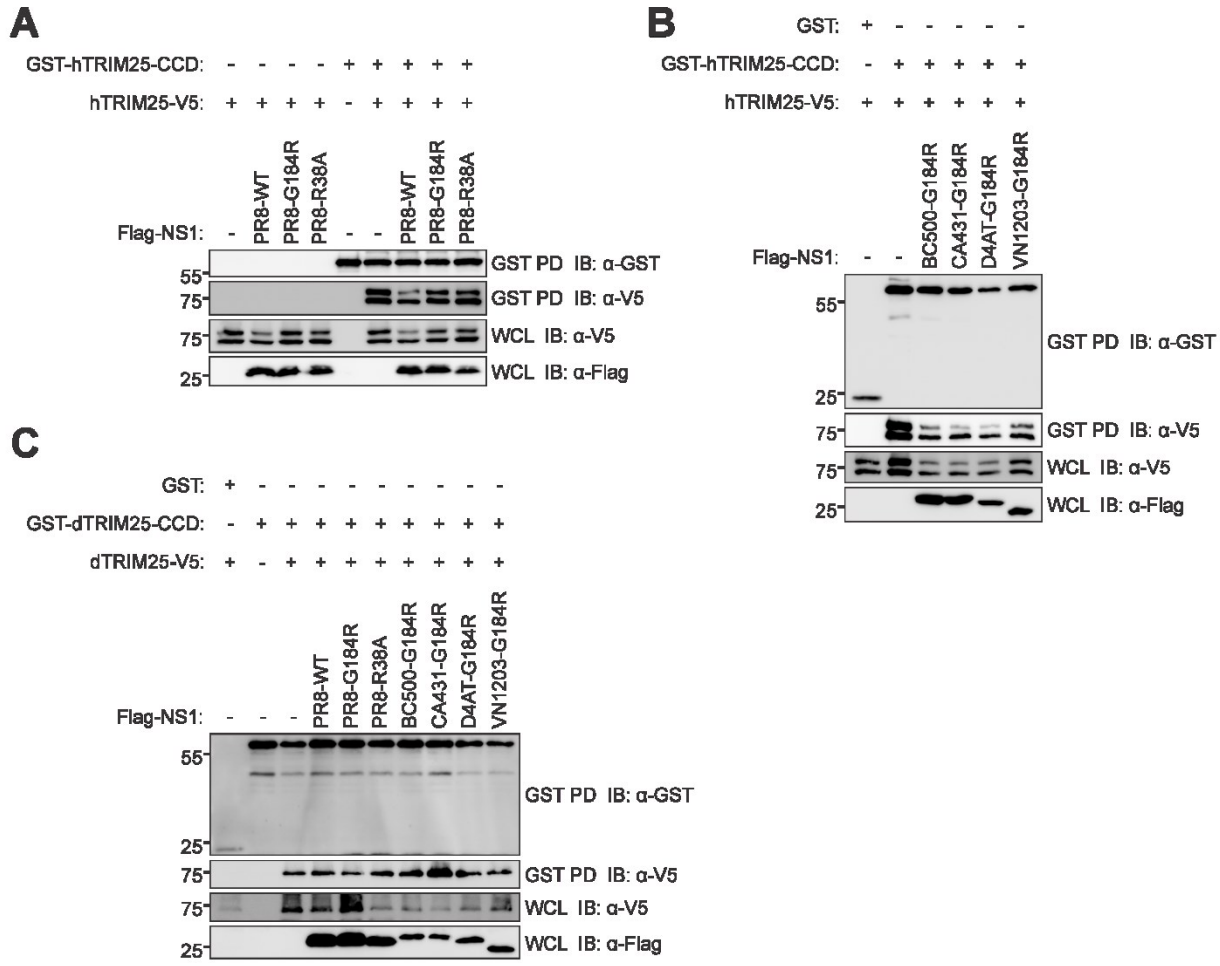



**Figure 3.9. NS1 proteins do not inhibit TRIM25 dimerization through its coiled-coil domain.**

(A and B) HEK293T cells were transfected with a GST-tagged coiled-coil domain of human TRIM25 (GST-hTRIM25-CCD) or empty GST vector, and V5-tagged human TRIM25 (hTRIM25-V5) together with the indicated Flag-tagged NS1 proteins. Clarified whole cell lysates (WCL) were subjected to GST pulldown (GST PD), followed by immunoblotting (IB) with anti-GST, anti-V5, and anti-Flag antibodies. (C) DF-1 cells were transfected with a GST-tagged coiled-coil domain of duck TRIM25 (GST-hTRIM25-CCD) or empty GST vector, and V5-tagged duck TRIM25 (dTRIM25-V5) together with the indicated Flag-tagged NS1 proteins. GST pulldown and immunoblotting were performed as in (A). Each experiment was repeated at least three times.

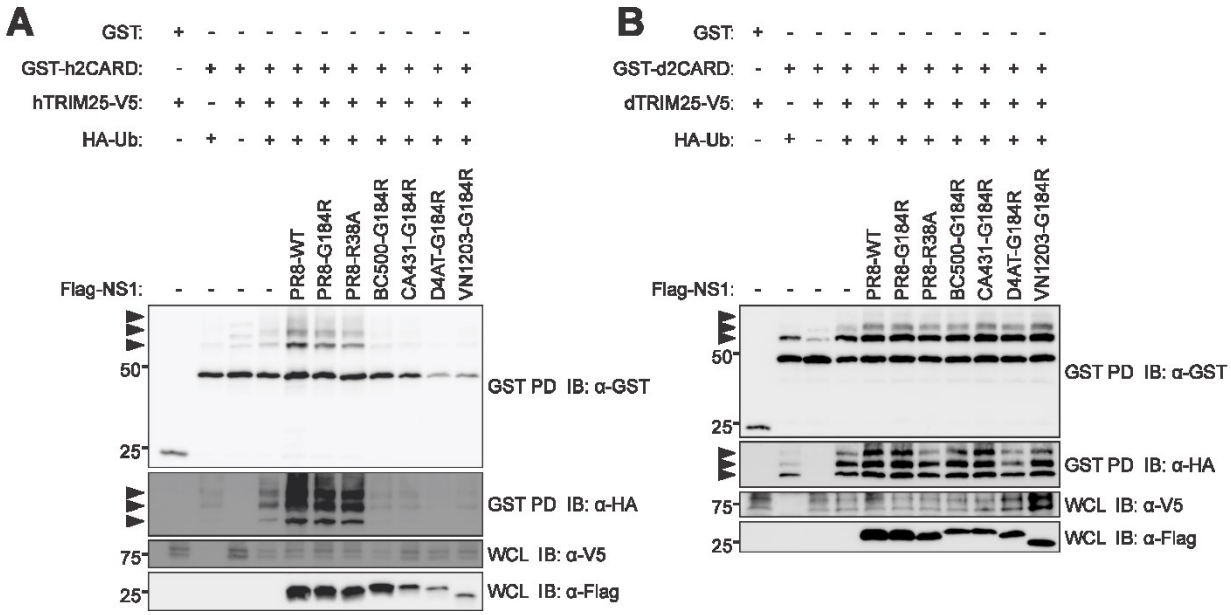



**Figure 3.10. Avian NS1 proteins inhibit human RIG-I-CARD ubiquitination, but not duck RIG-I-CARD ubiquitination.**

(A) HEK293T cells were transfected with GST-tagged human RIG-I CARD domains (GST-h2CARD) or empty GST vector, together with HA-tagged ubiquitin (HA-Ub), V5-tagged human TRIM25 (hTRIM25-V5), and the indicated Flag-tagged NS1 proteins (Flag-NS1). Clarified whole cell lysates (WCL) were subjected to GST pulldown (GST PD), followed by immunoblotting (IB) with anti-GST, anti-HA, anti-V5, and anti-Flag antibodies. (B) DF-1 cells were transfected with GST-tagged duck RIG-I CARD domains (GST-d2CARD) or empty GST vector, together with HA-tagged ubiquitin (HA-Ub), V5-tagged duck TRIM25 (dTRIM25-V5), and the indicated Flag-tagged NS1 proteins (Flag-NS1). GST pulldown and immunoblotting were performed as in (A). Each experiment was repeated at least three times.

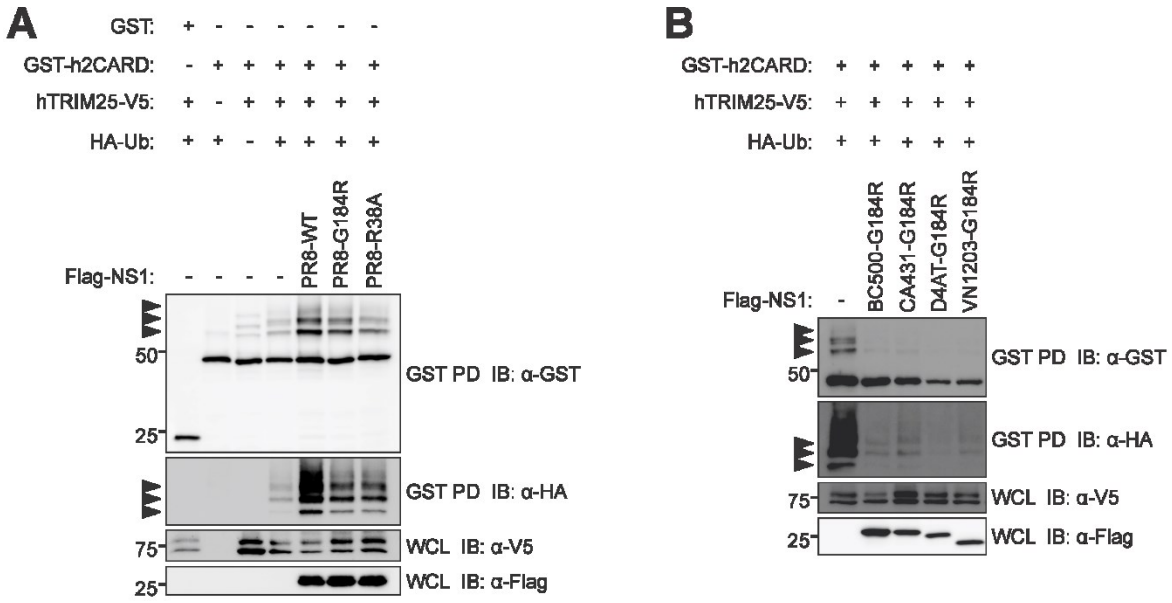



**Figure 3.11. PR8 NS1 increases human RIG-I-CARD ubiquitination, while avian NS1 proteins decrease it.**

(A and B) HEK293T cells were transfected with GST-tagged human RIG-I CARD domains (GST-h2CARD) or empty GST vector, together with HA-tagged ubiquitin (HA-Ub), V5-tagged human TRIM25 (hTRIM25-V5), and the indicated Flag-tagged NS1 proteins (Flag-NS1). Clarified whole cell lysates (WCL) were subjected to GST pulldown (GST PD), followed by immunoblotting (IB) with anti-GST, anti-HA, anti-V5, and anti-Flag antibodies. Each experiment was repeated at least three times.

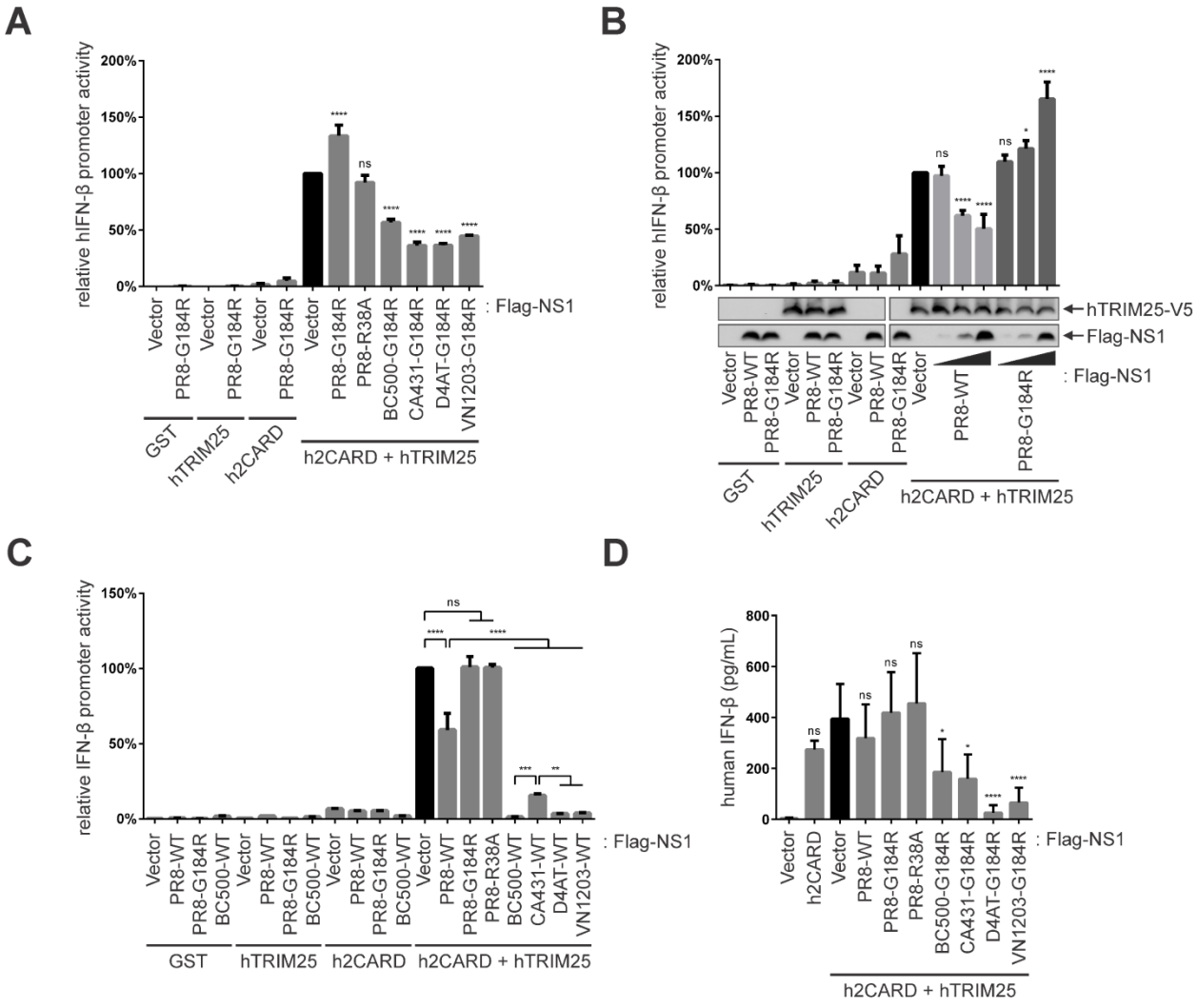



**Figure 3.12. Human interferon-beta inhibition assays.**

(A) HEK293T cells were transfected with 5 ng of GST-tagged human RIG-I CARD domains (h2CARD) or empty GST vector, 150 ng of V5-tagged human TRIM25 (hTRIM25), and 500 ng of Flag-tagged NS1 proteins (Flag-NS1), as indicated, along with human IFN-β luciferase reporter vector and control *Renilla* luciferase reporter vector. 24 hours post-transfection, cells were lysed and the IFN-β promoter activity was measured by dual luciferase assay. (B) HEK293T cells were transfected and treated as in (A), but with increasing amounts of either wild-type (WT) or G184R-mutant PR8 NS1 proteins (50 ng, 100 ng, 500 ng). hTRIM25-V5 and Flag-NS1 protein expression was confirmed by Western blot. (C) HEK293T cells were transfected and treated as in (A).

Bars show mean ± standard deviation of three independent experiments (n=3) in each case. Interferon promoter activity of each sample group was normalized to cells stimulated with RIG-I-CARD + TRIM25 in the absence of NS1 (black bar). Means were compared using one-way ANOVA with Dunnett's (A and B) or Tukey's (C) multiple comparisons post-hoc tests (*,

P≤0.05; **, P≤0.01; ***, P≤0.001; ****, P≤0.0001; ns, non-significant). Total DNA quantity was always kept constant with empty vector.
(D) Secreted interferon quantification. HEK293T cells were transfected with 500 ng of GST-tagged human RIG-I CARD domains (h2CARD) or empty GST vector, 1 µg of V5-tagged human TRIM25 (hTRIM25), and 2 µg of Flag-tagged NS1 proteins (Flag-NS1), as indicated. 24 hours-post transfection, cell culture supernatants were collected, cleared by centrifugation, and sent for interferon-beta protein quantification. Graph bars show mean ± standard deviation of six independent experiments (n=6). Means were compared using one-way ANOVA with a Dunnett's multiple comparisons post-hoc test (*, P≤0.05; ****, P≤0.0001; ns, non-significant).

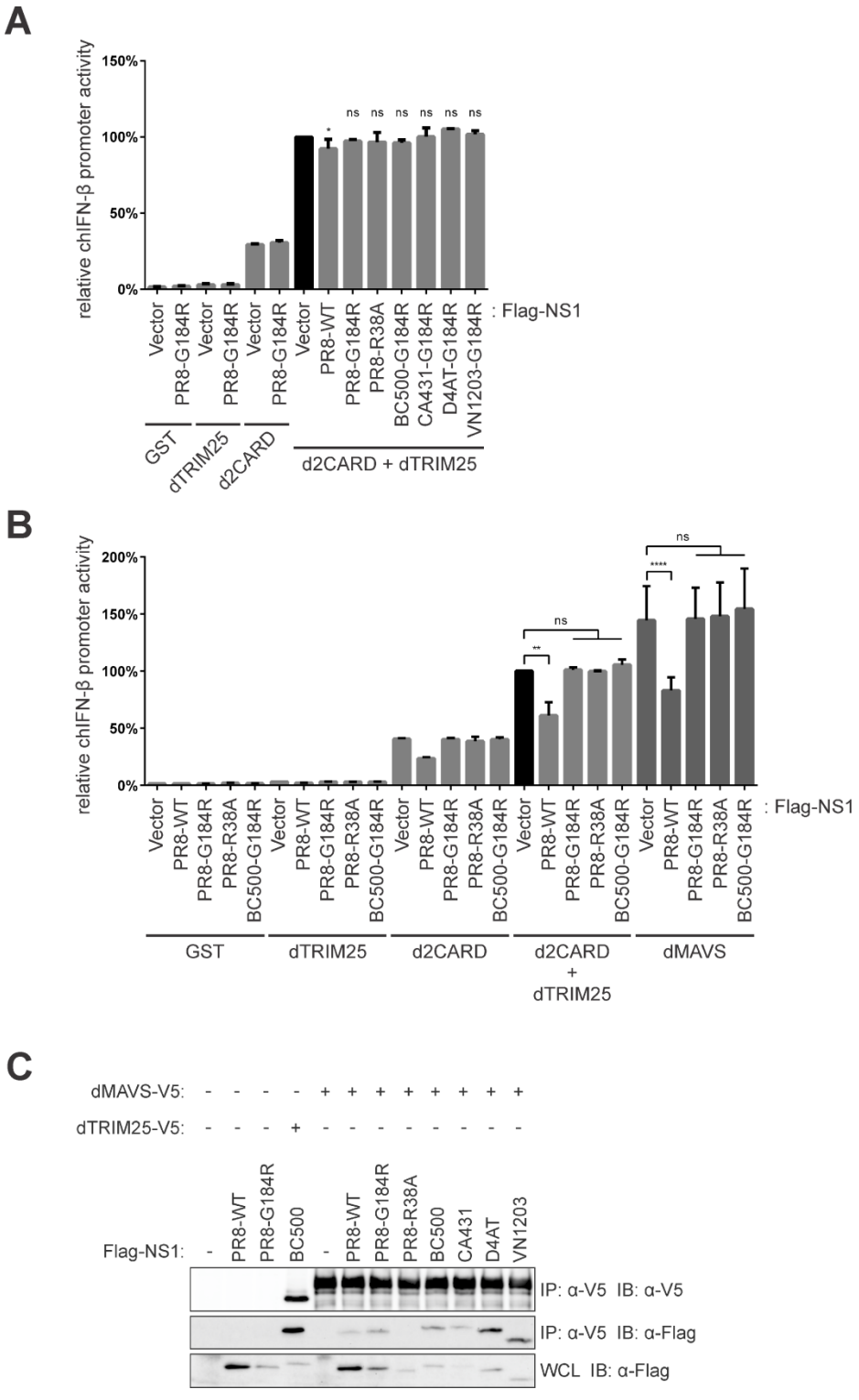



**Figure 3.13. Chicken interferon-beta inhibition assays in DF-1 cells.**

(A) DF-1 cells were transfected with 5ng of GST-tagged duck RIG-I CARD domains (d2CARD) or empty GST vector, 150 ng of V5-tagged duck TRIM25 (dTRIM25), and 500ng of Flag-tagged NS1 proteins (Flag-NS1), as indicated, along with chicken IFN-β luciferase reporter vector and

control *Renilla* luciferase reporter vector. 24 hours post-transfection, cells were lysed and the IFN-β promoter activity was measured by dual luciferase assay. Graph bars show mean ± standard deviation of three independent experiments (n=3). Interferon promoter activity of each sample group was normalized to cells stimulated with RIG-I-CARD + TRIM25 in the absence of NS1 (black bar). Means were compared using one-way ANOVA with a Dunnett's multiple comparisons post-hoc test (*, $P \leq 0.05$; ns, non-significant).
(B) DF-1 cells were transfected and treated as in (A), but also including 250ng of V5-tagged duck MAVS (dMAVS) where indicated. Bars show mean ± standard deviation of one experiment (n=1). Interferon promoter activity of each sample group was normalized to cells stimulated with RIG-I-CARD + TRIM25 in the absence of NS1 (black bar). Means were compared using one-way ANOVA with a Tukey's multiple comparisons post-hoc test (**, $P \leq 0.01$; ****, $P \leq 0.0001$; ns, non-significant). Total DNA quantity was always kept constant with empty vector.
(C) DF-1 cells were transfected with V5-tagged duck MAVS (dMAVS-V5) together with the indicated Flag-tagged NS1 proteins (Flag-NS1). Clarified whole cell lysates (WCL) were subjected to anti-V5 immunoprecipitation (IP), followed by immunoblotting (IB) with anti-V5 and anti-Flag antibodies. dTRIM25-V5 was used as a positive control. This experiment was repeated twice.

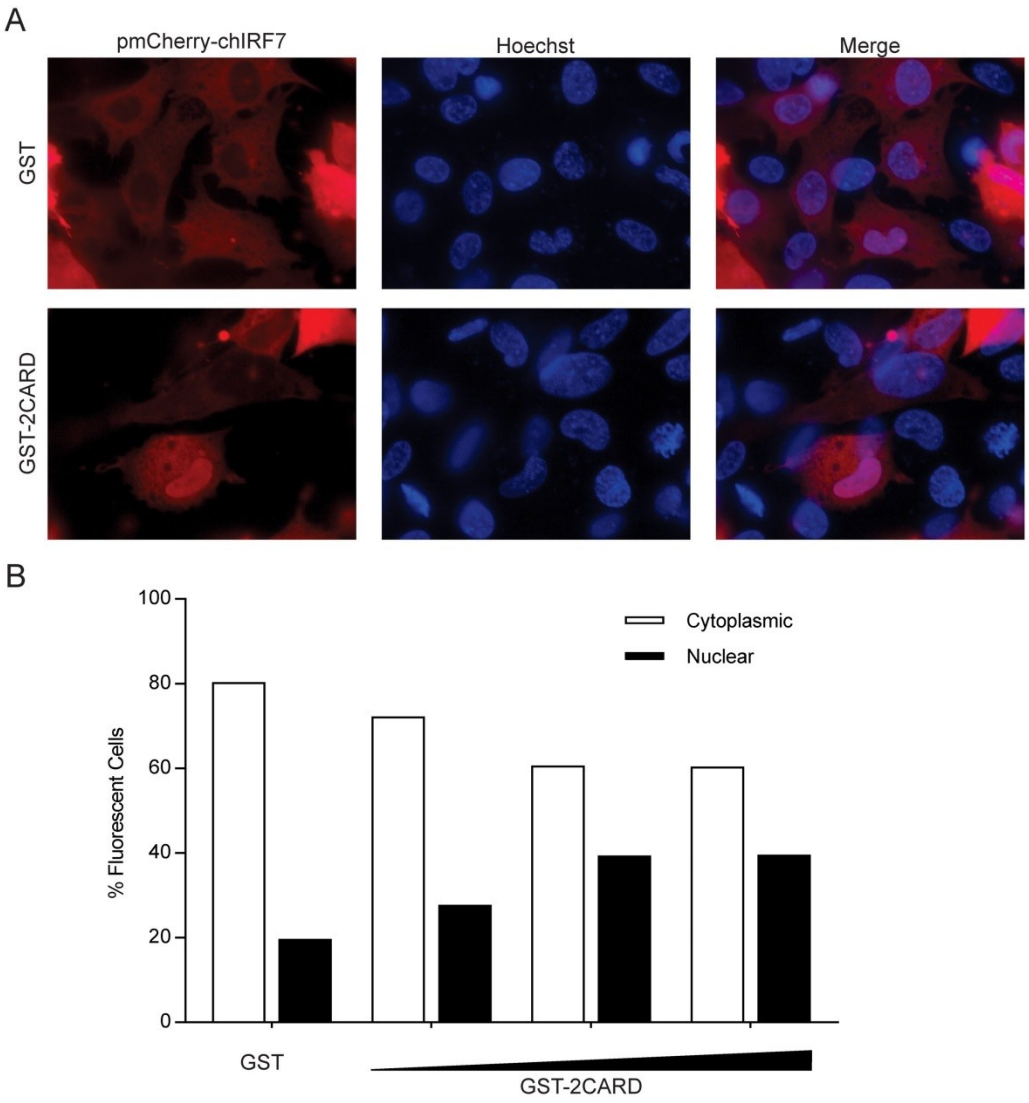



**Figure 3.14. Nuclear translocation of chicken IRF7 in DF-1 cells.**
(A) DF-1 cells were co-transfected with mCherry-tagged IRF7 (chIRF7) and empty GST vector or GST-tagged duck RIG-I-CARD (GST-2CARD). Representative images show IRF7 mCherry fluorescence (red), nuclear staining (blue), and a merge. (B) DF-1 cells were transfected with increasing amounts of GST-2CARD expression plasmids (100 ng, 500 ng, 1000 ng) and stained as in A. One hundred cells were counted from each group, using multiple images of multiple fields of view chosen at random, and the percentage of cells containing nuclear chIRF7 was tabulated. Counting data is from one representative of three independent experiments. *This figure, which appears in (Xiao et al., 2018) was made by Adam Caulfield and Matthew Reeves under my supervision.*

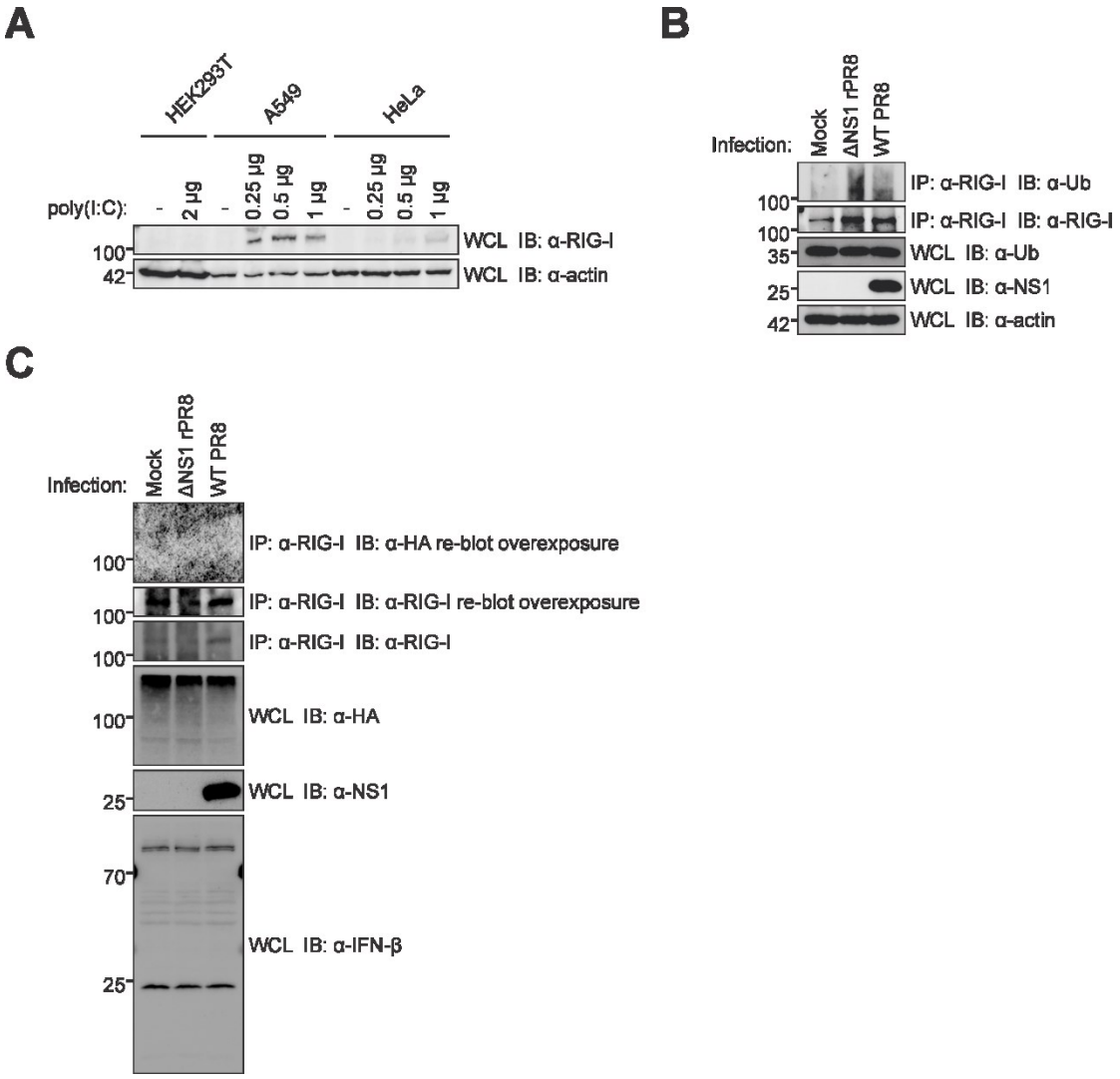



**Figure 3.15. Endogenous human RIG-I expression and ubiquitination in infected cells.** (A) HEK293T, A549, and HeLa cells were stimulated overnight with the indicated quantities of poly (I:C), then lysed. Whole cell lysates (WCL) were immunoblotted (IB) with anti-RIG-I and anti-β-actin antibodies. This experiment was done once. (B) A549 cells were either mock-infected or infected with influenza A/PR/8/34 WT or ΔNS1 rPR8 virus at an MOI of 2 for 11 h. Clarified whole cell lysates (WCL) were subjected to anti-RIG-I immunoprecipitation (IP), followed by immunoblotting (IB) with anti-ubiquitin (anti-Ub), anti-RIG-I, anti-NS1, and anti-β-actin antibodies. This experiment was performed three times. (C) At 36 h post-transfection with HA-tagged-ubiquitin (HA-Ub), A549 cells were either mock-infected or infected with influenza A/PR/8/34 WT or ΔNS1 rPR8 virus at an MOI of 2 for 11 h. Clarified whole cell lysates (WCL) were subjected to anti-RIG-I immunoprecipitation (IP), followed by immunoblotting (IB) with anti-HA, anti-RIG-I, anti-NS1, and anti-IFN-β antibodies. This experiment was performed twice.

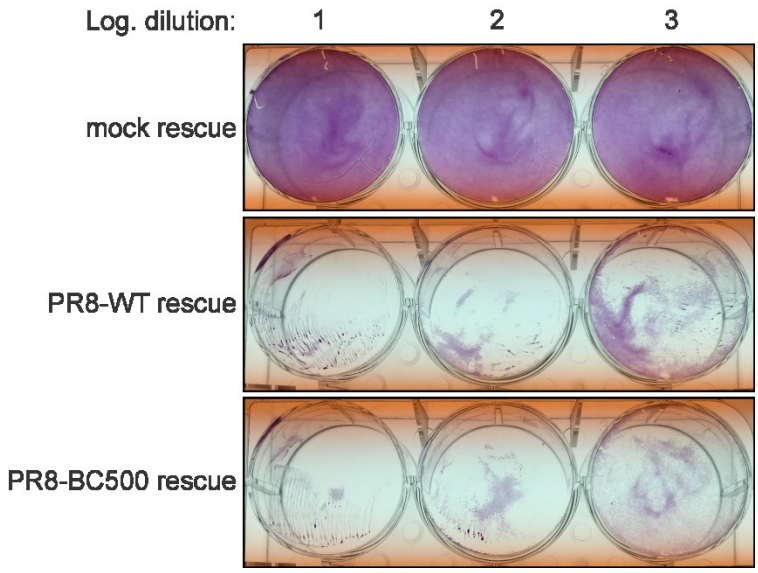



**Figure 3.16. Recombinant virus plaque assay in MDCK cells.**
Viruses were generated by transfecting a co-culture of HEK293T and MDCK cells according to the Martínez-Sobrido and García-Sastre protocol (Martínez-Sobrido and García-Sastre, 2010). In mock rescue, cells were transfected with no DNA. PR8-WT recombinant was assembled from plasmids encoding all PR8 gene segments. PR8-BC500 virus encodes BC500 NS1 in a PR8 genetic background. Supernatants from the viral rescue transfections were collected and centrifuged to remove cellular debris. Cleared viral supernatants were serially diluted tenfold and used to infect fresh monolayers of MDCK cells for plaque assay. Dilutions indicated at the top. This experiment was performed once.

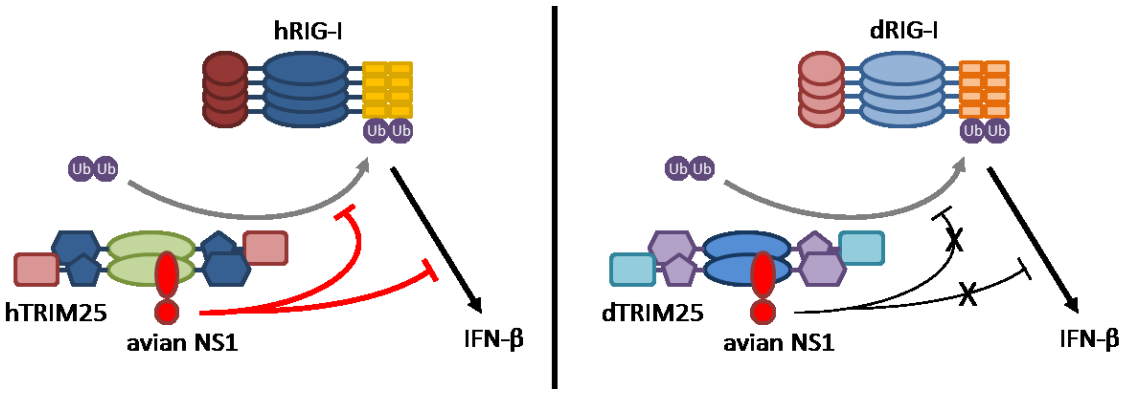



**Figure 3.17. Avian influenza NS1 proteins inhibit human, but not duck, RIG-I-CARD ubiquitination and interferon signaling.**
NS1 proteins from two HPAI strains (allele A) and two LPAI strain (allele B) interact with human TRIM25 and inhibit human RIG-I-CARD ubiquitination and signaling in HEK293T cells. They also associate with duck TRIM25, but do not inhibit duck RIG-I-CARD ubiquitination and signaling in DF-1 cells.


**Figure 3.18. Human TRIM25 amino acid residues that interact with NS1 are not well-conserved in duck TRIM25.**

Peptide alignment with highlighted residues of human TRIM25 that interact directly with NS1 according to a crystal structure (Koliopoulos et al., 2018).

# Chapter 4 PR8 PB1-F2 inhibits duck MAVS signaling and duck RIG-I-CARD ubiquitination.

## 4.1 Introduction

PB1-F2 is a small accessory protein encoded by most influenza A strains in the second reading frame of the PB1 gene (Zell et al., 2007). It a small non-structural protein of 87-90 amino acids, translated from the 4th start codon in the +1 alternate open reading frame, located 120 bp downstream of the first start codon of PB1 gene (Chen et al., 2001). Although not essential for viral replication, PB1-F2 may have contributed to the virulence of the Hong Kong 1997 H5N1 and the 1918 influenza outbreaks (Conenello et al., 2007). Modulation of virulence by PB1-F2 in mammalian cells may occur through multiple mechanisms, such as promoting apoptosis, modulating innate immune responses, and exacerbating secondary bacterial infections (reviewed in Kamal et al., 2017). Another important function of PB1-F2 is increasing the activity of the viral polymerase, which enhances replication and pathogenicity (Krumbholz et al., 2011; Marjuki et al., 2010). The large number of functions and interactions described appears to vary with context. For example, PB1-F2 proteins have been shown to inhibit type-I interferon and pro-inflammatory cytokine induction in infected mice and ferrets (Dudek et al., 2011; Meunier and von Messling, 2012), but can also contribute to the induction of interferon-beta in human respiratory epithelial cells (Le Goffic et al., 2010) and activate the NLRP3 inflammasome in mice (McAuley et al., 2013). In chickens, comparison of PB1-F2-expressing and knock-out viruses showed that PB1-F2 reduced inflammatory responses in the lungs and reduced pathogenicity in an H5N1 background (Leymarie et al., 2014) and prolonged viral shedding in a low-pathogenicity H9N2 background (James et al., 2016a).

The effects of specific PB1-F2 proteins on viral pathogenesis depend in part on their variable length and amino acid polymorphisms, both of which demonstrate host-species adaptations (Kosik et al., 2013). Full-length PB1-F2 proteins are widely conserved in avian viruses (James et al., 2016a). As avian influenza A strains adapt to pig and human hosts, the PB1-F2 proteins tend to acquire truncations (Pancucharova and Russ, 2006; Zell et al., 2007). The amino acid residue at position 66 contributes to virulence. Mammalian and human influenza A strains share a consensus N66 residue, while avian strains share a consensus S66 (Kosik et al., 2013). The virulent Hong Kong 1997 H5N1 influenza strain and 1918 influenza both carry PB1-F2 proteins that encode S66 (Conenello et al., 2007). PB1-F2 N66S mutation in an HPAI H5N1 recombinant virus increased virulence and central nervous system infiltration in mice, but did not affect pathogenicity in juvenile ducks (Schmolke et al., 2011).

It is known that PB1-F2 proteins are expressed naturally in infected duck embryonic fibroblasts (Schmolke et al., 2011), but little is known about their function in these hosts. Several studies have observed a direct interaction between the 87-amino-acid PB1-F2 of the PR8 strain and human MAVS, which dampened type I interferon signalling (Varga et al., 2012; Varga and Palese, 2011). To investigate species-specificity and adaptation, we asked whether PR8 PB1-F2 would also interact with duck MAVS and interfere in the duck RIG-I signaling pathway. We found that PR8 PB1-F2 co-localizes and interacts with duck MAVS in DF-1 cells and inhibits type I interferon induction. We also show that PR8 PB1-F2, and not PR8 NS1, abrogates TRIM25-mediated ubiquitination of human and duck RIG-I CARD domains.

## 4.2 Results

The data in this chapter was assembled in collaboration with my colleagues Dr. Yanna Xiao and Adam Moghrabi. To avoid confusion, figures that consist predominantly of experiments performed by my colleagues appear separately in the Appendix, and are designated as figures S1-S3.

### *4.2.1 Duck MAVS shares only 28% amino acid sequence identity with human MAVS.*

To compare duck MAVS to that of different species, we did an alignment of duck, chicken, human and mouse MAVS. The alignment shows that duck and chicken MAVS are quite divergent from human and mouse MAVS (Fig S1A). Duck MAVS shares low amino acid identity with chicken MAVS (58%) and human MAVS (28%). However, the CARD domain and the transmembrane (TM) domain of duck and chicken MAVS are more conserved, at 80.5% and 80% identity respectively. The proline-rich region of duck MAVS only shares 49% amino acid identity with its counterpart in chicken MAVS. Of the numerous known sites for post-translational modification of mammalian MAVS (Liu and Gao, 2018), only a few appear to be conserved in ducks. Sites for attachment of K48-linked ubiquitination near residues K7 and K420 in human MAVS, leading to proteasomal degradation after infection, are conserved between species (Castanier et al., 2012; Du et al., 2015). A serine-rich cluster is present in the duck sequence near Serine 496 and contains the motif HLGLS, which resembles the consensus *p*L*x*IS motif containing (human) Serine 442 that becomes phosphorylated by IKKβ and TBK1 upon viral infection, leading to recruitment and phosphorylation of IRF3 (Liu et al., 2015b). To investigate the relationship between duck and other vertebrate MAVS, including available avian sequences, a phylogenetic tree was constructed in MEGA7. As expected, duck MAVS grouped

with other avian MAVS sequences, with the closest evolutionary distance to chicken MAVS (Fig S1B).

### *4.2.2 Duck MAVS localizes to mitochondria in DF-1 cells and interacts with duck RIG-I CARD domains in the cytoplasm.*

To investigate the distribution of duck MAVS in DF-1 cells, we transfected them with V5-tagged duck MAVS expression constructs and visualized them by immunofluorescence at 24h post-transfection. Visually, MAVS co-localized with the mitochondria, stained with MitoTracker Red (Fig S2A) Co-localization of V5-MAVS with mitochondria was quantitatively analyzed using Pearson's correlation coefficient (Pearson's R). The mean Pearson's R value was 0.79 (Fig S2B), which indicated a high signal correlation. Mitochondrial localization is consistent with mammalian MAVS (Seth et al., 2005), despite very different amino acid sequences in their transmembrane domains, which also serve as mitochondrial membrane targeting sequences.

To confirm that duck MAVS interacts with duck RIG-I-CARD, we investigated the co-localization of these two proteins with confocal microscopy. The confocal images showed that overexpressed GST on its own was diffusely distributed within the whole cytoplasm, not related to the distribution of V5-MAVS, whereas duck RIG-I CARD domains (GST-2CARD) had a similar staining pattern to duck MAVS in DF-1 cells (Fig S2C). The mean Pearson's R value for co-localization between duck MAVS and duck RIG-I-CARD was 0.83 (Fig S2D). The high signal correlation between V5-MAVS and GST-2CARD suggests that they co-localize in DF-1 cells. In co-immunoprecipitation, V5-MAVS was pulled down by GST-2CARD, but not by GST alone (Fig S2E). We confirmed that the RIG-I-CARD :: MAVS interaction induces type-I interferon signaling by dual luciferase assay (Fig S2F). Compared to pcDNA3.1 vector only,

overexpression of V5-MAVS stimulated chIFN-β promoter activity approximately 8-fold in DF-1 cells, presumably because the overexpressed MAVS protein auto-assembles into the helical assembly needed for signaling (Fig S2F). The constitutively-active RIG-I CARD domain fragment on its own increased promoter activity approximately 50-fold through interaction with endogenous chicken MAVS (Barber et al., 2010). Co-expression of V5-MAVS and GST-2CARD induced the strongest chIFN-β promoter activity, suggesting functional interaction between duck MAVS and duck RIG-I 2CARD, though it could simply be additive. That RIG-I CARD domains induce interferon through functional MAVS interactions is demonstrated by the fact that duck RIG-I CARD domains (GST-d2CARD), but not human RIG-I CARD domains (GST-h2CARD), stimulate the IFN-β promoter in chicken cells, while GST-h2CARD is active in human HEK293T cells (Fig S2G and H). It is known that the RIG-I :: MAVS interacting surfaces are sufficiently diverged between humans and ducks that they do not interact across species (Wu et al., 2014).

### *4.2.3 Amino acid residue 120 in duck MAVS (122 in human MAVS) contributes to species-specific MAVS-CARD : RIG-I-CARD interactions.*

The MAVS-CARD :: RIG-I-CARD interface is poorly conserved between humans and ducks, but the overall structure is conserved such that the tandem T175K/T176E mutations on duck RIG-I permit interaction with human MAVS in HEK293T cells (Wu et al., 2014). Other residues contributing to interactions between RIG-I and MAVS have not been fully explored. We previously noted that D122, a RIG-I residue critically involved in the human RIG-I :: MAVS interaction, is replaced by A120 in ducks (Miranzo-Navarro and Magor, 2014). Notably, the mutant D122A of human RIG-I can form 2CARD tetramers, but does not engage human MAVS to initiate signalling (Peisley et al., 2014a). To investigate whether altering these homologous

residues would allow duck RIG-I to signal in human cells, and vice versa, we created the appropriate mutant 2CARD domains and investigated their ability to induce signaling activity compared to the wild-types in DF-1 and HEK293T cells. The wildtype d2CARD induced high chIFN-β promoter activity, while the A120D mutant d2CARD induced significantly less activity in DF-1 cells. However, neither the wildtype nor mutant h2CARD constructs induced significant promoter activity in avian cells (Fig 4.1A). In human cells, the RIG-I d2CARD mutation A120D increased relative human IFN-β promoter activity compared to the wildtype (Fig 4.1B), while the h2CARD mutation D122A almost completely abrogated relative IFN-β promoter activity in HEK293T cells. We confirmed that both sets of wild type and mutant GST-2CARD fusion proteins were expressed in DF-1 cells (Fig 4.1C) and HEK293T cells (Fig 4.1D) by Western blot. All GST-2CARD proteins were expressed, but the wild type h2CARD was expressed at a lower quantity than the other proteins in DF-1 cells.

### *4.2.4 PR8 PB1-F2 interacts with duck MAVS in the cytoplasm of DF-1 cells and inhibits type-I interferon signaling.*

PR8 PB1-F2 is targeted to the mitochondria in human cells, and interacts with mitochondrial proteins, like MAVS and Tom40 (a major mitochondrial outer membrane import channel) (Yoshizumi et al., 2014). To examine the distribution of PR8 PB1-F2 in avian cells, we transfected a Flag-tagged PB1-F2 expression vector into DF-1 cells and performed confocal microscopy and co-localization analysis. The distribution of PB1-F2 in HeLa cells served as the positive control. The confocal images show that PB1-F2 had a clearly similar staining pattern to the MitoTracker dye in both DF-1 cells and HeLa cells (Fig S3A). The co-localization of PR8 PB1-F2 with mitochondria in DF-1 cells was also quantitatively analyzed, with a mean Pearson's R value of 0.82 (Fig S3B). The high signal correlation between PB1-F2 and MitoTracker

staining indicated that PR8 PB1-F2 is also distributed on mitochondria of DF-1 cells, consistent with its distribution in human cells. To determine whether PB1-F2 interacts with mallard duck MAVS, we co-transfected DF-1 cells with Flag-tagged PB1-F2 and V5-MAVS. PB1-F2 had a similar staining pattern to duck MAVS (Fig S3C). The average Pearsons's R value of 0.67 indicated moderate co-localization (Fig S3D). PB1-F2 co-immunoprecipitated with V5-MAVS, but not with another V5-tagged duck protein, the PRY/SPRY domain fragment of duck TRIM27-L (Blaine et al., 2015), suggesting a specific interaction (Fig S3E).

To investigate whether PR8 PB1-F2 inhibits interferon induction through MAVS, as it does in human cells (Varga et al., 2011), we performed dual luciferase reporter assays. PB1-F2 inhibited chIFN-β promoter activity downstream of duck RIG-I CARD domains (Fig S3F and G) and downstream of full-length duck RIG-I (Fig S3H). Similarly, PR8 PB1-F2 inhibited interferon promoter activity downstream of duck MAVS by over 50% (Fig S3I). Wild-type PR8 NS1 also inhibited interferon signaling in both cases, while the empty expression vector and the Flag tag on their own had no effect.

### *4.2.5 PR8 PB1-F2 inhibits TRIM25-mediated ubiquitination of both human and duck RIG-I CARD domains.*

We accidentally stumbled across a surprising result in previous RIG-I-CARD ubiquitination studies when we included PB1-F2, initially as a negative control protein in place of influenza NS1. We found that in HEK293T cells co-expressing GST-h2CARD and HA-tagged ubiquitin, PR8 PB1-F2 visibly decreased h2CARD ubiquitination by endogenous TRIM25 (Fig 4.2A). When exogenous hTRIM25-V5 was added, a similar pattern was obtained, with PB1-F2 markedly decreasing TRIM25-mediated ubiquitination (Fig 4.2B). Under the confocal microscope, in HEK293T cells co-expressing Flag-PB1-F2 and hTRIM25-V5, PB1-F2

had a punctate distribution in the cytoplasm, and the large puncta appear to exclude the otherwise diffusely cytoplasmic hTRIM25-V5 (Fig 4.2C). A slightly negative average Pearson's coefficient indicates lack of co-localization (Fig 4.2D). In DF-1 cells transfected with GST-d2CARD, dTRIM25-V5, and HA-tagged ubiquitin, PB1-F2 also suppressed d2CARD ubiquitination (Fig 4.3).

**4.3 Discussion**

Duck MAVS localizes to the mitochondria of chicken DF-1 cells and overexpression induces IFN-β reporter activity, indicating duck MAVS function is conserved in chicken cells, even though it shares a low amino acid identity with chicken MAVS. PR8 PB1-F2 interacts with duck MAVS in the cytoplasm of DF-1 cells and inhibits interferon-β promoter activity downstream of both duck RIG-I and duck MAVS (Fig 4.4).

Signaling of duck MAVS was recently shown in duck embryonic fibroblast cells (Li et al., 2016), however, transfections were much more reproducible in DF-1 cells. Duck MAVS co-localized and co-precipitated with duck RIG-I CARD domains in DF-1 cells. Their co-expression induced chicken IFN-β promoter activity more strongly than either protein on its own, suggesting a productive interaction. In contrast, human RIG-I CARD domains did not induce IFN-β in avian cells, suggesting that h2CARD cannot interact with chicken MAVS. A co-transfection of h2CARD with duck MAVS should be done to demonstrate this more definitively. The inverse is also true (Wu et al., 2014). This is partly due to an amino acid polymorphism on RIG-I-CARD that lies at the interface between MAVS and RIG-I (A120 in duck MAVS, homologous to D122 in human MAVS) that we previously noted (Miranzo-Navarro and Magor, 2014). Mutation of the alanine residue in duck 2CARD to aspartate partially restored its activity in human cells, while decreasing its activity in avian cells. Activity was only partially restored

because of the presence of other polymorphisms at the interface. Previously, we showed that tandem mutation of the residues T175K/T176E in d2CARD allowed it to engage the appropriate residues on the CARD of human MAVS and restore filament formation and immunostimulatory activity (Wu et al., 2014). The additive effects of all three mutations could be interesting to investigate.

Using the reconstituted RIG-I – MAVS signaling pathway in chicken DF-1 cells, we investigated the function of PR8 PB1-F2. We observed the mitochondrial distribution of recombinant PB1-F2, consistent with its localization in HeLa cells (Chen et al., 2001; Varga et al., 2011). We also demonstrated co-localization and immunoprecipitation with duck MAVS, suggesting that the interaction with PB1-F2 is conserved. PB1-F2 has been shown to bind to the C-terminal region containing the transmembrane domain of mammalian MAVS (Varga et al., 2012), which is poorly conserved between avian and mammalian orthologues. Remarkably, although duck MAVS shares only 28% identity with human MAVS, PR8 PB1-F2 appears to interact with both proteins. The C-terminal region of MAVS includes the transmembrane domain and a conserved phosphorylation motif that allows MAVS to recruit IRF3 (Liu et al., 2015b). In their investigation of PR8 PB1-F2 and human MAVS, Varga et al. (2012) proposed that PB1-F2 could be interfering with the correct formation of MAVS-associated signaling complexes, but also showed that mitochondrial membrane potential was disrupted by PB1-F2. Mitochondrial membrane potential is necessary for proper MAVS signaling (Koshiba et al., 2011). Varga et al. (2012) suggested that both mechanisms, direct disruption of MAVS complex formation by PB1-F2 and disruption through membrane potential loss, could be contributing to signaling suppression. Another group confirmed that PR8 PB1-F2 disrupts mitochondrial membrane potential and observed translocation of PB1-F2 into the mitochondrial inner membrane space

(Yoshizumi et al., 2014). Both the interaction with MAVS and the disruption of membrane potential depend on the C-terminus of PB1-F2. It is unclear in our results which of these two mechanisms, and to what extent, is contributing to the interferon inhibition in the duck RIG-I pathway. PB1-F2 decreased signaling stimulated by overexpression of either duck MAVS or duck RIG-I. Therefore, if interference occurs at the level of MAVS, this suggests that PB1-F2 can interact with both duck and chicken MAVS. Mitochondrial membrane potential perturbations could be investigated using a tetramethylrhodamine ethyl ester (TMRE) dye (Varga et al., 2012).

Unexpectedly, we observed that overexpression of PB1-F2 inhibits TRIM25-mediated ubiquitination of RIG-I-CARD, in human and avian cells. It is particularly surprising that PB1-F2 inhibited duck RIG-I-CARD ubiquitination because none of the NS1 proteins investigated in the previous chapter did. Several possibilities must be considered. PB1-F2 may directly block ubiquitination by direct interaction with TRIM25, but this seems unlikely given their lack of co-localization in HEK293T cells. The fact that PB1-F2 aggregates in the cell cytoplasm exclude TRIM25 suggests that PB1-F2 could perhaps sequester RIG-I CARD domains away from TRIM25. This possibility could be investigated by checking for co-localization between PB1-F2 and RIG-I CARD domains with confocal microscopy. No mitochondrial staining was done on the TRIM25 co-expression slides (Fig 4.3C), so it is unclear if mitochondrial co-localization is disrupted (Fig S3A). Alternatively, loss of ubiquitination may be a consequence of negative feedback from disrupted MAVS or the loss of mitochondrial membrane potential. RIG-I signaling is tightly regulated in cells through a complex network of positive and negative feedback mechanisms that involve several different de-ubiquitinating enzymes (reviewed in Quicke et al., 2017). Indirect and reciprocal negative regulatory mechanisms have also been

described (Gack et al., 2008; Wu et al., 2017), including the induction of autophagy through MAVS (Lee et al., 2018). Finally, it is always possible that some non-biologically-relevant, technical limitation of the assay is responsible for unexpected results. The first steps towards investigating this more thoroughly would be to check for co-localization between PB1-F2 and human and duck RIG-I CARD domains, and to investigate RIG-I ubiquitination and signaling in cells infected with recombinant viruses, as discussed in the previous chapter. In particular, wild-type and del-NS1 PR8 viruses will help to discriminate between the effects of PB1-F2 and NS1 on RIG-I ubiquitination in a more biologically-relevant context.

In conclusion, we demonstrate that the function of MAVS in the RIG-I signaling pathway is conserved in ducks, and that PR8 PB1-F2 may interfere in this signaling pathway as it does in human cells. It is unclear whether PB1-F2 in DF-1 cells perturbs MAVS signaling complex assembly, mitochondrial membrane potential, RIG-I ubiquitination, or a combination of these things, but future experiments will likely clarify this issue. In this context, PR8 PB1-F2 demonstrates a conservation of function across diverse host species.

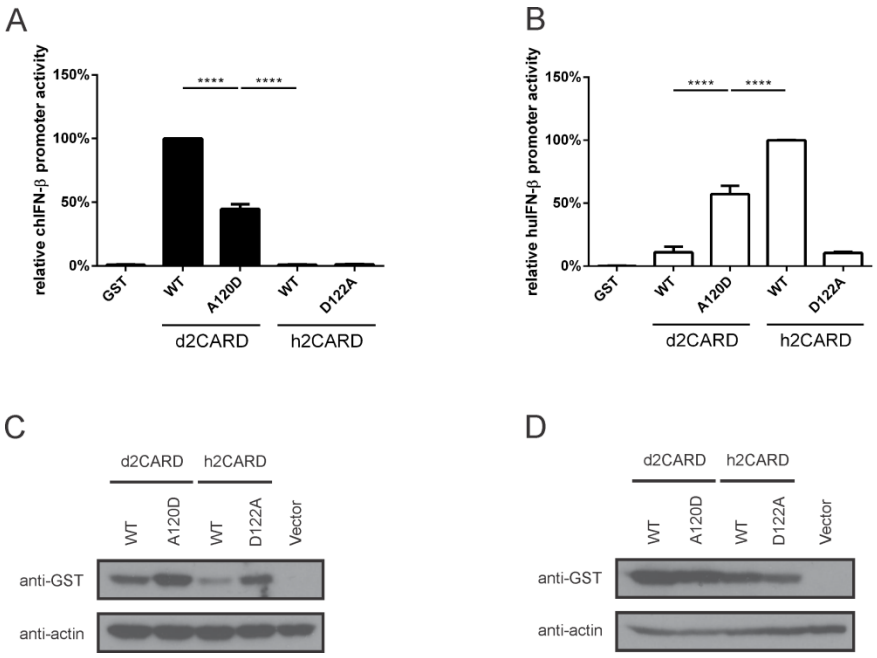



**Figure 4.1. Amino acid residue 120 in duck RIG-I CARD domains (122 in human RIG-I-CARD) contributes to species-specific MAVS-CARD :: RIG-I-CARD interactions.**
(A) d2CARD A120D mutation decreases IFN-β promoter activity in DF-1 cells, while h2CARD wildtype and the complementary D122A mutant remain inactive. (B) RIG-I d2CARD A120D mutant is more active than wildtype d2CARD in HEK293T cells, while the corresponding mutation in h2CARD nearly abrogates IFN-β promoter activity. Graph bars show mean ± SD of three independent experiments (n=3). Means were compared using one-way ANOVA with a Tukey's multiple comparisons post-hoc test (****, $p \leq 0.0001$). Total transfected DNA quantity was always kept constant with empty vector. (C and D) All GST-2CARD proteins are expressed in DF-1 cells (C) and HEK293T cells (D). Whole cell lysates were immunoblotted with anti-GST and anti-actin antibodies. *This figure was made by Chase Stevens under my supervision.*

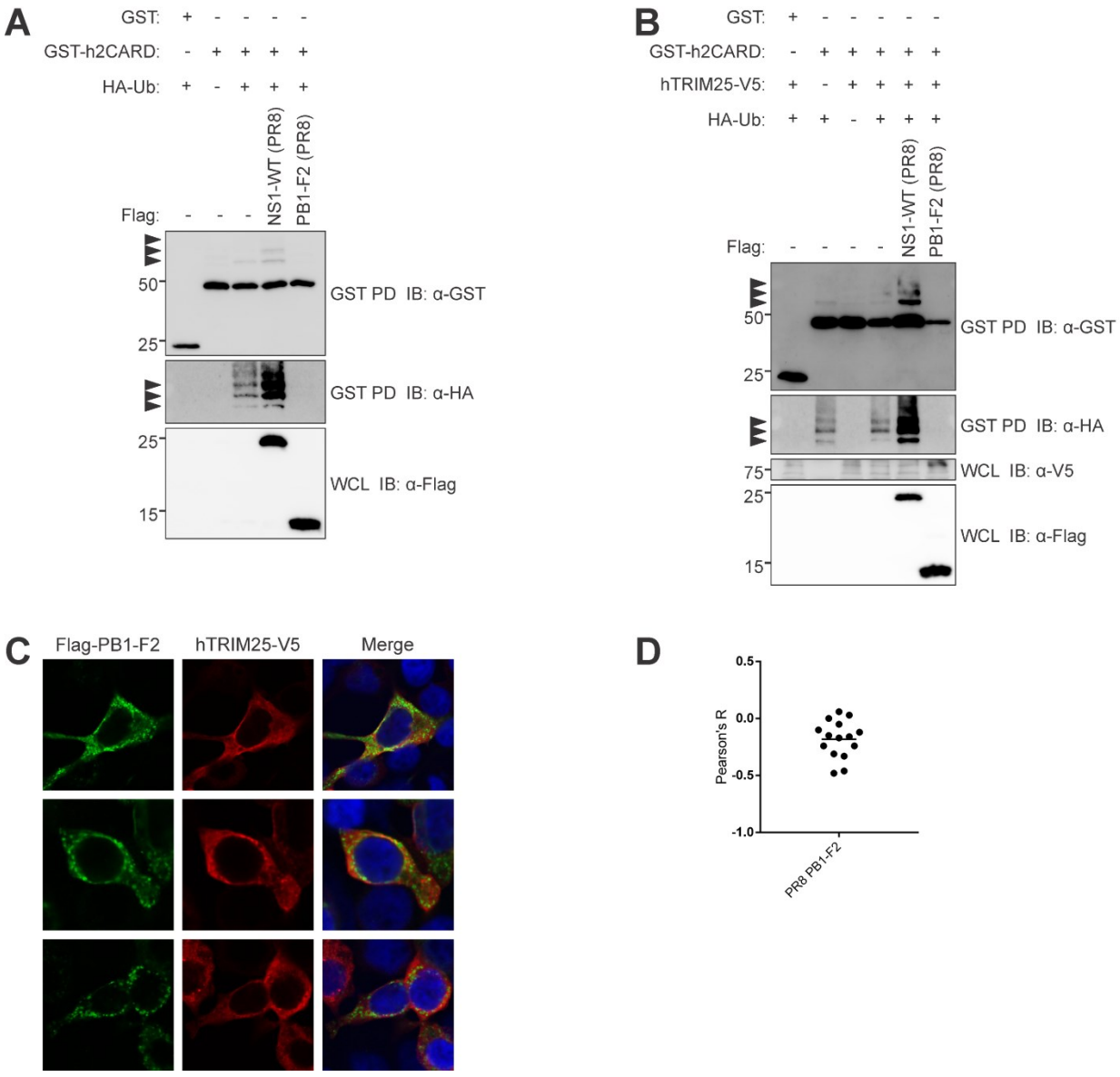



**Figure 4.2. PR8 PB1-F2 inhibits TRIM25-mediated ubiquitination of human RIG-I CARD domains.**

(A) HEK293T cells were transfected with GST-tagged human RIG-I CARD domains (GST-h2CARD) or empty GST vector, together with HA-tagged ubiquitin (HA-Ub) and the indicated Flag-tagged proteins (NS1 or PB1-F2). Clarified whole cell lysates (WCL) were subjected to GST pulldown (GST PD), followed by immunoblotting (IB) with anti-HA, anti-Flag, and anti-GST antibodies. (B) HEK293T cells were transfected as in (A), but with the addition of V5-tagged human TRIM25 (hTRIM25-V5), as indicated. (C) HEK293T cells overexpressing hTRIM25-V5 and Flag-PR8-PB1-F2 proteins were fixed, stained, and imaged using confocal microscopy. Panels show staining with Hoechst 33324 (blue), anti-Flag antibodies (green), anti-V5 antibodies (red), and a merged image. (D) Co-localization of Flag-PR8-PB1-F2 with

hTRIM25-V5 was quantified using Pearson's correlation coefficient (Pearson's R). Bar shows mean value from 15 analyzed cells.

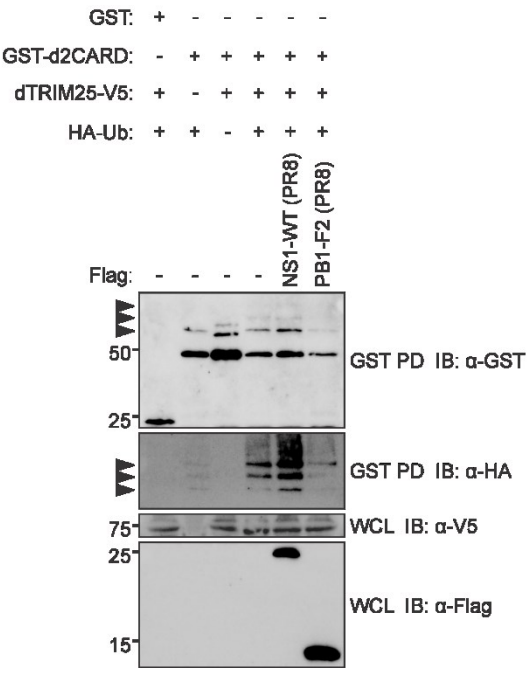



**Figure 4.3. PR8 PB1-F2 inhibits TRIM25-mediated ubiquitination of duck RIG-I CARD domains.**

DF-1 cells were transfected with GST-tagged duck RIG-I CARD domains (GST-d2CARD) or empty GST vector, together with HA-tagged ubiquitin (HA-Ub), V5-tagged duck TRIM25 (dTRIM25-V5), and the indicated Flag-tagged proteins (NS1 or PB1-F2). Clarified whole cell lysates (WCL) were subjected to GST pulldown (GST PD), followed by immunoblotting (IB) with anti-HA, anti-Flag, and anti-GST antibodies.

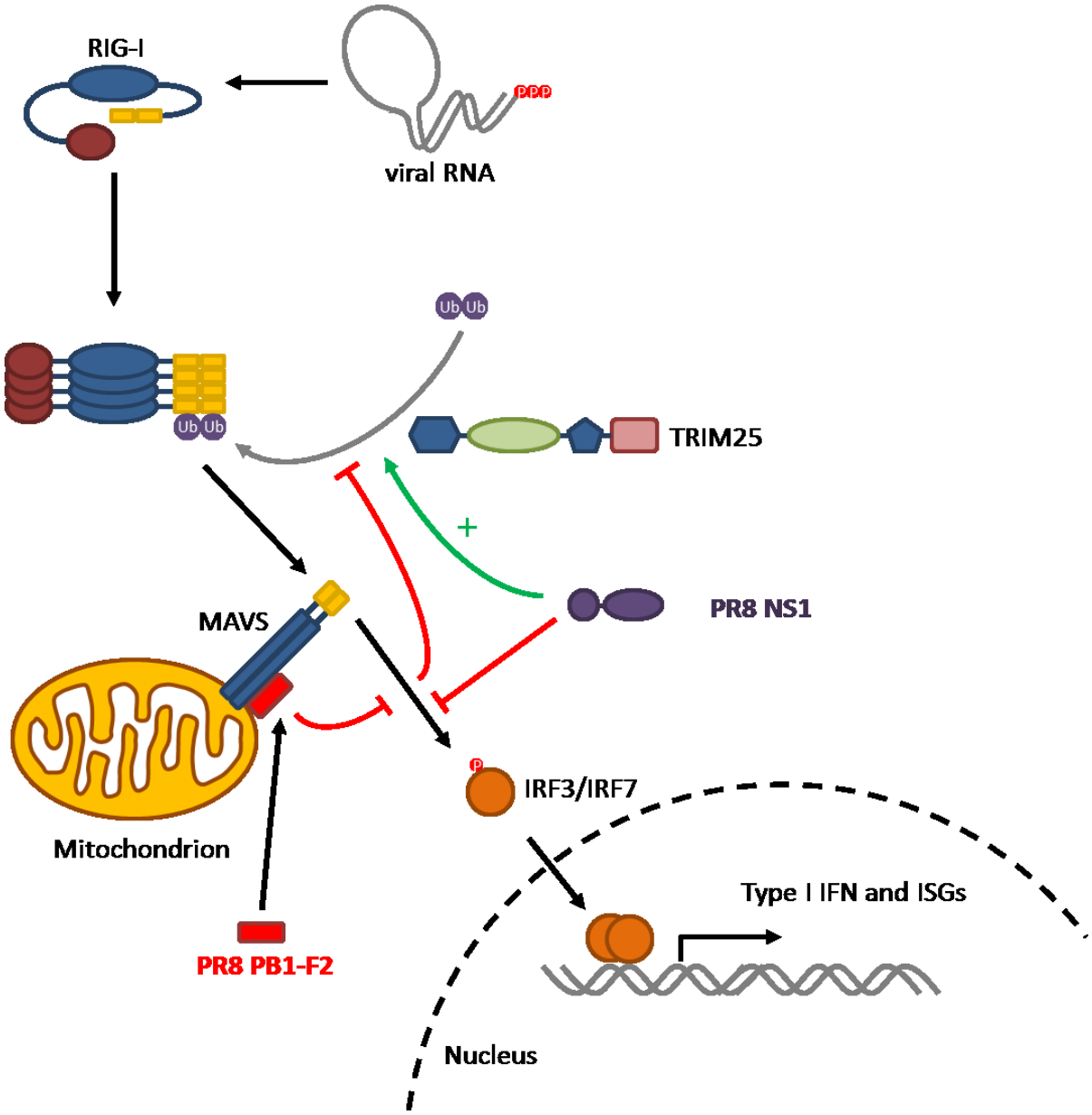



**Figure 4.4. Influenza A/Puerto Rico/8/1934 (PR8) PB1-F2 inhibits ubiquitination of RIG-I CARD domains and signaling downstream of MAVS.**
PR8 PB1-F2 interacts with duck MAVS in the cytoplasm of DF-1 cells and inhibits interferon-β promoter activity downstream of duck RIG-I and duck MAVS. PR8 PB1-F2 also decreases TRIM25-mediated K63-linked ubiquitination of both human and duck RIG-I CARD domains, whereas PR8 NS1 appears to increase it *in vitro*.

# Chapter 5 Influenza A PB2 proteins interact with duck MAVS and inhibit interferon signaling.

## 5.1 Introduction

When influenza A viruses cross the species barrier from birds to mammals, several genetic mutations are required to help adapt the virus to a new host (Schrauwen and Fouchier, 2014). Several adaptive mutations often occur in the multi-subunit IAV polymerase responsible for transcription and replication of viral RNA (Arai et al., 2016; Miotto et al., 2008). For example, several mutations in the polymerase basic protein 2 (PB2) – G590S, Q591K/R, E627K, and D701N – improve poor avian polymerase activity in mammalian cells or increase virulence in mammals (Hussein et al., 2016; Manz et al., 2016; Mehle and Doudna, 2009; Subbarao et al., 1993; Zhu et al., 2015). Other adaptations are required for PB2 to interact with host co-factor ANP32, which has significant species-specific differences (Hou et al., 2011; Long et al., 2016). The IAV polymerase is composed of three subunits, PB2, polymerase basic protein 1 (PB1), and polymerase acidic protein (PA). Variation in all three polymerase subunits can affect virulence; however, PB2 is the primary polymerase determinant of IAV virulence and host specificity (Neumann and Kawaoka, 2015).

PB2 proteins in mammalian cells also have the ability to inhibit the IFN-β response during IAV infection (Graef et al., 2010; Iwai et al., 2010). PB2 localizes primarily to the nucleus of infected cells to form the IAV polymerase, but a fraction of newly-synthesized PB2 is imported into the mitochondrial matrix of infected cells via a conserved N-terminal motif (Carr et al., 2006; Long and Fodor, 2016). Mitochondrial localization of PB2 correlates with a

mammalian adaptive residue at N9 within the mitochondrial-targeting signal (Graef et al., 2010). Avian adapted strains, including H5N1 strains, encode D9 and show no mitochondrial association in mammalian cells (Graef et al., 2010). At the mitochondria, PB2 has at least two roles that affect virulence. First, PB2 inhibits IFN-β signaling by interacting with mammalian MAVS (Graef et al., 2010; Iwai et al., 2010; Patel et al., 2013). This interaction involves amino acid positions 1-37 on the PB2 protein and positions 1-150 on human MAVS (Patel et al., 2013). PB2 proteins encoding either N9 or D9 both interact with human MAVS *in vitro*, despite differences in mitochondrial localization, but reduced mitochondrial localization does correlate with reduced interferon inhibition capacity (Graef et al., 2010). Presumably, PB2 proteins with functional mitochondrial targeting through residue N9 are more readily available to interact with MAVS. However, it seems that there must be a second, MAVS-independent function, since PB2 proteins are also imported into the mitochondrial matrix, whereas MAVS proteins are located on the mitochondrial outer membrane, and mutating PB2 residue 9 to prevent this attenuated a virus in MAVS-knockout mouse embryonic fibroblasts (Long and Fodor, 2016). Mutating an avian H5N1 virus to bear the human residue N9 increased its replication and pathogenicity in mice (Kim et al., 2010).

It is not known whether PB2 interferes with MAVS signaling in the natural host, mallard ducks, or how residue 9 affects function. It is known that H5N1 PB2 expression in chicken cells inhibits IFN-β signaling (Liniger et al., 2012). The N-terminal 150 amino acids of MAVS include the CARD and part of the proline-rich region (PRR) that recruits downstream signaling molecules TRAF2 and TRAF6 (Seth et al., 2005; Xu et al., 2005). The CARD domain alone is insufficient for PB2 interaction, implicating the PRR (Patel et al., 2013). MAVS from ducks shares only 28% overall amino acid identity with human MAVS (Wu et al., 2014), and the PRR

is poorly conserved between mammals and ducks (Li et al., 2016). We demonstrate that PB2 proteins from 2 LPAI strains, 2 HPAI strains, one pandemic H1N1/09 strain, and the mouse-adapted strain PR8, all interact with duck MAVS and inhibit duck MAVS-mediated IFN-β signaling in DF-1 cells. Amino acid polymorphism that distinguish avian-adapted and human-adapted PB2 proteins, at positions 9, 64, and 81, did not affect interaction with duck MAVS *in vitro*.

## 5.2 Results

### *5.2.1 Modeling influenza PB2 reveals three species-specific adaptive residues near the human MAVS binding interface.*

To examine the function of PB2 in avian cells, we cloned PB2 genes from 6 influenza A strains of avian and mammalian origin (Table 1). Overall, the 6 PB2 proteins have a high degree of amino acid sequence conservation (> 98% identity). An alignment of the N-terminal region reveals three host-adaptive amino acid polymorphisms (Fig 5.1A). Mammalian-adapted PR8 PB2 differs from the avian-strain PB2 proteins at three N-terminal amino acid positions – 9, 64, and 81. Specific polymorphisms at these positions are associated with strains that transmit among wild birds versus strains that transmit among humans (Miotto et al., 2008), and residue N9 imparts mitochondrial localization to PB2 proteins in mammalian cells (Graef et al., 2010). PR8 PB2 encodes the human-consensus residues N9, T64, and M81. The HPAI and LPAI avian PB2 proteins differ amongst themselves by a conservative substitution at position 64 – BC500 and M546 PB2 proteins encode M64; D4AT and VN1203 PB2 proteins encode I64. The 2009 pandemic A(H1N1)pdm09 strain, though a swine-origin virus that transmitted successfully among humans, is a multiple-reassortant that contains an avian PB2 gene (Smith et al., 2009b), and bears the avian-consensus residues. To visualize the locations of these adaptive residues, we

modeled the PR8 PB2 amino acid sequence on the recently published bat influenza A PB2 crystal structure (4WSB) (Fig 5.1B). The three adaptive residues (N9, T64, M81) reside within the same domain, but only one of them – N9 – falls within the first 37 amino acids of PB2 that are essential for binding human MAVS (Patel et al., 2013)(Fig 5.1A-C). However, their locations suggest that they could potentially participate in a common protein-protein interface (Fig. 1C). The PB2 proteins from the human pandemic pdm09 strain and from the VN1203 strain, which is an avian H5N1 strain that was isolated from a fatal human case, also contain mutations that increase polymerase activity in human cells – Q591R in pdm09 and E627K in VN1203 – but this is beyond the current scope of this study (Manz et al., 2016; Mehle and Doudna, 2009).

### *5.2.2 PB2 proteins from human-adapted and avian-adapted influenza A strains interact with duck MAVS.*

In human cells, PB2 proteins bearing either of the species-specific adaptive residues at position 9 co-precipitate with human MAVS (Graef et al., 2010). We investigated whether PB2 proteins also interact with duck MAVS, and whether the polymorphisms at positions 9, 64, and 81 affect this potential interaction. Co-immunoprecipitations showed that PB2 proteins bearing both the avian-consensus and the human-consensus residue trios interact with duck MAVS (Fig 5.2). In the first co-immunoprecipitations, done by a past student, M546 PB2 did not appear to interact with MAVS, despite being identical to BC500 PB2 in the first 100 amino acids (Fig 5.2A). When I repeated the experiment, I showed that M546 PB2 does interact with duck MAVS (Fig 5.2B), and so does avian PB2 from a 2009 pandemic isolate A/Halifax/210/2009 (H1N1) (pdm09) (Fig 5.2C). Thus, PB2 proteins from human-adapted and avian-adapted influenza A strains interact with duck MAVS *in vitro*, regardless of polymorphisms at positions 9, 64, and 81.

### *5.2.3 PB2 proteins from human-adapted and avian-adapted influenza A strains inhibit interferon-beta promoter induction by duck MAVS in DF-1 cells.*

To investigate whether the *in vitro* interaction of PB2 proteins with duck MAVS correlates with inhibition of type-I interferon signaling, I performed dual luciferase assays measuring chicken IFN-β promoter activity downstream of transfected duck MAVS in DF-1 cells (Fig 5.3). Overexpressed duck MAVS induced interferon promoter activity, which was suppressed by the positive control wild-type PR8 NS1 and unaffected by the negative control protein duck Riplet (a ubiquitin ligase that acts in the RIG-I pathway upstream of MAVS). All the PB2 proteins significantly inhibited MAVS signal transduction. The HPAI and LPAI PB2 proteins had comparable activities. The mammalian PR8 PB2 and the avian pdm09 PB2 suppressed interferon signaling more effectively than the avian HPAI and LPAI proteins.

## 5.3 Discussion

In mammalian cells, PB2 proteins from different strains are known to inhibit IFN-β signaling downstream of MAVS to varying degrees based on their host origin, and the amino acid residue at position 9 (Graef et al., 2010; Iwai et al., 2010; Long and Fodor, 2016; Patel et al., 2013). In these preliminary results, I show that PB2 proteins from avian and mammalian sources also interact with duck MAVS *in vitro* and inhibit type-I interferon signaling in DF-1 cells (Fig 5.4).

It is interesting that the PB2-MAVS interaction is conserved in the duck host despite the low degree of sequence conservation with human MAVS, especially in the N-terminal proline-rich region. To determine if binding to duck MAVS occurs in the same region, co-precipitations can be done with truncated MAVS mutants. Just as the natural polymorphism at position 9 in PB2 does not affect *in vitro* interaction with human MAVS, so it did not affect co-precipitation

with duck MAVS. Additionally, two other N-terminal host-adaptive polymorphisms in PB2, at positions 64 and 81, do not appear to influence association with duck MAVS. This can be explained in two different ways. It is possible that duck MAVS interacts with the first 37 amino acids of PB2 proteins, just as human MAVS (Patel et al., 2013), and there is some plasticity in this interaction. Alternatively, it is possible that duck MAVS interacts with another region of PB2 that is conserved between avian and mammalian strains. Experiments with truncated PB2 mutants could help to discriminate between these possibilities.

Certain limitations of the co-immunoprecipitation technique must be kept in mind when interpreting this data. It is understood that co-precipitation of proteins from a cell lysate does not necessarily imply direct interaction, but it is also important to remember that the system itself is somewhat artificial because the molar ratios of the overexpressed proteins probably do not reflect their actual biological proportions in an infected cell. Factors like bait:prey ratio and buffer stringency, as well as other manipulations, may affect the results. This is exemplified in figure 5.2, where my colleague and I obtained different results with respect to PB2 from the M546 strain, even though we used the same protocol and buffer recipes. Testing a series of different buffer stringencies and establishing a loss-of-function mutant negative control would be helpful to bolster these results.

The interferon inhibition luciferase assay showed that all of the PB2 proteins inhibited MAVS-induced signaling to some extent, but produced an unclear pattern with respect to PB2 species adaptation. PR8 PB2 encodes N9 and inhibited IFN-β promoter activity to a greater extent than the LPAI and HPAI PB2 proteins, which all encode D9 and performed similarly to each other. This resembles data from mammalian cells (Graef et al., 2010), assuming that residue N9 also imparts mitochondrial localization in chicken cells. We have preliminary data suggesting

that residue N9 does increase mitochondrial localization in chicken cells and may affect interferon inhibition, but the results are inconclusive. The experiments need to be optimized and repeated.

Surprisingly, avian-origin pdm09(H1N1) PB2, bearing the avian consensus residues at positions 9, 64, and 81, inhibited interferon to the same extent as PR8 PB2, more effectively than the other avian PB2 proteins in the set. The pdm09(H1N1) PB2 has two unique amino acid residues in the N-terminal region – an arginine at position 52 (R52) in place of the lysine (K52) in the rest of the PB2 sequences, and an aspartic acid at position 65 (D65) in place of the glutamic acid (E65) in the rest of the PB2 sequences. These polymorphisms do not seem to affect *in vitro* interaction with duck MAVS, but could potentially influence mitochondrial localization or interferon inhibition in other ways. There are also several other unique polymorphisms in the pdm09(H1N1) PB2 protein, closer to the C-terminus, whose contributions could also be investigated. To explore these possibilities, the mitochondrial localization of pdm09(H1N1) PB2 in avian cells should be examined and mutagenesis studies should be performed to discover the contributions of specific amino acids, if any. I must also consider that the enhanced inhibition could be simply due to greater PB2 protein expression in the pdm09-transfected groups in my luciferase assays, since protein expression was not verified.

In conclusion, avian-adapted and human-adapted PB2 proteins appear to interact with duck MAVS and inhibit type-I interferon transduction. Additional experiments will need to be performed to discover if the mechanisms of binding and interferon inhibition are analogous to what has been described in mammalian cells. Recombinant viral infection studies should follow, comparing wild-type and point-mutant PB2 proteins in identical backgrounds to substantiate the contributions of the described polymorphisms.

**Table 5.1. Influenza A virus strains used to clone PB2.**

| Strain name | Abbreviation | Virus Type | Source |
|---|---|---|---|
| A/duck/British Columbia/500/2005 (H5N2) | BC500 | avian-LPAI | duck |
| A/duck/Memphis/546/1974 (H11N9) | M546 | avian-LPAI | duck |
| A/duck/D4AT/Thailand/71.1/2004 (H5N1) | D4AT | avian-HPAI | duck |
| A/Vietnam/1203/2004 (H5N1) | VN1203 | avian-HPAI | human |
| A/Halifax/210/2009 (H1N1) | pdm09 | human, triple-reassortant | human |
| A/Puerto Rico/8/1934 (H1N1) | PR8 | human, mouse-adapted | human |

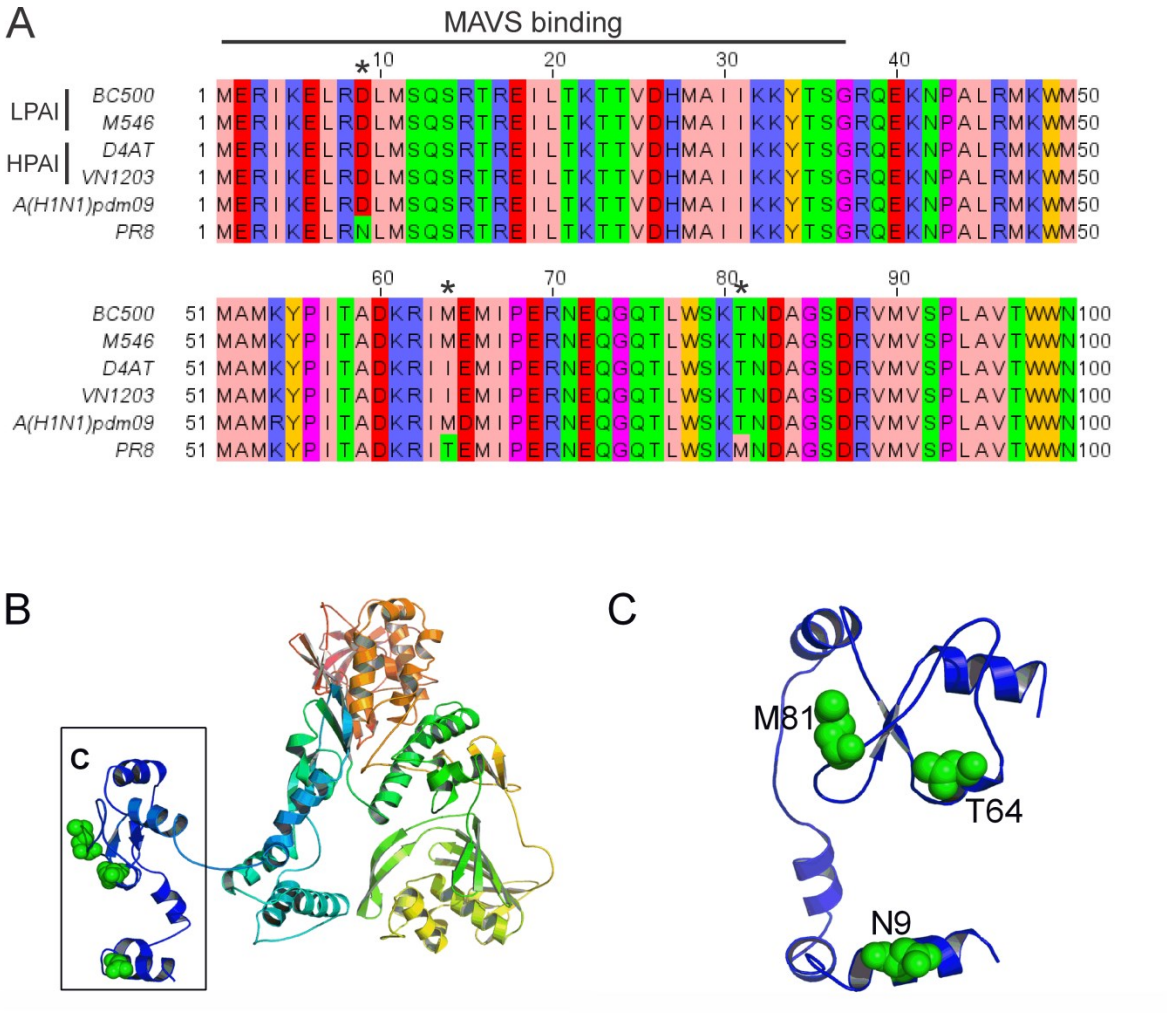



**Figure 5.1. Map of adaptive residues within the PB2 structure.**
(A) Alignment of the first 100 residues of PB2 from 6 influenza strains, 2 low-pathogenic avian influenza strains (LPAI), 2 highly pathogenic avian influenza strains (HPAI), a pandemic H1N1/09 strain (A(H1N1)pdm09, A/Halifax/210/2009 (H1N1)), and the mouse-adapted PR8. The residues are coloured according to their physiochemical properties based on the Zappo colour scheme in Jalview. The known PB2 residues involved in MAVS binding are overlined, and the PB2 residues involved in host adaptation are indicated with asterisks. Influenza PB2 has three adaptive residues that localize near the MAVS-PB2 interaction domain. PR8 PB2 bears the amino acids commonly found in human-to-human transmitting viruses (Miotto et al., 2008). (B) Modeled influenza PB2 (PR8) is shown from the N-terminus (blue) to the C-terminus (red). PB2 (PR8) was modeled to the bat influenza A PB2 crystal structure (4WSB) template using the SWISS-MODEL protein structure homology-modelling server. (C) The first 103 residues of PB2 (PR8) are displayed with the location of three adaptive residues (green) that differ between mammalian and avian influenza strains indicated. *Panels B and C were prepared by David Guy Tetrault and Dr. Katharine Magor.*

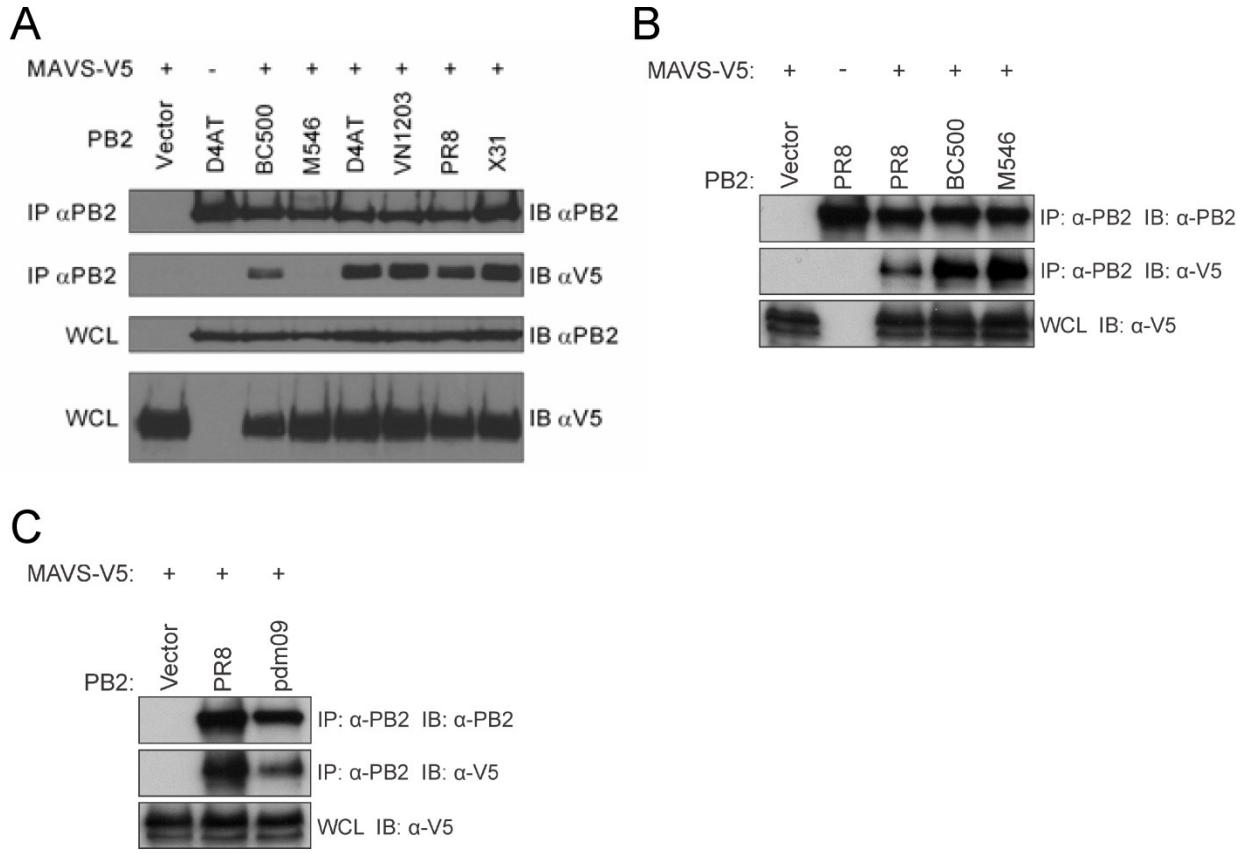



**Figure 5.2. PB2 proteins interact with duck MAVS regardless of the amino acid residues at positions 9, 64, and 81.**

(A-C) DF-1 cells were transfected with duck MAVS-V5 and the indicated PB2 proteins for 24 h. Pre-cleared whole cell lysates (WCL) were subjected to anti-PB2 immunoprecipitation (IP), followed by immunoblotting (IB) with anti-PB2 and anti-V5 antibodies. X31 PB2 panel A is from a reassortant strain derived from PR8 and human vaccine strain A/Aichi/68 (H3N2) (Kilbourne, 1969); it is identical to PR8 PB2. Experiments in panels B and C were performed twice. *Panel A was made by David Guy Tetrault.*

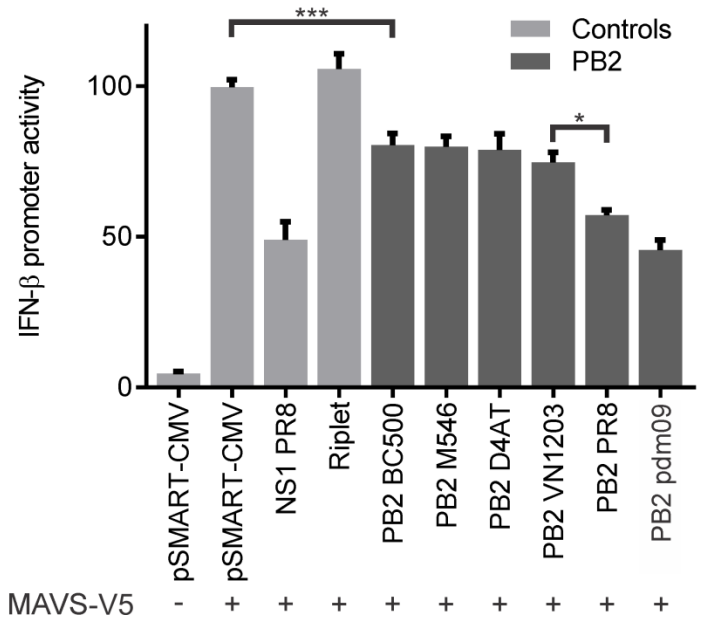



**Figure 5.3. PB2 proteins inhibit interferon-beta promoter induction by duck MAVS.** (A) DF-1 cells were transfected with 25 ng of V5-tagged duck MAVS (MAVS-V5) or empty vector, and 500 ng of PB2 expression constructs or of the indicated controls (NS1, Riplet, pSMART-CMV vector), along with chicken IFN-β luciferase reporter vector and control *Renilla* luciferase reporter vector. pSMART-CMV is the vector backbone of the PB2 expression constructs. 24 hours post-transfection, cells were lysed and the IFN-β promoter activity was measured by dual luciferase assay. Graph bars show mean ± standard deviation of three independent experiments (n=3). Means were compared using one-way ANOVA with a Tukey's multiple comparisons post-hoc test (*, $P \leq 0.05$; ***, $P \leq 0.001$).

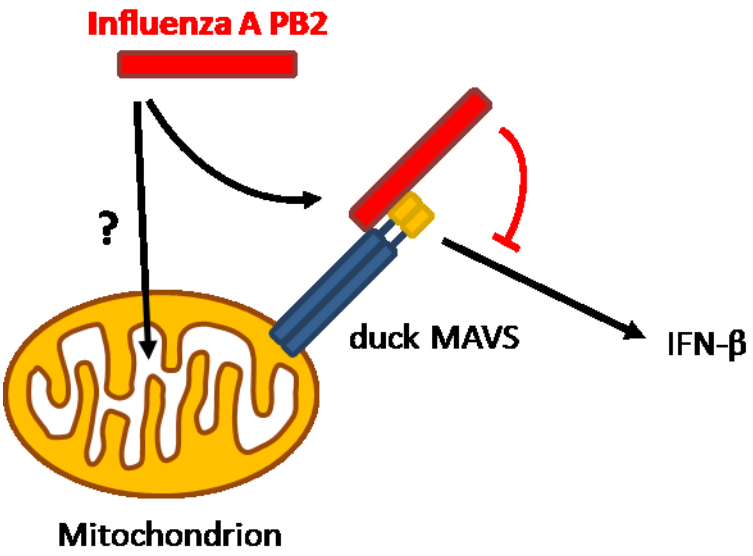



**Figure 5.4. Influenza A virus PB2 proteins interact with duck MAVS and inhibit interferon signal transduction.**
Avian-adapted and human-adapted PB2 proteins associate with duck MAVS *in vitro* irrespective of host-adaptive polymorphisms in their N-termini (positions 9, 64, and 81). The exact mechanism(s) of inhibition and whether PB2 proteins are imported into the inner mitochondrial matrix in avian cells remains unclear.

# Chapter 6 General discussion

Influenza A viruses are important pathogens of humans and animals. Virtually all IAV strains that we know of descend from a common ancestor that arose in mallard ducks, the primordial hosts of IAV. Mallard ducks today serve as a reservoir for the maintenance, diversification, and spread of IAV strains all over the world. They are more resistant than other animals and birds to disease caused by almost all strains of IAV. The ability of IAVs from different hosts to genetically reassort in co-infected 'mixing vessel' animals poses a serious pandemic threat, particularly when human and swine IAVs acquire new avian genetic elements. IAVs in mammals and in avian hosts display divergent evolution. All of the viral proteins acquire species-specific polymorphisms that probably contribute to viral adaptation. Comparing the molecular interactions of IAVs in different hosts, and the functional significance of some of these species-specific polymorphisms may reveal mechanisms of viral adaptation and host susceptibility or disease resistance. The classical understanding of host-virus co-evolution suggests a natural progression toward efficient viral replication that minimizes damage to the host, benefiting both host and virus (Arien et al., 2005; Webster et al., 1992). Relatively little is known about IAV host-pathogen interactions in mallard ducks. In this thesis, I focused on interactions between IAV and innate immunity, particularly the RIG-I signaling pathway, which is critical for limiting viral spread during the earliest points of infection. I investigated three known mechanisms of type-I interferon signaling suppression by IAV identified in mammals, and assessed whether they are also present in ducks.

The findings described in this thesis should be considered in the context of what is already known about the co-adaptation between mallard ducks and IAV. The primary site of IAV replication in ducks is the intestine, not the airway as it is in mammals. LPAI viruses replicate to

high titres in duck intestines and do not induce appreciable immune responses or apparent morbidity in the animals (Hinshaw et al., 1980; Webster et al., 1978). In chickens, LPAI viruses also replicate in the lower intestine, but also frequently infect the airways and kidneys (Claes et al., 2013; Condobery and Slemons, 1992; Guan et al., 2013; Slemons and Swayne, 1995). HPAI viruses do replicate in the airways and other organs of ducks, but ducks are more resistant compared to other hosts and have a less pronounced pro-inflammatory response than other birds (reviewed in Evseev and Magor, 2019). Additionally, HPAI viruses display weaker endothelial tropism in ducks than in galliform birds, which is also linked to the induction of inflammation (Short et al., 2014). Mallards appear able to tolerate a certain amount of systemic HPAI replication, particularly strikingly in the brain and central nervous system, and still successfully clear the virus (Bingham et al., 2009). In humans, viral replication in CNS is associated with severe symptoms and sequelae (Studahl, 2003). These apparent macroscopic co-adaptations provide the context for the molecular investigations in this thesis.

To date, only one previous study has examined the species-specificity of NS1 interference with various hosts' TRIM25 proteins (Rajsbaum et al., 2012). Rajsbaum and colleagues found that NS1 proteins from representative avian, human, and swine influenza A strains all bound human TRIM25 *in vitro*. In that study it was assumed that binding to TRIM25 necessarily leads to the suppression of RIG-I ubiquitination, although this was not directly verified. Thus, we know that NS1 proteins *can* inhibit human RIG-I ubiquitination (Gack et al., 2009) and that NS1 proteins from different host-species interacted with human TRIM25 (Rajsbaum et al., 2012). Rajsbaum et al. also reported that chicken TRIM25 interacted only with an avian NS1 protein, and that this interaction reduced interferon induction *in vitro*, even though chickens lack RIG-I and the target of chicken TRIM25 ubiquitination was unclear.

In Chapter 3, I show that various NS1 proteins do not appear to inhibit duck TRIM25-mediated ubiquitination of RIG-I-CARD and the resulting interferon signaling. It is possible that this lack of inhibition is a product of co-evolution that preserves the intestinal tropism of LPAI viruses in ducks, and limits the extra-intestinal replication of highly pathogenic strains. Using transgenic mice that express a luciferase reporter, Kallfass et al. (2013) found that lung epithelial cells were the first cells to produce IFN-β upon infection with an influenza A virus lacking a functional NS1 protein. By contrast, in reporter mice infected with the wild-type NS1-competent virus, the lung epithelial cells did not begin to produce visible interferon until 48 hours post-infection, and at that point the viral spread in lung tissues was extensive. Ducks acquire IAV naturally through their dabbling behaviour, and LPAI viruses initially infect the airways, but do not persist in that location. Although the preferred α-2,3-Gal sialic acid receptors for LPAIV hemagglutinin are abundant in both the airways and the lower intestine, both naturally and experimentally infected ducks display virus replication principally in the lower gut, where there is little innate immune signaling (Vanderven et al., 2012), and long-term shedding from the cloaca only (Costa et al., 2012; Daoust et al., 2011). Experiments with LPAIV subtypes H3N8, H5N2, H2N3, and H13N6 all show that the airways do get infected through the mucosa of the head, and mild tracheitis and pneumonia are detectable in the first two days after infection, but all the viruses become undetectable in tracheal swabs after 3 or 4 days and replicate for longer and to higher titres in the colon, cecum, and bursa of Fabricius, and (Daoust et al., 2013; França et al., 2012). It is possible that uninhibited RIG-I signaling in the airway epithelium limits IAV establishment in the lungs of ducks, while the intestinal tissue is more permissive. In ducks infected with BC500, an LPAI strain used in this study, there was no detectable viral shedding from the trachea at 3 days post infection (Vanderven et al., 2012).

The two evolutionary lineages of avian NS1 proteins, alleles A and B, diverged early in the evolution of IAV (Webster et al., 1992). The fact that representatives of both alleles did not inhibit RIG-I-CARD ubiquitination supports the supposition that this is specifically adaptive and maintained. To ascertain this, it would first be necessary to confirm that duck RIG-I ubiquitination does occur during actual infections with recombinant viruses encoding different NS1 proteins, as discussed in Chapter 3. Furthermore, the interactions between NS1 proteins and duck Riplet should also be examined. Riplet also activates RIG-I by ubiquitinating the C-terminal repressor domain (Oshiumi et al., 2009; Oshiumi et al., 2010). In their comparative study, Rajsbaum et al. (2012) showed that in mice, Riplet, but not TRIM25, was targeted by multiple NS1 proteins to reduce the induction of type-I interferon. They also found that NS1 proteins from two human viruses also targeted human Riplet, and supressed type-I interferon more effectively than NS1 proteins that targeted TRIM25 only. Thus, it will also be important to investigate duck Riplet function in the RIG-I pathway, and secondly, whether NS1 proteins interact with it to suppress type-I interferon induction.

Two strains of origin for my NS1 proteins were HPAI H5N1 viruses. Data from several experimental infections of mallard ducks with these strains is available (Saito et al., 2018; Sturm-Ramirez et al., 2005; Vanderven et al., 2012). Both viruses can cause severe disease, and can be fatal to ducks (Sturm-Ramirez et al., 2005), but in those ducks that survive, there is a rapid and brief induction of type-I interferon and a sustained upregulation of ISGs (Saito et al., 2018). It is tempting to say that uninhibited RIG-I signaling is contributing to survival in these cases. However, this kind of interpretation is complicated by the fact that circulating leukocytes also produce type-I interferon in infected animals. In Kallfass and colleagues' study of transgenic mouse lungs, they noted that when IFN-β is supressed by NS1 in epithelial cells and in lung-

resident $CD11^-$ macrophages, infiltrating $CD11^+$ cells produce IFN-β (Kallfass et al., 2013). Because of the complexity of whole-organism immune responses, and the high degree of entanglement and cross-talk between innate immune signaling pathways, it is difficult to clearly discriminate between protective and deleterious responses. Sustained induction of pro-inflammatory cytokines is usually accompanied by similarly high and prolonged induction of type-I interferons (Cao et al., 2017; Moulin et al., 2011; Penski et al., 2011; Soubies et al., 2010a) and higher viral titres usually correlate with higher interferon induction (Hammerbeck et al., 2007; Wyde et al., 1982). Li et al. (2018) showed that the internal genes (not NS1) of an HPAI H5N1 strain allowed a recombinant virus to replicate to high titres in murine myeloid cells, which resulted in severe disease and elevated type-I interferon secretion by those cells. For these reasons many studies correlate high IFN-α induction with pathogenicity. Two elements appear to determine the effectiveness of a type-I interferon response – that it occurs early at the site of infection to delay initial viral establishment, and that strong activation does not persist. Both of these conditions are met in ducks that survive HPAI H5N1 infections (Fleming-Canepa et al., 2019; Saito et al., 2018). However, measuring whole tissue mRNA levels at 24-hour intervals does not provide the resolution needed to distinguish which cells are secreting interferon, especially during the earliest times of infection. It would be interesting to adapt the transgenic interferon-reporter visualization techniques described by Kallfass et al. (2013) to ducks, and investigate interferon production in duck lung epithelial cells immediately upon infection with different viruses.

The need to introduce a G184R mutation into the avian NS1 proteins in my study highlights a limitation of the reductionist approach. Reductionist experiments aim to isolate and characterize individual protein functions and interactions in absence of other variables. They do

not recapitulate the additive, synergistic, or competitive effects of multiple functions of a single protein. In order to investigate interference in the RIG-I pathway, I had to abrogate the interaction of the avian NS1 proteins with host CPSF4, with unintended consequences. A systematic comparison of several NS1 mutations in a PR8 background, by Steidle et al. (2010), showed that in human A549 cells and in mice, the G184R mutation does not affect the levels of interferon induction. In contrast, I found that it did, and the possible reasons for this and how to test them experimentally are discussed in detail in Chapter 3. But this observation also raises a more fundamental question, about the relative importance and contributions of different NS1 functions during infection. The species-adaptive value of CPSF4 interaction is not clear. Studies suggest that avian-origin NS1 proteins tend to evolve away from this function in non-human mammals (Chauché et al., 2018; Dankar et al., 2011). Sequence analysis suggests that CPSF4-interacting residues are not strictly maintained in either human or avian hosts, and there are notable examples of pandemic or HPAI avian viruses whose NS1 proteins lack this function, as discussed in the introductory chapter of this thesis. Studies show that removing or rescuing the interaction with CPSF4 has different and contradictory effects on pathogenicity, depending on the genetic background of the virus (Chauché et al., 2018; Dankar et al., 2011; Hale et al., 2010b; Spesock et al., 2011; Steidle et al., 2010). It appears that the loss of CPSF4 binding in seasonal human strains enhances viral replication and pathogenicity in mice (Dankar et al., 2011; Hale et al., 2010b), but the opposite is true for an H5N1 strain (Spesock et al., 2011). Steidle et al. (2010) found that rescuing CPSF4 interaction in the PR8 strain did not significantly affect interferon induction or replication efficiency of the virus in interferon-reporter mice. The spontaneous loss of CPSF4 interaction by the equine H3N8 virus lineage appears to correlate with enhanced replication in horses (Chauché et al., 2018). In ducks, we see sustained induction

of ISG mRNA when they are infected with viruses whose NS1 proteins would be expected to interact with duck CPSF4, such as the strains used in this study (Saito et al., 2018). However, the ISG induction at the tissue level could be due to non-infected cells being activated by type-I interferons secreted by leukocytes. Here too, it would be interesting to couple recombinant viral work with a cell-by-cell visual reporter system in live animals, like the interferon-reporter mice described by Kallfass et al. (2013), to look at interferon and ISG responses in infected tissues and individual cells early during infection.

In Chapter 4, in collaboration with my colleagues Dr. Yanna Xiao and Adam Moghrabi, I investigated whether the IAV accessory protein PB1-F2 from the PR8 strain would interact with duck MAVS and suppress interferon induction, as it does in human cells. PB1-F2 first came to our attention with the discovery that mutations therein can affect viral polymerase efficiency and attenuate an avian HPAI H5N1 virus in ducks (Marjuki et al., 2010). It was also discovered that PB1-F2 proteins could help to inhibit type-I interferon induction in human cells by interacting directly with MAVS (Varga and Palese, 2011; Varga et al., 2012). Relatively little is known about the species-specificity of PB1-F2 besides that fact that most avian viruses tend to conserve full-length PB1-F2 proteins (James et al., 2016b), while truncations are common in strains that enter the human pool (Pancucharova and Russ, 2006; Zell et al., 2007). We showed that recombinant duck MAVS induces interferon promoter activity in the DF-1 chicken cell line and that PR8 PB1-F2 co-localizes with and suppresses duck MAVS signaling. We were surprised to find that PR8 PB1-F2 also inhibited TRIM25-mediated ubiquitination of both duck and human RIG-I CARD domains, a function that had been previously ascribed only to IAV NS1 proteins. Continuation of this research requires first the corroboration of these observations with recombinant PR8 virus infection studies, as described in Chapter 4. We need to determine the

relative contributions of PR8 NS1 and PB1-F2 proteins to the ubiquitination of both duck and human RIG-I CARD domains during actual infections, which can be accomplished by removing NS1. Then, it would be interesting to investigate interferon inhibition (and polymerase activity in a mini-replicon system (Marjuki et al., 2010)) by other PB1-F2 proteins from human and avian viral sources. Recombinant viral studies to test different PB1-F2 proteins in the same viral backbone would be more difficult to accomplish, since PB1-F2 is expressed from an alternate reading frame of the PB1 polymerase component and altering polymerase subunits can have profound effects on viral fitness. Mutants lacking PB1-F2 expression, made by mutating the possible start codons, have been described.

Many studies have investigated species-specific adaptations of the polymerase components, like PB2. Many of them have focussed on polymorphisms that enhance polymerase activity in different hosts (Hussein et al., 2016; Manz et al., 2016; Mehle and Doudna, 2009; Subbarao et al., 1993; Zhu et al., 2015). A secondary, immunomodulatory function of PB2 was also discovered that occurs through two distinct but cooperative mechanisms. In human cells, PB2 proteins interact with MAVS to suppress interferon induction, but may also be trafficked into the inner mitochondrial matrix, which enhances interferon suppression (Graef et al., 2010; Iwai et al., 2010). Mitochondrial trafficking depends on a single polymorphism that is maintained in the human IAV pool. In Chapter 5, my colleague David Tetrault and I showed that several avian and one human PB2 proteins all associate with duck MAVS irrespective of the substitution at position 9 or at two other species-specific polymorphism sites. However, interferon inhibition efficiencies differed between the PB2 proteins and suggest the involvement of one or more polymorphisms in mitochondrial targeting, as in human cells. As described in the chapter discussion, immediate future experiments should confirm that mitochondrial trafficking

in avian cells also differs according to the identity of PB2 residue 9. Mutagenesis studies will help to determine which polymorphisms are responsible for the differences in interferon inhibition that we observed.

Taken together, these data fit into several plausible models of viral interference in the RIG-I pathway and beyond. Most likely, NS1, PB1-F2, and PB2 proteins work co-operatively to inhibit the human and duck RIG-I signaling pathways at several points. In human cells, NS1 interacts with TRIM25 to inhibit RIG-I CARD ubiquitination, while in avian cells duck TRIM25 activity is not inhibited by NS1 interaction. PB1-F2 inhibits the RIG-I pathway a little further downstream by associating with MAVS or a MAVS-containing complex, in both human and avian cells. A fraction of PB2 proteins is also targeted to the mitochondria and associates with MAVS to also suppress signaling. These complementary inhibitory mechanisms may occur simultaneously in infected cells, or may occur at different times during the viral replication cycle, with varying degrees of overlap. Theoretically, this has a co-operative effect to suppress the signaling of this important innate viral detection pathway. Importantly, the PB2 and PB1-F2 functions appear to be at least partially conserved between the species (human and duck), unlike the NS1 inhibition of human TRIM25 activity. However, NS1 is multifunctional, and I show evidence that another immune-inhibitory function of NS1, possibly outside of the RIG-I pathway and associated with NS1 residue 184, is conserved between the species. It is also possible that the association between NS1 and duck TRIM25 inhibits some other unaccounted-for function of duck TRIM25, which may be relevant for viral replication but has escaped my attention.

Two potential explanations can be given regarding PR8 PB1-F2 and its inhibition of both human and duck RIG-I-CARD ubiquitination by TRIM25, given the following caveat: In Chapters 3 and 5, my colleagues and I investigated and compared NS1 and PB2 proteins from

several different strains of IAV. In contrast, in Chapter 4 we investigated only the interactions of PB1-F2 from the PR8 strain. Whereas interferon inhibition by PB1-F2 proteins from other strains has been studied in human cells (reviewed by Kamal et al., 2017) and in avian cells by Dr. Yanna Xiao, Chapter 4 of this thesis contains the first investigation of the effect of PB1-F2 on TRIM25 function, and only with that single strain. We do not yet know whether other PB1-F2 proteins also inhibit RIG-I-CARD ubiquitination by TRIM25. Thus, two or three models can be proposed based on two different assumptions. The fact that PR8 PB1-F2 inhibited K63-linked ubiquitination by both human and duck TRIM25 proteins, unlike all the NS1 proteins investigated in Chapter 3, suggests that this occurs through a distinct mechanism. If the mechanism involves negative feedback from inhibition at the level of MAVS, we can assume that this would be conserved in other PB1-F2 proteins that similarly inhibit MAVS. In human cells this provides a co-operative inhibition, and it ducks cells, presumably compensates for the inability of NS1 proteins to achieve the same. The other possibility is that inhibiting RIG-I-CARD ubiquitination is not a common function of PB1-F2 proteins. It is interesting that this inhibition by PB1-F2 was discovered in a strain whose NS1 variant was unable to inhibit RIG-I-CARD ubiquitination in both human and duck cells. In Chapter 3, I hypothesize that this lack of function of NS1 is due to a polymorphism at position 55, and point out that residue K55, encoded in my PR8 NS1, is far more common in human and porcine NS1 proteins than in avian isolates. It is possible, therefore, that PR8 PB1-F2 evolved a mechanism that compensates for NS1 loss of function in that aspect, and perhaps this occurs in other NS1-K55-encoding strains as well. Thus, either inhibition of RIG-I-CARD ubiquitination is common to all PB1-F2 proteins that inhibit MAVS signaling, or it might be associated with the acquisition of K55 in the NS1

proteins of mammalian strains, or it might be totally independent. Investigating several other PB1-F2 proteins from different strains would allow us to differentiate between these models.

Insofar as these models represent reality, they raise some interesting evolutionary questions. For example, the fact that multiple different avian NS1 proteins inhibit RIG-I-CARD ubiquitination in human cells, but not in avian cells, suggests that duck TRIM25 may have evolved to escape this inhibition. This is interesting because viruses evolve much faster than their hosts. Has this adaptation in ducks occurred recently in evolutionary time? Is NS1 unable to evolve to counter this because of some constraints on its own evolution in this host, or is this escape from inhibition compensated by other viral immune-antagonistic mechanisms in duck cells, for example by PB1-F2? More broadly, is maximum possible innate immune inhibition not necessarily advantageous for co-evolutionary balance in the natural host? A theme that seems to emerge repeatedly in the literature is that avian influenza A virus strains are more potent inhibitors of innate immune signaling in human cells than human-adapted IAV strains. If NS1 residue K55 reduces RIG-I inhibition in human cells, why is this residue common in mammalian NS1 proteins? Finally, it is important to keep the null hypothesis in mind – that some or all of the results presented here are artifacts of the techniques and not necessarily representative of biological activities in actual infections. Until these results are corroborated by data from infection experiments, it is important to remember that these are speculative models.

Viral success in a host depends not only on interactions with host proteins, but also on the well-matched activities of its own proteins that must work together. A good example is the balancing of HA and NA activities that is required for efficient viral budding (de Vries et al., 2020), or ribonucleoprotein components, which interact with each other and acquire mutations at these interacting interfaces in new hosts (Chen et al., 2006; Finkelstein et al., 2007). The link

between individual viral protein functions and overall viral fitness or phenotype is imprecise and conditional. Each data chapter in this thesis investigated a different viral protein in isolation, making it difficult to combine all of the results into a single comparable dataset without an unreasonable amount of assumptions. Furthermore, host immune responses are complex and multilayered, and exhibit individual variation. Therefore, all of the findings in this thesis, and the potential findings from the future experiments that I suggested, exist in multiple hierarchically-nested layers of complexity. The relationship of ducks with IAV, sometimes described as "evolutionary stasis," is a dynamic equilibrium, where new changes may be mitigated or balanced by other changes, such that there is no single optimal adapted state, but multiple context-dependent ones. In general then, future studies should aim to couple phylogenetic analyses with functional protein work and *in vivo* studies to create a more comprehensive mosaic from which conclusions can be drawn.

## Bibliography


Abdelwhab, E.S.M., Veits, J., and Mettenleiter, T.C. (2013). Genetic changes that accompanied shifts of low pathogenic avian influenza viruses toward higher pathogenicity in poultry. Virulence *4*, 441-452.

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*, 647-662.

Allen, H., McCauley, J., Waterfield, M., and Gething, M.J. (1980). Influenza virus RNA segment 7 has the coding capacity for two polypeptides. Virology *107*, 548-551.

Allen, I.C., Scull, M.A., Moore, C.B., Holl, E.K., McElvania-TeKippe, E., Taxman, D.J., Guthrie, E.H., Pickles, R.J., and Ting, J.P.Y. (2009). The NLRP3 Inflammasome Mediates In Vivo Innate Immunity to Influenza A Virus through Recognition of Viral RNA. Immunity *30*, 556-565.

Alsharifi, M., Müllbacher, A., and Regner, M. (2008). Interferon type I responses in primary and secondary infections. Immunol Cell Biol *86*, 239-245.

Anhlan, D., Grundmann, N., Makalowski, W., Ludwig, S., and Scholtissek, C. (2011). Origin of the 1918 pandemic H1N1 influenza A virus as studied by codon usage patterns and phylogenetic analysis. Rna *17*, 64-73.

Aragón, T., Luna, S.d.l., Novoa, I., Carrasco, L., Ortín, J., and Nieto, A. (2000). Eukaryotic Translation Initiation Factor 4GI Is a Cellular Target for NS1 Protein, a Translational Activator of Influenza Virus. Mol Cell Biol.

Arai, Y., Kawashita, N., Daidoji, T., Ibrahim, M.S., El-Gendy, E.M., Takagi, T., Takahashi, K., Suzuki, Y., Ikuta, K., Nakaya, T.*, et al.* (2016). Novel Polymerase Gene Mutations for Human Adaptation in Clinical Isolates of Avian H5N1 Influenza Viruses. PLoS Pathog *12*, e1005583.

Arien, K.K., Troyer, R.M., Gali, Y., Colebunders, R.L., Arts, E.J., and Vanham, G. (2005). Replicative fitness of historical and recent HIV-1 isolates suggests HIV-1 attenuation over time. Aids *19*, 1555-1564.

Arimori, Y., Nakamura, R., Yamada, H., Shibata, K., Maeda, N., Kase, T., and Yoshikai, Y. (2013). Type I interferon limits influenza virus-induced acute lung injury by regulation of excessive inflammation in mice. Antiviral Res *99*, 230-237.

Arimoto, K.-i., Takahashi, H., Hishiki, T., Konishi, H., Fujita, T., and Shimotohno, K. (2007). Negative regulation of the RIG-I signaling by the ubiquitin ligase RNF125. PNAS *104*, 7500-7505.

Ayllon, J., and García-Sastre, A. (2015). The NS1 Protein: A Multitasking Virulence Factor. In Influenza Pathogenesis and Control - Volume II, M.B.A. Oldstone, and R.W. Compans, eds. (Cham: Springer International Publishing), pp. 73-107.

Baltimore, D. (1971). Expression of animal virus genomes. Bacteriol Rev *35*, 235-241.

Barber, M.R.W., Aldridge, J.R., Fleming-Canepa, X., Wang, Y.-D., Webster, R.G., and Magor, K.E. (2013). Identification of avian RIG-I responsive genes during influenza infection. Mol Immunol *54*, 89-97.

Barber, M.R.W., Aldridge, J.R., Webster, R.G., and Magor, K.E. (2010). Association of RIG-I with innate immunity of ducks to influenza. Proc Natl Acad Sci USA *107*, 5913-5918.

Baskin, C.R., Bielefeldt-Ohmann, H., Tumpey, T.M., Sabourin, P.J., Long, J.P., Garcia-Sastre, A., Tolnay, A.E., Albrecht, R., Pyles, J.A., Olson, P.H.*, et al.* (2009). Early and sustained innate immune response defines pathology and death in nonhuman primates infected by highly pathogenic influenza virus. Proc Natl Acad Sci U S A *106*, 3455-3460.

Bauernfeind, F.G., Horvath, G., Stutz, A., Alnemri, E.S., MacDonald, K., Speert, D., Fernandes-Alnemri, T., Wu, J., Monks, B.G., Fitzgerald, K.A.*, et al.* (2009). Cutting edge: NF-kappaB activating pattern recognition and cytokine receptors license NLRP3 inflammasome activation by regulating NLRP3 expression. J Immunol *183*, 787-791.

Baum, A., Sachidanandam, R., and García-Sastre, A. (2010). Preference of RIG-I for short viral RNA molecules in infected cells revealed by next-generation sequencing. PNAS *107*, 16303-16308.

Bean, W.J. (1984). Correlation of influenza A virus nucleoprotein genes with host species. Virology *133*, 438-442.

Beaton, A.R., and Krug, R.M. (1986). Transcription antitermination during influenza viral template RNA synthesis requires the nucleocapsid protein and the absence of a 5' capped end. Proc Natl Acad Sci U S A *83*, 6282-6286.

Bergsbaken, T., Fink, S.L., and Cookson, B.T. (2009). Pyroptosis: host cell death and inflammation. Nat Rev Microbiol *7*, 99-109.

Bertram, S., Glowacka, I., Steffen, I., Kühl, A., and Pöhlmann, S. (2010). Novel insights into proteolytic cleavage of influenza virus hemagglutinin. Reviews in Medical Virology *20*, 298-310.

Billiau, A. (2006). Anti-inflammatory properties of Type I interferons. Antiviral Res *71*, 108-116.

Bingham, J., Green, D.J., Lowther, S., Klippel, J., Burggraaf, S., Anderson, D.E., Wibawa, H., Hoa, D.M., Long, N.T., Vu, P.P.*, 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 Pathology *38*, 267-278.

Blaine, A.H., Miranzo-Navarro, D., Campbell, L.K., Aldridge, J.R., Webster, R.G., and Magor, K.E. (2015) Duck TRIM27-L enhances MAVS signaling and is absent in chickens and turkeys. Mol Immunol 67, 607-615.

Bornholdt, Z.A., and Prasad, B.V.V. (2008). X-ray structure of NS1 from a highly pathogenic H5N1 influenza virus. Nature *456*, 985-988.

Bouvier, N.M. (2015). Animal models for influenza virus transmission studies: A historical perspective. Curr Opin Virol *13*, 101-108.

Burggraaf, S., Karpala, A.J., Bingham, J., Lowther, S., Selleck, P., Kimpton, W., and Bean, A.G.D. (2014). H5N1 infection causes rapid mortality and high cytokine levels in chickens compared to ducks. Virus Res *185*, 23-31.

Cagle, C., To, T.L., Nguyen, T., Wasilenko, J., Adams, S.C., Cardona, C.J., Spackman, E., Suarez, D.L., and Pantin-Jackwood, M.J. (2011). Pekin and Muscovy ducks respond differently to vaccination with a H5N1 highly pathogenic avian influenza (HPAI) commercial inactivated vaccine. Vaccine *29*, 6549-6557.

Cagle, C., Wasilenko, J., Adams, S.C., Cardona, C.J., To, T.L., Nguyen, T., Spackman, E., Suarez, D.L., Smith, D., Shepherd, E.*, et al.* (2012). Differences in Pathogenicity, Response to Vaccination, and Innate Immune Responses in Different Types of Ducks Infected with a Virulent H5N1 Highly Pathogenic Avian Influenza Virus from Vietnam. Avian Diseases *56*, 479-487.

Cao, Y., Huang, Y., Xu, K., Liu, Y., Li, X., Xu, Y., Zhong, W., and Hao, P. (2017). Differential responses of innate immunity triggered by different subtypes of influenza a viruses in human and avian hosts. BMC Med Genomics *10*.

Carr, S.M., Carnero, E., Garcia-Sastre, A., Brownlee, G.G., and Fodor, E. (2006). Characterization of a mitochondrial-targeting signal in the PB2 protein of influenza viruses. Virology *344*, 492-508.

Carrillo, B., Choi, J.M., Bornholdt, Z.A., Sankaran, B., Rice, A.P., and Prasad, B.V.V. (2014). The Influenza A Virus Protein NS1 Displays Structural Polymorphism. J Virol *88*, 4113-4122.

Castanier, C., Zemirli, N., Portier, A., Garcin, D., Bidere, N., Vazquez, A., and Arnoult, D. (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.

CDC (Centers for Disease Control and Prevention) (2019). Types of Influenza Viruses. <https://www.cdc.gov/flu/about/viruses/types.htm> Accessed on January 20, 2020.

Chambers, T.M., Yamnikova, S., Kawaoka, Y., Lvov, D.K., and Webster, R.G. (1989). Antigenic and molecular characterization of subtype H13 hemagglutinin of influenza virus. Virology *172*, 180-188.

Chauché, C., Nogales, A., Zhu, H., Goldfarb, D., Ahmad Shanizza, A.I., Gu, Q., Parrish, C.R., Martínez-Sobrido, L., Marshall, J.F., and Murcia, P.R. (2018). Mammalian Adaptation of an Avian Influenza A Virus Involves Stepwise Changes in NS1. J Virol *92*.

Chen, G.W., Chang, S.C., Mok, C.K., Lo, Y.L., Kung, Y.N., Huang, J.H., Shih, Y.H., Wang, J.Y., Chiang, C., Chen, C.J*., et al.* (2006). Genomic signatures of human versus avian influenza A viruses. Emerg Infect Dis *12*, 1353-1360.

Chen, H., Smith, G.J.D., Zhang, S.Y., Qin, K., Wang, J., Li, K.S., Webster, R.G., Peiris, J.S.M., and Guan, Y. (2005). Avian flu: H5N1 virus outbreak in migratory waterfowl. Nature *436*, 191-192.

Chen, R., and Holmes, E.C. (2006). Avian influenza virus exhibits rapid evolutionary dynamics. Mol Biol Evol *23*, 2336-2341.

Chen, S., Cheng, A., and Wang, M. (2013). Innate sensing of viruses by pattern recognition receptors in birds. Vet Res *44*, 82.

Chen, S.N., Zou, P.F., and Nie, P. (2017). Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) in fish: current knowledge and future perspectives. Immunology *151*, 16-25.

Chen, W., Calvo, P.A., Malide, D., Gibbs, J., Schubert, U., Bacik, I., Basta, S., O'Neill, R., Schickli, J., Palese, P*., et al.* (2001). A novel influenza A virus mitochondrial protein that induces cell death. Nat Med *7*, 1306-1312.

Chen, Y., Xu, Q., Li, Y., Liu, R., Huang, Z., Wang, B., and Chen, G. (2016). Gene expression profile after activation of RIG-I in 5'ppp-dsRNA challenged DF1. Developmental & Comparative Immunology *65*, 191-200.

Cheng, Y., Huang, Q., Ji, W., Du, B., Fu, Q., An, H., Li, J., Wang, H., Yan, Y., Ding, C.*, 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*, 183-193.

Cheung, C.Y., Poon, L.L.M., Lau, A.S., Luk, W., Lau, Y.L., Shortridge, K.F., Gordon, S., Guan, Y., and Peiris, J.S.M. (2002). Induction of proinflammatory cytokines in human macrophages by influenza A (H5N1) viruses: a mechanism for the unusual severity of human disease? Lancet *360*, 1831-1837.

Childs, K., Stock, N., Ross, C., Andrejeva, J., Hilton, L., Skinner, M., Randall, R., and Goodbourn, S. (2007). mda-5, but not RIG-I, is a common target for paramyxovirus V proteins. Virology *359*, 190-200.

Claes, G., Welby, S., Van Den Berg, T., Van Der Stede, Y., Dewulf, J., Lambrecht, B., and Marche, S. (2013). The impact of viral tropism and housing conditions on the transmission of three H5/H7 low pathogenic avian influenza viruses in chickens. Epidemiol Infect *141*, 2428-2443.

Clingan, J.M., and Matloubian, M. (2013). B cell-intrinsic TLR7 signaling is required for optimal B cell responses during chronic viral infection. J Immunol *191*, 810-818.

Colman, P.M., Varghese, J.N., and Laver, W.G. (1983). Structure of the catalytic and antigenic sites in influenza virus neuraminidase. Nature *303*, 41-44.

Colonna, M., Trinchieri, G., and Liu, Y.-J. (2004). Plasmacytoid dendritic cells in immunity. Nat Immunol *5*, 1219-1226.

Compans, R.W., Content, J., and Duesberg, P.H. (1972). Structure of the ribonucleoprotein of influenza virus. J Virol *10*, 795-800.

Condobery, P.K., and Slemons, R.D. (1992). Biological properties of waterfowl-origin type A influenza viruses in chickens. Avian Dis *36*, 17-23.

Conenello, G.M., Zamarin, D., Perrone, L.A., Tumpey, T., and Palese, P. (2007). A single mutation in the PB1-F2 of H5N1 (HK/97) and 1918 influenza A viruses contributes to increased virulence. PLoS Pathog *3*, 1414-1421.

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*, 1-5.

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*, 519-529.

Costa, T., Chaves, A.J., Valle, R., Darji, A., van Riel, D., Kuiken, T., Majó, N., and Ramis, A. (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.

Costa, T.P., Brown, J.D., Howerth, E.W., and Stallknecht, D.E. (2011). Variation in viral shedding patterns between different wild bird species infected experimentally with low-pathogenicity avian influenza viruses that originated from wild birds. Avian Pathology *40*, 119-124.

Cros, J.F., Garcia-Sastre, A., and Palese, P. (2005). An unconventional NLS is critical for the nuclear import of the influenza A virus nucleoprotein and ribonucleoprotein. Traffic *6*, 205-213.

Cui, X.-F., Imaizumi, T., Yoshida, H., Borden, E.C., and Satoh, K. (2004). Retinoic acid-inducible gene-I is induced by interferon-gamma and regulates the expression of interferon-gamma stimulated gene 15 in MCF-7 cells. Biochem Cell Biol *82*, 401-405.

D'Cruz, A.A., Kershaw, N.J., Chiang, J.J., Wang, M.K., Nicola, N.A., Babon, J.J., Gack, M.U., and Nicholson, S.E. (2013). Crystal structure of the TRIM25 B30.2 (PRYSPRY) domain: a key component of antiviral signalling. Biochem J *456*, 231-240.

Damjanovic, D., Small, C.L., Jeyanathan, M., McCormick, S., and Xing, Z. (2012). Immunopathology in influenza virus infection: uncoupling the friend from foe. Clin Immunol *144*, 57-69.

Dankar, S.K., Miranda, E., Forbes, N.E., Pelchat, M., Tavassoli, A., Selman, M., Ping, J., Jia, J., and Brown, E.G. (2013). Influenza A/Hong Kong/156/1997(H5N1) virus NS1 gene mutations F103L and M106I both increase IFN antagonism, virulence and cytoplasmic localization but differ in binding to RIG-I and CPSF30. Virol J *10*, 243.

Dankar, S.K., Wang, S., Ping, J., Forbes, N.E., Keleta, L., Li, Y., and Brown, E.G. (2011). Influenza A virus NS1 gene mutations F103L and M106I increase replication and virulence. Virol J *8*, 13.

Daoust, P.-Y., Kibenge, F.S.B., Fouchier, R.A.M., van de Bildt, M.W.G., 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*, 401-409.

Daoust, P.Y., van de Bildt, M., van Riel, D., van Amerongen, G., Bestebroer, T., Vanderstichel, R., Fouchier, R.A.M., and Kuiken, T. (2013). Replication of 2 Subtypes of Low-Pathogenicity Avian Influenza Virus of Duck and Gull Origins in Experimentally Infected Mallard Ducks. Vet Pathol *50*, 548-559.

Das, K., Ma, L.-C., Xiao, R., Radvansky, B., Aramini, J., Zhao, L., Marklund, J., Kuo, R.-L., Twu, K.Y., Arnold, E.*, et al.* (2008). Structural basis for suppression of a host antiviral response by influenza A virus. Proc Natl Acad Sci USA *105*, 13093-13098.

de Vries, E., Du, W., Guo, H., and de Haan, C.A.M. (2020). Influenza A Virus Hemagglutinin-Neuraminidase-Receptor Balance: Preserving Virus Motility. Trends Microbiol *28*, 57-67.

Dias, A., Bouvier, D., Crepin, T., McCarthy, A.A., Hart, D.J., Baudin, F., Cusack, S., and Ruigrok, R.W. (2009). The cap-snatching endonuclease of influenza virus polymerase resides in the PA subunit. Nature *458*, 914-918.

Diebold, S.S., Kaisho, T., Hemmi, H., Akira, S. and e Sousa, C.R. (2004). Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science *303*, 1529-1531.

Donelan, N.R., Basler, C.F., and Garcia-Sastre, A. (2003). A recombinant influenza A virus expressing an RNA-binding-defective NS1 protein induces high levels of beta interferon and is attenuated in mice. J Virol *77*, 13257-13266.

Du, J., Zhang, D., Zhang, W., Ouyang, G., Wang, J., Liu, X., Li, S., Ji, W., Liu, W., and Xiao, W. (2015). pVHL Negatively Regulates Antiviral Signaling by Targeting MAVS for Proteasomal Degradation. J Immunol *195*, 1782-1790.

Dudek, S.E., Wixler, L., Nordhoff, C., Nordmann, A., Anhlan, D., Wixler, V., and Ludwig, S. (2011). The influenza virus PB1-F2 protein has interferon antagonistic activity. Biol Chem *392*, 1135-1144.

Duesberg, P.H. (1969). Distinct subunits of the ribonucleoprotein of influenza virus. J Mol Biol *42*, 485-499.

Evseev, D., and Magor, K.E. (2019). Innate Immune Responses to Avian Influenza Viruses in Ducks and Chickens. Veterinary Sciences *6*, 5.

Fan, X., and Jin, T. (2019). Structures of RIG-I-Like Receptors and Insights into Viral RNA Sensing. Adv Exp Med Biol *1172*, 157-188.

Fanning, A.S., and Anderson, J.M. (1996). Protein-protein interactions: PDZ domain networks. Curr Biol *6*, 1385-1388.

Fechter, P., Mingay, L., Sharps, J., Chambers, A., Fodor, E., and Brownlee, G.G. (2003). Two aromatic residues in the PB2 subunit of influenza A RNA polymerase are crucial for cap binding. J Biol Chem *278*, 20381-20388.

Feldman, S., Stein, D., Amrute, S., Denny, T., Garcia, Z., Kloser, P., Sun, Y., Megjugorac, N., and Fitzgerald-Bocarsly, P. (2001). Decreased Interferon-α Production in HIV-Infected

Patients Correlates with Numerical and Functional Deficiencies in Circulating Type 2 Dendritic Cell Precursors. Clinical Immunology *101*, 201-210.

Feldman, S.B., Milone, M.C., Kloser, P., and Fitzgerald-Bocarsly, P. (1995). Functional deficiencies in two distinct interferon alpha-producing cell populations in peripheral blood mononuclear cells from human immunodeficiency virus seropositive patients. J Leukoc Biol *57*, 214-220.

Fernandes-Alnemri, T., Wu, J., Yu, J.W., Datta, P., Miller, B., Jankowski, W., Rosenberg, S., Zhang, J., and Alnemri, E.S. (2007). The pyroptosome: a supramolecular assembly of ASC dimers mediating inflammatory cell death via caspase-1 activation. Cell Death Differ *14*, 1590-1604.

Finkelstein, D.B., Mukatira, S., Mehta, P.K., Obenauer, J.C., Su, X., Webster, R.G., and Naeve, C.W. (2007). Persistent host markers in pandemic and H5N1 influenza viruses. J Virol *81*, 10292-10299.

Finter, N.B. (1966). Interferon as an antiviral agent in vivo: quantitative and temporal aspects of the protection of mice against Semliki Forest virus. Br J Exp Pathol *47*, 361-371.

Finter, N.B. (1967). Interferon in mice: protection against small doses of virus. J Gen Virol *1*, 395-397.

Fleming-Canepa, X., Aldridge, J.R., Canniff, L., Kobewka, M., Jax, E., Webster, R.G., and Magor, K.E. (2019). Duck innate immune responses to high and low pathogenicity H5 avian influenza viruses. Vet Microbiol *228*, 101-111.

Food and Agriculture Organization of the United Nations (2018). Approaches to controlling, preventing and eliminating H5N1 Highly Pathogenic Avian Influenza in endemic countries. <http://www.fao.org/docrep/014/i2150e/i2150e00.htm> Accessed on January 15, 2020.

França, M., Stallknecht, D.E., Poulson, R., Brown, J., and Howerth, E.W. (2012). The Pathogenesis of Low Pathogenic Avian Influenza in Mallards. Avian Diseases *56*, 976-980.

Furuse, Y., Suzuki, A., Kamigaki, T., and Oshitani, H. (2009). Evolution of the M gene of the influenza A virus in different host species: large-scale sequence analysis. Virol J *6*, 67.

Gack, M.U., Albrecht, R.A., Urano, T., Inn, K.-S., Huang, I.C., Carnero, E., Farzan, M., Inoue, S., Jung, J.U., and García-Sastre, A. (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*, 439-449.

Gack, M.U., Kirchhofer, A., Shin, Y.C., Inn, K.S., Liang, C., Cui, S., Myong, S., Ha, T., Hopfner, K.P., and Jung, J.U. (2008). Roles of RIG-I N-terminal tandem CARD and splice variant in TRIM25-mediated antiviral signal transduction. Proc Natl Acad Sci U S A *105*, 16743-16748.

Gack, M.U., Shin, Y.C., Joo, C.-H., Urano, T., Liang, C., Sun, L., Takeuchi, O., Akira, S., Chen, Z., Inoue, S.*, et al.* (2007). TRIM25 RING-finger E3 ubiquitin ligase is essential for RIG-I-mediated antiviral activity. Nature *446*, 916-920.

Gammelin, M., Altmuller, A., Reinhardt, U., Mandler, J., Harley, V.R., Hudson, P.J., Fitch, W.M., and Scholtissek, C. (1990). Phylogenetic analysis of nucleoproteins suggests that human influenza A viruses emerged from a 19th-century avian ancestor. Mol Biol Evol *7*, 194-200.

Gao, D., Yang, Y.K., Wang, R.P., Zhou, X., Diao, F.C., Li, M.D., Zhai, Z.H., Jiang, Z.F., and Chen, D.Y. (2009). REUL is a novel E3 ubiquitin ligase and stimulator of retinoic-acid-inducible gene-I. PLoS ONE *4*, e5760.

Gao, P., Xiang, B., Li, Y., Li, Y., Sun, M., Kang, Y., Xie, P., Chen, L., Lin, Q., Liao, M.*, et al.* (2018). Therapeutic Effect of Duck Interferon-Alpha Against H5N1 Highly Pathogenic Avian Influenza Virus Infection in Peking Ducks. Journal of Interferon & Cytokine Research *38*, 145-152.

Garaigorta, U., Falcon, A.M., and Ortin, J. (2005). Genetic analysis of influenza virus NS1 gene: a temperature-sensitive mutant shows defective formation of virus particles. J Virol *79*, 15246-15257.

García-Sastre, A., Egorov, A., Matassov, D., Brandt, S., Levy, D.E., Durbin, J.E., Palese, P., and Muster, T. (1998). Influenza A virus lacking the NS1 gene replicates in interferon-deficient systems. Virology *252*, 324-330.

Germundsson, A., Madslien, K.I., Hjortaas, M.J., Handeland, K., and Jonassen, C.M. (2010). Prevalence and subtypes of influenza A viruses in wild waterfowl in Norway 2006-2007. Acta Vet Scand *52*, 28.

Glezen, P.W., Schmier, J.K., Kuehn, C.M., Ryan, K.J., and Oxford, J. (2013). The burden of influenza B: a structured literature review. Am J Public Health *103*, e43-51.

Gobl, A.E., Funa, K., and Alm, G.V. (1988). Different induction patterns of mRNA for IFN-alpha and -beta in human mononuclear leukocytes after in vitro stimulation with herpes simplex virus-infected fibroblasts and Sendai virus. J Immunol *140*, 3605-3609.

Golebiewski, L., Liu, H., Javier, R.T., and Rice, A.P. (2011). The Avian Influenza Virus NS1 ESEV PDZ Binding Motif Associates with Dlg1 and Scribble To Disrupt Cellular Tight Junctions. J Virol *85*, 10639-10648.

Gorman, O.T., Bean, W.J., Kawaoka, Y., Donatelli, I., Guo, Y.J., and Webster, R.G. (1991). Evolution of influenza A virus nucleoprotein genes: implications for the origins of H1N1 human and classical swine viruses. J Virol *65*, 3704-3714.

Gorman, O.T., Bean, W.J., Kawaoka, Y., and Webster, R.G. (1990a). Evolution of the nucleoprotein gene of influenza A virus. J Virol *64*, 1487-1497.

Gorman, O.T., Donis, R.O., Kawaoka, Y., and Webster, R.G. (1990b). Evolution of influenza A virus PB2 genes: implications for evolution of the ribonucleoprotein complex and origin of human influenza A virus. J Virol *64*, 4893-4902.

Govorkova, E.A., Rehg, J.E., Krauss, S., Yen, H.-L., Guan, Y., Peiris, M., Nguyen, T.D., Hanh, T.H., Puthavathana, P., Long, H.T.*, et al.* (2005). Lethality to ferrets of H5N1 influenza viruses isolated from humans and poultry in 2004. J Virol *79*, 2191-2198.

Graef, K.M., Vreede, F.T., Lau, Y.F., McCall, A.W., Carr, S.M., Subbarao, K., and Fodor, E. (2010). The PB2 subunit of the influenza virus RNA polymerase affects virulence by interacting with the mitochondrial antiviral signaling protein and inhibiting expression of beta interferon. J Virol *84*, 8433-8445.

Greenspan, D., Palese, P., and Krystal, M. (1988). Two nuclear location signals in the influenza virus NS1 nonstructural protein. J Virol *62*, 3020-3026.

Gresser, I., Tovey, M.G., Bandu, M.E., Maury, C., and Brouty-Boye, D. (1976a). Role of interferon in the pathogenesis of virus diseases in mice as demonstrated by the use of anti-interferon serum. I. Rapid evolution of encephalomyocarditis virus infection. J Exp Med *144*, 1305-1315.

Gresser, I., Tovey, M.G., Maury, C., and Bandu, M.T. (1976b). Role of interferon in the pathogenesis of virus diseases in mice as demonstrated by the use of anti-interferon serum. II. Studies with herpes simplex, Moloney sarcoma, vesicular stomatitis, Newcastle disease, and influenza viruses. J Exp Med *144*, 1316-1323.

WHO/OIE/FAO H5N1 Evolution Working Group. (2012). Continued evolution of highly pathogenic avian influenza A (H5N1): updated nomenclature. Influenza Other Respir Viruses *6*, 1-5.

Guan, J., Fu, Q., Chan, M., and Spencer, J.L. (2013). Aerosol transmission of an avian influenza H9N2 virus with a tropism for the respiratory tract of chickens. Avian Dis *57*, 645-649.

Guan, Y., Poon, L.L., Cheung, C.Y., Ellis, T.M., Lim, W., Lipatov, A.S., Chan, K.H., Sturm-Ramirez, K.M., Cheung, C.L., Leung, Y.H.*, et al.* (2004). H5N1 influenza: a protean pandemic threat. Proc Natl Acad Sci U S A *101*, 8156-8161.

Guan, Y., Vijaykrishna, D., Bahl, J., Zhu, H., Wang, J., and Smith, G.J. (2010). The emergence of pandemic influenza viruses. Protein Cell *1*, 9-13.

Guarda, G., Braun, M., Staehli, F., Tardivel, A., Mattmann, C., Förster, I., Farlik, M., Decker, T., Du Pasquier, Renaud A., Romero, P.*, et al.* (2011). Type I Interferon Inhibits Interleukin-1 Production and Inflammasome Activation. Immunity *34*, 213-223.

Guo, Y., Wang, M., Kawaoka, Y., Gorman, O., Ito, T., Saito, T., and Webster, R.G. (1992). Characterization of a new avian-like influenza A virus from horses in China. Virology *188*, 245-255.

Hagau, N., Slavcovici, A., Gonganau, D.N., Oltean, S., Dirzu, D.S., Brezoszki, E.S., Maxim, M., Ciuce, C., Mlesnite, M., Gavrus, R.L.*, et al.* (2010). Clinical aspects and cytokine response in severe H1N1 influenza A virus infection. Crit Care *14*, R203.

Hale, B.G., Kerry, P.S., Jackson, D., Precious, B.L., Gray, A., Killip, M.J., Randall, R.E., and Russell, R.J. (2010a). Structural insights into phosphoinositide 3-kinase activation by the influenza A virus NS1 protein. Proc Natl Acad Sci U S A *107*, 1954-1959.

Hale, B.G., Knebel, A., Botting, C.H., Galloway, C.S., Precious, B.L., Jackson, D., Elliott, R.M., and Randall, R.E. (2009). CDK/ERK-mediated phosphorylation of the human influenza A virus NS1 protein at threonine-215. Virology *383*, 6-11.

Hale, B.G., Randall, R.E., Ortín, J., and Jackson, D. (2008). The multifunctional NS1 protein of influenza A viruses. J Gen Virol *89*, 2359-2376.

Hale, B.G., Steel, J., Medina, R.A., Manicassamy, B., Ye, J., Hickman, D., Hai, R., Schmolke, M., Lowen, A.C., Perez, D.R.*, et al.* (2010b). Inefficient control of host gene expression by the 2009 pandemic H1N1 influenza A virus NS1 protein. J Virol *84*, 6909-6922.

Haller, O., Acklin, M., and Staeheli, P. (1987). Influenza Virus Resistance of Wild Mice: Wild-Type and Mutant Mx Alleles Occur at Comparable Frequencies. Journal of Interferon Research *7*, 647-656.

Haller, O., Arnheiter, H., Gresser, I., and Lindenmann, J. (1981). Virus-specific interferon action. Protection of newborn Mx carriers against lethal infection with influenza virus. J Exp Med *154*, 199-203.

Hammerbeck, D.M., Burleson, G.R., Schuller, C.J., Vasilakos, J.P., Tomai, M., Egging, E., Cochran, F.R., Woulfe, S., and Miller, R.L. (2007). Administration of a dual toll-like receptor 7 and toll-like receptor 8 agonist protects against influenza in rats. Antiviral Res *73*, 1-11.

Han, H., Cui, Z.-Q., Wang, W., Zhang, Z.-P., Wei, H.-P., Zhou, Y.-F., and Zhang, X.-E. (2010). New regulatory mechanisms for the intracellular localization and trafficking of influenza A virus NS1 protein revealed by comparative analysis of A/PR/8/34 and A/Sydney/5/97. Journal of General Virology *91*, 2907-2917.

Hause, B.M., Collin, E.A., Liu, R., Huang, B., Sheng, Z., Lu, W., Wang, D., Nelson, E.A., and Li, F. (2014). Characterization of a novel influenza virus in cattle and Swine: proposal for a new genus in the Orthomyxoviridae family. mBio *5*, e00031-00014.

Hessen, M.T. (2009). In the clinic. Influenza. Ann Intern Med *151*, ICT5-1-ICT5-15; quiz ICT15-16.

Hinshaw, V.S., Webster, R.G., and Turner, B. (1980). The perpetuation of orthomyxoviruses and paramyxoviruses in Canadian waterfowl. Can J Microbiol *26*, 622-629.

Hoffmann, E., Stech, J., Guan, Y., Webster, R.G., and Perez, D.R. (2001). Universal primer set for the full-length amplification of all influenza A viruses. Arch Virol *146*, 2275-2289.

Horimoto, T., and Kawaoka, Y. (1994). Reverse genetics provides direct evidence for a correlation of hemagglutinin cleavability and virulence of an avian influenza A virus. J Virol *68*, 3120-3128.

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*, 448-461.

Huang, X., Liu, T., Muller, J., Levandowski, R.A., and Ye, Z. (2001). Effect of influenza virus matrix protein and viral RNA on ribonucleoprotein formation and nuclear export. Virology *287*, 405-416.

Hughey, P.G., Compans, R.W., Zebedee, S.L., and Lamb, R.A. (1992). Expression of the influenza A virus M2 protein is restricted to apical surfaces of polarized epithelial cells. J Virol *66*, 5542-5552.

Hulse-Post, D.J., Sturm-Ramirez, K.M., Humberd, J., Seiler, P., Govorkova, E.A., Krauss, S., Scholtissek, C., Puthavathana, P., Buranathai, C., Nguyen, T.D*., et al.* (2005). Role of domestic ducks in the propagation and biological evolution of highly pathogenic H5N1 influenza viruses in Asia. Proc Natl Acad Sci USA *102*, 10682-10687.

Hussein, I.T., Ma, E.J., Hill, N.J., Meixell, B.W., Lindberg, M., Albrecht, R.A., Bahl, J., and Runstadler, J.A. (2016). A point mutation in the polymerase protein PB2 allows a reassortant H9N2 influenza isolate of wild-bird origin to replicate in human cells. Infect Genet Evol *41*, 279-288.

Ichinohe, T., Pang, I.K., and Iwasaki, A. (2010). Influenza virus activates inflammasomes via its intracellular M2 ion channel. Nat Immunol *11*, 404-410.

Imai, Y., Kuba, K., Neely, G.G., Yaghubian-Malhami, R., Perkmann, T., van Loo, G., Ermolaeva, M., Veldhuizen, R., Leung, Y.H.C., Wang, H*., et al.* (2008). Identification of Oxidative Stress and Toll-like Receptor 4 Signaling as a Key Pathway of Acute Lung Injury. Cell *133*, 235-249.

Imaizumi, T., Aratani, S., Nakajima, T., Carlson, M., Matsumiya, T., Tanji, K., Ookawa, K., Yoshida, H., Tsuchida, S., McIntyre, T.M*., et al.* (2002). Retinoic acid-inducible gene-I is induced in endothelial cells by LPS and regulates expression of COX-2. Biochem Biophys Res Commun *292*, 274-279.

Imaizumi, T., Hatakeyama, M., Yamashita, K., Yoshida, H., Ishikawa, A., Taima, K., Satoh, K., Mori, F., and Wakabayashi, K. (2004). Interferon-gamma induces retinoic acid-inducible gene-I in endothelial cells. Endothelium *11*, 169-173.

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. Vet Immunol Immunopathol *104*, 117-127.

Ito, T., Gorman, O.T., Kawaoka, Y., Bean, W.J., and Webster, R.G. (1991). Evolutionary analysis of the influenza A virus M gene with comparison of the M1 and M2 proteins. J Virol *65*, 5491-5498.

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 Immun *26*, 221-229.

Iwai, A., Shiozaki, T., Kawai, T., Akira, S., Kawaoka, Y., Takada, A., Kida, H., and Miyazaki, T. (2010). Influenza A virus polymerase inhibits type I interferon induction by binding to interferon beta promoter stimulator 1. J Biol Chem *285*, 32064-32074.

Iwasaki, A., and Pillai, P.S. (2014). Innate immunity to influenza virus infection. Nat Rev Immunol *14*, 315-328.

Jackson, D., Hossain, M.J., Hickman, D., Perez, D.R., and Lamb, R.A. (2008). A new influenza virus virulence determinant: The NS1 protein four C-terminal residues modulate pathogenicity. PNAS *105*, 4381-4386.

Jagger, B.W., Wise, H.M., Kash, J.C., Walters, K.A., Wills, N.M., Xiao, Y.L., Dunfee, R.L., Schwartzman, L.M., Ozinsky, A., Bell, G.L.*, et al.* (2012). An overlapping protein-coding region in influenza A virus segment 3 modulates the host response. Science *337*, 199-204.

James, J., Howard, W., Iqbal, M., Nair, V.K., Barclay, W.S., and Shelton, H. (2016a). Influenza A virus PB1-F2 protein prolongs viral shedding in chickens lengthening the transmission window. The Journal of General Virology *97*, 2516-2527.

James, J., Howard, W., Iqbal, M., Nair, V.K., Barclay, W.S., and Shelton, H. (2016b). Influenza A virus PB1-F2 protein prolongs viral shedding in chickens lengthening the transmission window. J Gen Virol *97*, 2516-2527.

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*, 447.

Jiao, P., Tian, G., Li, Y., Deng, G., Jiang, Y., Liu, C., Liu, W., Bu, Z., Kawaoka, Y., and Chen, H. (2008). A Single-Amino-Acid Substitution in the NS1 Protein Changes the Pathogenicity of H5N1 Avian Influenza Viruses in Mice. J Virol *82*, 1146-1154.

Jiao, P.R., Wei, L.M., Cheng, Y.Q., Yuan, R.Y., Han, F., Liang, J., Liu, W.L., Ren, T., Xin, C.A., and Liao, M. (2012). Molecular cloning, characterization, and expression analysis of the Muscovy duck Toll-like receptor 3 (MdTLR3) gene. Poult Sci *91*, 2475-2481.

Jones, I.M., Reay, P.A., and Philpott, K.L. (1986). Nuclear location of all three influenza polymerase proteins and a nuclear signal in polymerase PB2. EMBO J *5*, 2371-2376.

Jones, L.V., Compans, R.W., Davis, A.R., Bos, T.J., and Nayak, D.P. (1985). Surface expression of influenza virus neuraminidase, an amino-terminally anchored viral membrane glycoprotein, in polarized epithelial cells. Mol Cell Biol *5*, 2181-2189.

Jørgensen, S.E., Christiansen, M., Ryø, L.B., Gad, H.H., Gjedsted, J., Staeheli, P., Mikkelsen, J.G., Storgaard, M., Hartmann, R., and Mogensen, T.H. (2018). Defective RNA sensing by RIG-I in severe influenza virus infection. Clinical & Experimental Immunology *192*, 366-376.

Jourdain, E., Gunnarsson, G., Wahlgren, J., Latorre-Margalef, N., Bröjer, C., Sahlin, S., Svensson, L., Waldenström, J., Lundkvist, Å., and Olsen, B. (2010). Influenza Virus in a Natural Host, the Mallard: Experimental Infection Data. PLoS ONE *5*.

Jureka, A.S., Kleinpeter, A.B., Cornilescu, G., Cornilescu, C.C., and Petit, C.M. (2015). Structural Basis for a Novel Interaction between the NS1 Protein Derived from the 1918 Influenza Virus and RIG-I. Structure *23*, 2001-2010.

Kallfass, C., Lienenklaus, S., Weiss, S., and Staeheli, P. (2013). Visualizing the Beta Interferon Response in Mice during Infection with Influenza A Viruses Expressing or Lacking Nonstructural Protein 1. J Virol *87*, 6925-6930.

Kamal, R.P., Alymova, I.V., and York, I.A. (2017). Evolution and Virulence of Influenza A Virus Protein PB1-F2. Int J Mol Sci *19*.

Kamitani, T., Kito, K., Nguyen, H.P., and Yeh, E.T. (1997). Characterization of NEDD8, a developmentally down-regulated ubiquitin-like protein. J Biol Chem *272*, 28557-28562.

Kankkunen, P., Teirila, L., Rintahaka, J., Alenius, H., Wolff, H., and Matikainen, S. (2010). (1,3)-beta-glucans activate both dectin-1 and NLRP3 inflammasome in human macrophages. J Immunol *184*, 6335-6342.

Kanneganti, T.D., Body-Malapel, M., Amer, A., Park, J.H., Whitfield, J., Franchi, L., Taraporewala, Z.F., Miller, D., Patton, J.T., Inohara, N.*, et al.* (2006). Critical role for Cryopyrin/Nalp3 in activation of caspase-1 in response to viral infection and double-stranded RNA. J Biol Chem *281*, 36560-36568.

Karpala, A.J., Lowenthal, J.W., and Bean, A.G. (2008). Activation of the TLR3 pathway regulates IFNβ production in chickens. Developmental & Comparative Immunology *32*, 435-444.

Kato, H., Takeuchi, O., Sato, S., Yoneyama, M., Yamamoto, M., Matsui, K., Uematsu, S., Jung, A., Kawai, T., Ishii, K.J.*, et al.* (2006). Differential roles of MDA5 and RIG-I helicases in the recognition of RNA viruses. Nature *441*, 101-105.

Kawaoka, Y., Chambers, T.M., Sladen, W.L., and Webster, R.G. (1988). Is the gene pool of influenza viruses in shorebirds and gulls different from that in wild ducks? Virology *163*, 247-250.

Kawaoka, Y., Krauss, S., and Webster, R.G. (1989). Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. J Virol *63*, 4603-4608.

Khaperskyy, D.A., Emara, M.M., Johnston, B.P., Anderson, P., Hatchette, T.F., and McCormick, C. (2014). Influenza a virus host shutoff disables antiviral stress-induced translation arrest. PLoS Pathog *10*, e1004217.

Khaperskyy, D.A., and McCormick, C. (2015). Timing Is Everything: Coordinated Control of Host Shutoff by Influenza A Virus NS1 and PA-X Proteins. J Virol *89*, 6528-6531.

Khatchikian, D., Orlich, M., and Rott, R. (1989). Increased viral pathogenicity after insertion of a 28S ribosomal RNA sequence into the haemagglutinin gene of an influenza virus. Nature *340*, 156-157.

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

Kilbourne, E.D. (1969). Future influenza vaccines and the use of genetic recombinants. Bull World Health Organ *41*, 643-645.

Kilpatrick, A.M., Chmura, A.A., Gibbons, D.W., Fleischer, R.C., Marra, P.P., and Daszak, P. (2006). Predicting the global spread of H5N1 avian influenza. Proc Natl Acad Sci USA *103*, 19368-19373.

Kim, J.-K., Negovetich, N.J., Forrest, H.L., and Webster, R.G. (2009). Ducks: the "Trojan horses" of H5N1 influenza. Influenza Other Respir Viruses *3*, 121-128.

Kim, J.-K., Seiler, P., Forrest, H.L., Khalenkov, A.M., Franks, J., Kumar, M., Karesh, W.B., Gilbert, M., Sodnomdarjaa, R., Douangngeun, B.*, et al.* (2008). Pathogenicity and Vaccine Efficacy of Different Clades of Asian H5N1 Avian Influenza A Viruses in Domestic Ducks. J Virol *82*, 11374-11382.

Kim, J.H., Hatta, M., Watanabe, S., Neumann, G., Watanabe, T., and Kawaoka, Y. (2010). Role of host-specific amino acids in the pathogenicity of avian H5N1 influenza viruses in mice. J Gen Virol *91*, 1284-1289.

King, A.M., Lefkowitz, E., Adams, M.J., and Carstens, E.B. (2011). Virus taxonomy: ninth report of the International Committee on Taxonomy of Viruses, Vol 9 (Elsevier).

Kleyheeg, E., Slaterus, R., Bodewes, R., Rijks, J.M., Spierenburg, M.A.H., Beerens, N., Kelder, L., Poen, M.J., Stegeman, J.A., Fouchier, R.A.M.*, et al.* (2017). Deaths among Wild Birds during Highly Pathogenic Avian Influenza A(H5N8) Virus Outbreak, the Netherlands. Emerging Infect Dis *23*, 2050-2054.

Kochs, G., Koerner, I., Thiel, L., Kothlow, S., Kaspers, B., Ruggli, N., Summerfield, A., Pavlovic, J., Stech, J., and Staeheli, P. (2007). Properties of H7N7 influenza A virus strain SC35M lacking interferon antagonist NS1 in mice and chickens. Journal of General Virology *88*, 1403-1409.

Koerner, I., Kochs, G., Kalinke, U., Weiss, S., and Staeheli, P. (2007). Protective role of beta interferon in host defense against influenza A virus. J Virol *81*, 2025-2030.

Koliopoulos, M.G., Lethier, M., van der Veen, A.G., Haubrich, K., Hennig, J., Kowalinski, E., Stevens, R.V., Martin, S.R., Reis e Sousa, C., Cusack, S.*, et al.* (2018). Molecular mechanism of influenza A NS1-mediated TRIM25 recognition and inhibition. Nature Communications *9*.

Koshiba, T., Yasukawa, K., Yanagi, Y., and Kawabata, S. (2011). Mitochondrial membrane potential is required for MAVS-mediated antiviral signaling. Sci Signal *4*, ra7.

Kosik, I., Holly, J., and Russ, G. (2013). PB1-F2 expedition from the whole protein through the domain to aa residue function. Acta Virol *57*, 138-148.

Krishna, V.D., Roach, E., Zaidman, N.A., Panoskaltsis-Mortari, A., Rotschafer, J.H., O'Grady, S.M., and Cheeran, M.C.J. (2015). Differential Induction of Type I and Type III Interferons by Swine and Human Origin H1N1 Influenza A Viruses in Porcine Airway Epithelial Cells. PLoS ONE *10*.

Krug, R.M. (2014). Viral proteins that bind double-stranded RNA: countermeasures against host antiviral responses. J Interferon Cytokine Res *34*, 464-468.

Krumbholz, A., Philipps, A., Oehring, H., Schwarzer, K., Eitner, A., Wutzler, P., and Zell, R. (2011). Current knowledge on PB1-F2 of influenza A viruses. Med Microbiol Immunol *200*, 69-75.

Kumar, A., Vijayakumar, P., Gandhale, P.N., Ranaware, P.B., Kumar, H., Kulkarni, D.D., Raut, A.A., and Mishra, A. (2017). Genome-wide gene expression pattern underlying differential host response to high or low pathogenic H5N1 avian influenza virus in ducks. Acta Virol *61*, 66-76.

Kumar, M., Liu, H., and Rice, A.P. (2012). Regulation of Interferon-β by MAGI-1 and Its Interaction with Influenza A Virus NS1 Protein with ESEV PBM. PLoS ONE *7*.

Kumar, R., Halder, P., and Poddar, R. (2006). Adaptive molecular evolution of virulence genes of avian influenza - A virus subtype H5N1: An analysis of host radiation. Bioinformation *1*, 321-326.

Kuo, R.-L., and Krug, R.M. (2009). Influenza a virus polymerase is an integral component of the CPSF30-NS1A protein complex in infected cells. J Virol *83*, 1611-1616.

Kuo, R.L., Zhao, C., Malur, M., and Krug, R.M. (2010). Influenza A virus strains that circulate in humans differ in the ability of their NS1 proteins to block the activation of IRF3 and interferon-beta transcription. Virology *408*, 146-158.

Lamb, R.A., and Choppin, P.W. (1981). Identification of a second protein (M2) encoded by RNA segment 7 of influenza virus. Virology *112*, 729-737.

Lamb, R.A., Choppin, P.W., Chanock, R.M., and Lai, C.J. (1980). Mapping of the two overlapping genes for polypeptides NS1 and NS2 on RNA segment 8 of influenza virus genome. Proc Natl Acad Sci U S A *77*, 1857-1861.

Lamb, R.A., Lai, C.J., and Choppin, P.W. (1981). Sequences of mRNAs derived from genome RNA segment 7 of influenza virus: colinear and interrupted mRNAs code for overlapping proteins. Proc Natl Acad Sci U S A *78*, 4170-4174.

Laudert, E.A., Sivanandan, V., and Halvorson, D.A. (1993). Effect of intravenous inoculation of avian influenza virus on reproduction and growth in mallard ducks. J Wildl Dis *29*, 523-526.

Lazzarini, R.A., Keene, J.D., and Schubert, M. (1981). The origins of defective interfering particles of the negative-strand RNA viruses. Cell *26*, 145-154.

Le Goffic, R., Balloy, V., Lagranderie, M., Alexopoulou, L., Escriou, N., Flavell, R., Chignard, M., and Si-Tahar, M. (2006). Detrimental Contribution of the Toll-Like Receptor (TLR)3 to Influenza A Virus–Induced Acute Pneumonia. PLoS Pathog *2*.

Le Goffic, R., Bouguyon, E., Chevalier, C., Vidic, J., Da Costa, B., Leymarie, O., Bourdieu, C., Decamps, L., Dhorne-Pollet, S., and Delmas, B. (2010). Influenza A virus protein PB1-F2

exacerbates IFN-beta expression of human respiratory epithelial cells. J Immunol *185*, 4812-4823.

Lee, N.R., Ban, J., Lee, N.J., Yi, C.M., Choi, J.Y., Kim, H., Lee, J.K., Seong, J., Cho, N.H., Jung, J.U*., et al.* (2018). Activation of RIG-I-Mediated Antiviral Signaling Triggers Autophagy Through the MAVS-TRAF6-Beclin-1 Signaling Axis. Front Immunol *9*, 2096.

Lee, Y.-J., Kang, H.-M., Lee, E.-K., Song, B.-M., Jeong, J., Kwon, Y.-K., Kim, H.-R., Lee, K.-J., Hong, M.-S., Jang, I*., et al.* (2014). Novel Reassortant Influenza A(H5N8) Viruses, South Korea, 2014. Emerging Infect Dis *20*, 1087-1089.

Leemans, J.C., Cassel, S.L., and Sutterwala, F.S. (2011). Sensing damage by the NLRP3 inflammasome. Immunol Rev *243*, 152-162.

Leymarie, O., Embury-Hyatt, C., Chevalier, C., Jouneau, L., Moroldo, M., Da Costa, B., Berhane, Y., Delmas, B., Weingartl, H.M., and Le Goffic, R. (2014). PB1-F2 attenuates virulence of highly pathogenic avian H5N1 influenza virus in chickens. PLoS ONE *9*, e100679.

Li, H., Bradley, K.C., Long, J.S., Frise, R., Ashcroft, J.W., Hartgroves, L.C., Shelton, H., Makris, S., Johansson, C., Cao, B., Barclay, W.S. (2018). Internal genes of a highly pathogenic H5N1 influenza virus determine high viral replication in myeloid cells and severe outcome of infection in mice. PLOS Pathog *14*, e1006821.

Li, H., Zhai, Y., Fan, Y., Chen, H., Zhang, A., Jin, H., and Luo, R. (2016). Molecular cloning and functional characterization of duck mitochondrial antiviral-signaling protein (MAVS). Dev Comp Immunol *56*, 1-6.

Li, J., Zhang, K., Chen, Q., Zhang, X., Sun, Y., Bi, Y., Zhang, S., Gu, J., Li, J., Liu, D. and Liu, W. (2018). Three amino acid substitutions in the NS1 protein change the virus replication of H5N1 influenza virus in human cells. Virology *519*, 64-73.

Li, K., Chen, Z., Kato, N., Gale, M., and Lemon, S.M. (2005). Distinct poly(I-C) and virus-activated signaling pathways leading to interferon-beta production in hepatocytes. J Biol Chem *280*, 16739-16747.

Li, K.S., Guan, Y., Wang, J., Smith, G.J.D., Xu, K.M., Duan, L., Rahardjo, A.P., Puthavathana, P., Buranathai, C., Nguyen, T.D.*, et al.* (2004). Genesis of a highly pathogenic and potentially pandemic H5N1 influenza virus in eastern Asia. Nature *430*, 209-213.

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*, 13-21.

Li, S., Shi, Z., Jiao, P., Zhang, G., Zhong, Z., Tian, W., Long, L.P., Cai, Z., Zhu, X., Liao, M.*, et al.* (2010). Avian-origin H3N2 canine influenza A viruses in Southern China. Infect Genet Evol *10*, 1286-1288.

Liedmann, S., Hrincius, E.R., Anhlan, D., McCullers, J.A., Ludwig, S. and Ehrhardt, C. (2014). New virulence determinants contribute to the enhanced immune response and reduced virulence of an influenza A virus A/PR8/34 variant. J Infect Dis *209*, 532-541.

Linehan, M.M., Dickey, T.H., Molinari, E.S., Fitzgerald, M.E., Potapova, O., Iwasaki, A., and Pyle, A.M. (2018). A minimal RNA ligand for potent RIG-I activation in living mice. Science Advances *4*, e1701854.

Liniger, M., Moulin, H.R., Sakoda, Y., Ruggli, N., and Summerfield, A. (2012). Highly pathogenic avian influenza virus H5N1 controls type I IFN induction in chicken macrophage HD-11 cells: a polygenic trait that involves NS1 and the polymerase complex. Virol J *9*, 7.

Liu, B., and Gao, C. (2018). Regulation of MAVS activation through post-translational modifications. Curr Opin Immunol *50*, 75-81.

Liu, D., Shi, W., Shi, Y., Wang, D., Xiao, H., Li, W., Bi, Y., Wu, Y., Li, X., Yan, J.*, et al.* (2013). Origin and diversity of novel avian influenza A H7N9 viruses causing human infection: phylogenetic, structural, and coalescent analyses. Lancet *381*, 1926-1932.

Liu, G., Lu, Y., Liu, Q., and Zhou, Y. (2019). Inhibition of Ongoing Influenza A Virus Replication Reveals Different Mechanisms of RIG-I Activation. J Virol *93*.

Liu, G., Lu, Y., Raman, S.N.T., Xu, F., Wu, Q., Li, Z., Brownlie, R., Liu, Q., and Zhou, Y. (2018). Nuclear-resident RIG-I senses viral replication inducing antiviral immunity. Nature Communications *9*, 3199.

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 IFN Induction. J Virol, JVI.00232-00215.

Liu, H., Golebiewski, L., Dow, E.C., Krug, R.M., Javier, R.T., and Rice, A.P. (2010). The ESEV PDZ-Binding Motif of the Avian Influenza A Virus NS1 Protein Protects Infected Cells from Apoptosis by Directly Targeting Scribble. J Virol *84*, 11164-11174.

Liu, J., Lynch, P.A., Chien, C.Y., Montelione, G.T., Krug, R.M., and Berman, H.M. (1997). Crystal structure of the unique RNA-binding domain of the influenza virus NS1 protein. Nat Struct Biol *4*, 896-899.

Liu, S., Cai, X., Wu, J., Cong, Q., Chen, X., Li, T., Du, F., Ren, J., Wu, Y.T., Grishin, N.V.*, et al.* (2015b). Phosphorylation of innate immune adaptor proteins MAVS, STING, and TRIF induces IRF3 activation. Science *347*, aaa2630.

Liu, Y.-J. (2005). IPC: Professional Type 1 Interferon-Producing Cells and Plasmacytoid Dendritic Cell Precursors. Annual Review of Immunology *23*, 275-306.

Long, J.C., and Fodor, E. (2016). The PB2 subunit of the influenza A virus RNA polymerase is imported into the mitochondrial matrix. J Virol.

Long, J.S., Giotis, E.S., Moncorge, O., Frise, R., Mistry, B., James, J., Morisson, M., Iqbal, M., Vignal, A., Skinner, M.A.*, et al.* (2016). Species difference in ANP32A underlies influenza A virus polymerase host restriction. Nature *529*, 101-104.

Long, J.X., Peng, D.X., Liu, Y.L., Wu, Y.T., and Liu, X.F. (2008). Virulence of H5N1 avian influenza virus enhanced by a 15-nucleotide deletion in the viral nonstructural gene. Virus Genes *36*, 471-478.

Loo, Y.-M., Fornek, J., Crochet, N., Bajwa, G., Perwitasari, O., Martinez-Sobrido, L., Akira, S., Gill, M.A., García-Sastre, A., Katze, M.G.*, et al.* (2008). Distinct RIG-I and MDA5 Signaling by RNA Viruses in Innate Immunity. J Virol *82*, 335-345.

Loo, Y.M., and Gale, M., Jr. (2007). Influenza: fatal immunity and the 1918 virus. Nature *445*, 267-268.

Lu, Y., Wambach, M., Katze, M.G., and Krug, R.M. (1995). Binding of the influenza virus NS1 protein to double-stranded RNA inhibits the activation of the protein kinase that phosphorylates the elF-2 translation initiation factor. Virology *214*, 222-228.

Lucas, S.-M., Rothwell, N.J., and Gibson, R.M. (2006). The role of inflammation in CNS injury and disease. British Journal of Pharmacology *147*, S232-S240.

Ludwig, S., Schultz, U., Mandler, J., Fitch, W.M., and Scholtissek, C. (1991). Phylogenetic relationship of the nonstructural (NS) genes of influenza A viruses. Virology *183*, 566-577.

Ludwig, S., Wang, X., Ehrhardt, C., Zheng, H., Donelan, N., Planz, O., Pleschka, S., Garcia-Sastre, A., Heins, G., and Wolff, T. (2002). The influenza A virus NS1 protein inhibits activation of Jun N-terminal kinase and AP-1 transcription factors. J Virol *76*, 11166-11171.

Lund, J.M., Alexopoulou, L., Sato, A., Karow, M., Adams, N.C., Gale, N.W., Iwasaki, A., and Flavell, R.A. (2004). Recognition of single-stranded RNA viruses by Toll-like receptor 7. PNAS *101*, 5598-5603.

Ma, W., Brenner, D., Wang, Z., Dauber, B., Ehrhardt, C., Hogner, K., Herold, S., Ludwig, S., Wolff, T., Yu, 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. J Virol *84*, 2122-2133.

MacDonald, M.R.W., Xia, J., Smith, A.L., and Magor, K.E. (2008). The duck toll like receptor 7: Genomic organization, expression and function. Mol Immunol *45*, 2055-2061.

Magor, K.E., Miranzo Navarro, D., Barber, M.R.W., Petkau, K., Fleming-Canepa, X., Blyth, G.A.D., and Blaine, A.H. (2013). Defense genes missing from the flight division. Dev Comp Immunol *41*, 377-388.

Manz, B., de Graaf, M., Mogling, R., Richard, M., Bestebroer, T.M., Rimmelzwaan, G.F., and Fouchier, R.A. (2016). Multiple Natural Substitutions in Avian Influenza A Virus PB2 Facilitate Efficient Replication in Human Cells. J Virol *90*, 5928-5938.

Marc, D., Barbachou, S., and Soubieux, D. (2013). The RNA-binding domain of influenzavirus non-structural protein-1 cooperatively binds to virus-specific RNA sequences in a structure-dependent manner. In Nucleic Acids Res, pp. 434-449.

Marchenko, V.Y., Alekseev, A.Y., Sharshov, K.A., Petrov, V.N., Silko, N.Y., Susloparov, I.M., Tserennorov, D., Otgonbaatar, D., Savchenko, I.A., and Shestopalov, A.M. (2012). Ecology of Influenza Virus in Wild Bird Populations in Central Asia. Avian Diseases *56*, 234-237.

Mariathasan, S., Weiss, D.S., Newton, K., McBride, J., O'Rourke, K., Roose-Girma, M., Lee, W.P., Weinrauch, Y., Monack, D.M., and Dixit, V.M. (2006). Cryopyrin activates the inflammasome in response to toxins and ATP. Nature *440*, 228-232.

Marjuki, H., Scholtissek, C., Franks, J., Negovetich, N.J., Aldridge, J.R., Salomon, R., Finkelstein, D., and Webster, R.G. (2010). Three amino acid changes in PB1-F2 of highly pathogenic H5N1 avian influenza virus affect pathogenicity in mallard ducks. Arch Virol *155*, 925-934.

Martínez-Sobrido, L., and García-Sastre, A. (2010). Generation of Recombinant Influenza Virus from Plasmid DNA. J Vis Exp.

Martinon, F., Agostini, L., Meylan, E., and Tschopp, J. (2004). Identification of bacterial muramyl dipeptide as activator of the NALP3/cryopyrin inflammasome. Curr Biol *14*, 1929-1934.

Martinon, F., Petrilli, V., Mayor, A., Tardivel, A., and Tschopp, J. (2006). Gout-associated uric acid crystals activate the NALP3 inflammasome. Nature *440*, 237-241.

Matlin, K.S., Reggio, H., Helenius, A., and Simons, K. (1981). Infectious entry pathway of influenza virus in a canine kidney cell line. J Cell Biol *91*, 601-613.

Matrosovich, M., Zhou, N., Kawaoka, Y., and Webster, R. (1999). The Surface Glycoproteins of H5 Influenza Viruses Isolated from Humans, Chickens, and Wild Aquatic Birds Have Distinguishable Properties. J Virol *73*, 1146-1155.

McAuley, J.L., Tate, M.D., MacKenzie-Kludas, C.J., Pinar, A., Zeng, W., Stutz, A., Latz, E., Brown, L.E., and Mansell, A. (2013). Activation of the NLRP3 inflammasome by IAV virulence protein PB1-F2 contributes to severe pathophysiology and disease. PLoS Pathog *9*, e1003392.

Mehle, A. (2014). Unusual influenza A viruses in bats. Viruses *6*, 3438-3449.

Mehle, A., and Doudna, J.A. (2009). Adaptive strategies of the influenza virus polymerase for replication in humans. Proc Natl Acad Sci U S A *106*, 21312-21316.

Melén, K., Kinnunen, L., Fagerlund, R., Ikonen, N., Twu, K.Y., Krug, R.M., and Julkunen, I. (2007). Nuclear and Nucleolar Targeting of Influenza A Virus NS1 Protein: Striking Differences between Different Virus Subtypes. J Virol.

Meunier, I., and von Messling, V. (2012). PB1-F2 modulates early host responses but does not affect the pathogenesis of H1N1 seasonal influenza virus. J Virol *86*, 4271-4278.

Meyerson, N.R., Zhou, L., Guo, Y.R., Zhao, C., Tao, Y.J., Krug, R.M., and Sawyer, S.L. (2017). Nuclear TRIM25 Specifically Targets Influenza Virus Ribonucleoproteins to Block the Onset of RNA Chain Elongation. Cell Host Microbe *22*, 627-638.e627.

Mibayashi, M., Martinez-Sobrido, L., Loo, Y.M., Cardenas, W.B., Gale, M., Jr., and Garcia-Sastre, A. (2007). Inhibition of retinoic acid-inducible gene I-mediated induction of beta interferon by the NS1 protein of influenza A virus. J Virol *81*, 514-524.

Min, J.-Y., Li, S., Sen, G.C., and Krug, R.M. (2007). A site on the influenza A virus NS1 protein mediates both inhibition of PKR activation and temporal regulation of viral RNA synthesis. Virology *363*, 236-243.

Min, J.Y., and Krug, R.M. (2006). The primary function of RNA binding by the influenza A virus NS1 protein in infected cells: Inhibiting the 2'-5' oligo (A) synthetase/RNase L pathway. Proc Natl Acad Sci U S A *103*, 7100-7105.

Miotto, O., Heiny, A., Tan, T.W., August, J.T., and Brusic, V. (2008). Identification of human-to-human transmissibility factors in PB2 proteins of influenza A by large-scale mutual information analysis. BMC Bioinformatics *9 Suppl 1*, S18.

Miranzo-Navarro, D., and Magor, K.E. (2014). Activation of duck RIG-I by TRIM25 is independent of anchored ubiquitin. PLoS ONE *9*, e86968.

Monteagudo, P.L., Muñoz-Moreno, R., Fribourg, M., Potla, U., Mena, I., Marjanovic, N., Hartmann, B.M., Sealfon, S.C., García-Sastre, A., Ramos, I.*, et al.* (2019). Differential modulation of innate immune responses in human primary cells by influenza A viruses carrying human or avian non-structural 1 proteins. J Virol *Epub ahead of print*.

Monto, A.S. (1987). Influenza: quantifying morbidity and mortality. Am J Med *82*, 20-25.

Moulin, H.R., Liniger, M., Python, S., Guzylack-Piriou, L., Ocaña-Macchi, M., Ruggli, N., and Summerfield, A. (2011). High interferon type I responses in the lung, plasma and spleen during highly pathogenic H5N1 infection of chicken. Vet Res *42*, 6.

Munster, V.J., Baas, C., Lexmond, P., Waldenstrom, J., Wallensten, A., Fransson, T., Rimmelzwaan, G.F., Beyer, W.E., Schutten, M., Olsen, B.*, et al.* (2007). Spatial, temporal, and species variation in prevalence of influenza A viruses in wild migratory birds. PLoS Pathog *3*, e61.

Murakami, S., Horimoto, T., Nidom, C.A., Chen, H., Muramoto, Y., Yamada, S., Iwasa, A., Iwatsuki-Horimoto, K., Shimojima, M., Iwata, A. and Kawaoka, Y. (2008). Growth determinants for H5N1 influenza vaccine seed viruses in MDCK cells. J Virol *82*, 10502-10509.

Muramoto, Y., Noda, T., Kawakami, E., Akkina, R., and Kawaoka, Y. (2013). Identification of novel influenza A virus proteins translated from PA mRNA. J Virol *87*, 2455-2462.

Nath, S.T., and Nayak, D.P. (1990). Function of two discrete regions is required for nuclear localization of polymerase basic protein 1 of A/WSN/33 influenza virus (H1 N1). Mol Cell Biol *10*, 4139-4145.

Nemeroff, M.E., Barabino, S.M.L., Li, Y., Keller, W., and Krug, R.M. (1998). Influenza Virus NS1 Protein Interacts with the Cellular 30 kDa Subunit of CPSF and Inhibits 3′ End Formation of Cellular Pre-mRNAs. Mol Cell *1*, 991-1000.

Neumann, G., Hughes, M.T., and Kawaoka, Y. (2000). Influenza A virus NS2 protein mediates vRNP nuclear export through NES-independent interaction with hCRM1. EMBO J *19*, 6751-6758.

Neumann, G., and Kawaoka, Y. (2015). Transmission of influenza A viruses. Virology *479-480*, 234-246.

Nieto, A., de la Luna, S., Barcena, J., Portela, A., and Ortin, J. (1994). Complex structure of the nuclear translocation signal of influenza virus polymerase PA subunit. J Gen Virol *75 ( Pt 1)*, 29-36.

Njoto, E.N., Scotch, M., Bui, C.M., Adam, D.C., Chughtai, A.A., and MacIntyre, C.R. (2018). Phylogeography of H5N1 avian influenza virus in Indonesia. Transboundary and Emerging Diseases *65*.

Noah, D.L., Twu, K.Y., and Krug, R.M. (2003). Cellular antiviral responses against influenza A virus are countered at the posttranscriptional level by the viral NS1A protein via its binding to a cellular protein required for the 3' end processing of cellular pre-mRNAS. Virology *307*, 386-395.

Nogales, A., DeDiego, M.L., Topham, D.J., and Martínez-Sobrido, L. (2016). Rearrangement of Influenza Virus Spliced Segments for the Development of Live-Attenuated Vaccines. J Virol *90*, 6291-6302.

Nolan, T.F., Jr., Goodman, R.A., Hinman, A.R., Noble, G.R., Kendal, A.P., and Thacker, S.B. (1980). Morbidity and mortality associated with influenza B in the United States, 1979-1980. A report from the Center for Disease Control. J Infect Dis *142*, 360-362.

Noton, S.L., Medcalf, E., Fisher, D., Mullin, A.E., Elton, D., and Digard, P. (2007). Identification of the domains of the influenza A virus M1 matrix protein required for NP binding, oligomerization and incorporation into virions. J Gen Virol *88*, 2280-2290.

Nourry, C., Grant, S.G., and Borg, J.P. (2003). PDZ domain proteins: plug and play! Sci STKE *2003*, Re7.

O'Neill, R.E., Jaskunas, R., Blobel, G., Palese, P., and Moroianu, J. (1995). Nuclear import of influenza virus RNA can be mediated by viral nucleoprotein and transport factors required for protein import. J Biol Chem *270*, 22701-22704.

O.I.E. (World Organization for Animal Health) (2019). Health Manual of Diagnostic Tests and Vaccines for Terrestrial Animals 2019. <https://www.oie.int/standard-setting/terrestrial-manual/access-online/> Accessed on January 15, 2020.

Obenauer, J.C., Denson, J., Mehta, P.K., Su, X., Mukatira, S., Finkelstein, D.B., Xu, X., Wang, J., Ma, J., Fan, Y.*, et al.* (2006). Large-Scale Sequence Analysis of Avian Influenza Isolates. Science *311*, 1576-1580.

Okazaki, K., Kawaoka, Y., and Webster, R.G. (1989). Evolutionary pathways of the PA genes of influenza A viruses. Virology *172*, 601-608.

Olsen, B., Munster, V.J., Wallensten, A., Waldenström, J., Osterhaus, A.D.M.E., and Fouchier, R.A.M. (2006). Global Patterns of Influenza A Virus in Wild Birds. Science *312*, 384-388.

Opitz, B., Rejaibi, A., Dauber, B., Eckhard, J., Vinzing, M., Schmeck, B., Hippenstiel, S., Suttorp, N., and Wolff, T. (2007). IFNβ induction by influenza A virus is mediated by RIG-I which is regulated by the viral NS1 protein. Cellular Microbiology *9*, 930-938.

Orlich, M., Gottwald, H., and Rott, R. (1994). Nonhomologous recombination between the hemagglutinin gene and the nucleoprotein gene of an influenza virus. Virology *204*, 462-465.

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*, 807-817.

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*, 496-509.

Palese, P., and Compans, R.W. (1976). Inhibition of influenza virus replication in tissue culture by 2-deoxy-2,3-dehydro-N-trifluoroacetylneuraminic acid (FANA): mechanism of action. J Gen Virol *33*, 159-163.

Palese, P., Racaniello, V.R., Desselberger, U., Young, J., and Baez, M. (1980). Genetic structure and genetic variation of influenza viruses. Philos Trans R Soc Lond B Biol Sci *288*, 299-305.

Palese, P., Ritchey, M.B., and Schulman, J.L. (1977). Mapping of the influenza virus genome. II. Identification of the P1, P2, and P3 genes. Virology *76*, 114-121.

Palese, P., and Schulman, J.L. (1976). Mapping of the influenza virus genome: identification of the hemagglutinin and the neuraminidase genes. Proc Natl Acad Sci U S A *73*, 2142-2146.

Palese, P., Tobita, K., Ueda, M., and Compans, R.W. (1974). Characterization of temperature sensitive influenza virus mutants defective in neuraminidase. Virology *61*, 397-410.

Pancucharova, H., and Russ, G. (2006). PB1-F2 gene in influenza a viruses of different hemagglutinin subtype. Acta Virol *50*, 269-272.

Pantin-Jackwood, M.J., Costa-Hurtado, M., Shepherd, E., DeJesus, E., Smith, D., Spackman, E., Kapczynski, D.R., Suarez, D.L., Stallknecht, D.E., and Swayne, D.E. (2016). Pathogenicity and Transmission of H5 and H7 Highly Pathogenic Avian Influenza Viruses in Mallards. J Virol *90*, 9967-9982.

Parvin, J.D., Moscona, A., Pan, W.T., Leider, J.M., and Palese, P. (1986). Measurement of the mutation rates of animal viruses: influenza A virus and poliovirus type 1. J Virol *59*, 377-383.

Pasick, J., Berhane, Y., Joseph, T., Bowes, V., Hisanaga, T., Handel, K., and Alexandersen, S. (2015). Reassortant Highly Pathogenic Influenza A H5N2 Virus Containing Gene Segments Related to Eurasian H5N8 in British Columbia, Canada, 2014. Sci Rep *5*, 9484.

Pasick, J., Handel, K., Robinson, J., Copps, J., Ridd, D., Hills, K., Kehler, H., Cottam-Birt, C., Neufeld, J., Berhane, Y.*, et al.* (2005). Intersegmental recombination between the haemagglutinin and matrix genes was responsible for the emergence of a highly pathogenic H7N3 avian influenza virus in British Columbia. J Gen Virol *86*, 727-731.

Patel, D., Schultz, L.W., and Umland, T.C. (2013). Influenza A polymerase subunit PB2 possesses overlapping binding sites for polymerase subunit PB1 and human MAVS proteins. Virus Res *172*, 75-80.

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

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

Penski, N., Härtle, S., Rubbenstroth, D., Krohmann, C., Ruggli, N., Schusser, B., Pfann, M., Reuter, A., Gohrbandt, S., Hundt, J.*, et al.* (2011). Highly Pathogenic Avian Influenza Viruses Do Not Inhibit Interferon Synthesis in Infected Chickens but Can Override the Interferon-Induced Antiviral State ▽. J Virol *85*, 7730-7741.

Peper, R.L., and Van Campen, H. (1995). Tumor necrosis factor as a mediator of inflammation in influenza A viral pneumonia. Microbial Pathogenesis *19*, 175-183.

Perez, J.T., García-Sastre, A., and Manicassamy, B. (2013). Insertion of a GFP reporter gene in influenza virus. Curr Protoc Microbiol *Chapter 15*, Unit-15G.14.

Perry, A.K., Chen, G., Zheng, D., Tang, H., and Cheng, G. (2005). The host type I interferon response to viral and bacterial infections. Cell Res *15*, 407-422.

Philbin, V.J., Iqbal, M., Boyd, Y., Goodchild, M.J., Beal, R.K., Bumstead, N., Young, J., and Smith, A.L. (2005). Identification and characterization of a functional, alternatively spliced Toll-like receptor 7 (TLR7) and genomic disruption of TLR8 in chickens. Immunology *114*, 507-521.

Pichlmair, A., Schulz, O., Tan, C.P., Näslund, T.I., Liljeström, P., Weber, F., and Sousa, C.R.e. (2006). RIG-I-Mediated Antiviral Responses to Single-Stranded RNA Bearing 5'-Phosphates.

Pinto, L.H., and Lamb, R.A. (2006). The M2 proton channels of influenza A and B viruses. J Biol Chem *281*, 8997-9000.

Pothlichet, J., Meunier, I., Davis, B.K., Ting, J.P.Y., Skamene, E., Messling, V.v., and Vidal, S.M. (2013). Type I IFN Triggers RIG-I/TLR3/NLRP3-dependent Inflammasome Activation in Influenza A Virus Infected Cells. PLoS Pathog *9*, e1003256.

Qian, W., Wei, X., Guo, K., Li, Y., Lin, X., Zou, Z., Zhou, H., and Jin, M. (2017). The C-Terminal Effector Domain of Non-Structural Protein 1 of Influenza A Virus Blocks IFN-β Production by Targeting TNF Receptor-Associated Factor 3. Front Immunol *8*, 779.

Quicke, K.M., Diamond, M.S., and Suthar, M.S. (2017). Negative regulators of the RIG-I-like receptor signaling pathway. Eur J Immunol *47*, 615-628.

Rajsbaum, R., Albrecht, R.A., Wang, M.K., Maharaj, N.P., Versteeg, G.A., Nistal-Villán, E., García-Sastre, A., and Gack, M.U. (2012). Species-specific inhibition of RIG-I ubiquitination and IFN induction by the influenza A virus NS1 protein. PLoS Pathog *8*, e1003059.

Richardson, J.C., and Akkina, R.K. (1991). NS2 protein of influenza virus is found in purified virus and phosphorylated in infected cells. Arch Virol *116*, 69-80.

Ritchey, M.B., Palese, P., and Schulman, J.L. (1976). Mapping of the influenza virus genome. III. Identification of genes coding for nucleoprotein, membrane protein, and nonstructural protein. J Virol *20*, 307-313.

Roth, M.G., Compans, R.W., Giusti, L., Davis, A.R., Nayak, D.P., Gething, M.J., and Sambrook, J. (1983). Influenza virus hemagglutinin expression is polarized in cells infected with recombinant SV40 viruses carrying cloned hemagglutinin DNA. Cell *33*, 435-443.

Rott, R. (1992). The pathogenic determinant of influenza virus. Vet Microbiol *33*, 303-310.

Runstadler, J.A., Happ, G.M., Slemons, R.D., Sheng, Z.M., Gundlach, N., Petrula, M., Senne, D., Nolting, J., Evers, D.L., Modrell, A.*, 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*, 1901-1910.

Ryan, G.B., and Majno, G. (1977). Acute inflammation. A review. The American Journal of Pathology *86*, 183-276.

Saito, L.B., Diaz-Satizabal, L., Evseev, D., Fleming-Canepa, X., Mao, S., Webster, R.G., and Magor, K.E. (2018). IFN and cytokine responses in ducks to genetically similar H5N1 influenza A viruses of varying pathogenicity. Journal of General Virology *99*, 464-474.

Sakaki, H., Imaizumi, T., Matsumiya, T., Kusumi, A., Nakagawa, H., Kubota, K., Nishi, N., Nakamura, T., Hirashima, M., Satoh, K.*, et al.* (2005). Retinoic acid-inducible gene-I is induced by interleukin-1beta in cultured human gingival fibroblasts. Oral Microbiol Immunol *20*, 47-50.

Sánchez-Aparicio, M.T., Ayllón, J., Leo-Macias, A., Wolff, T., and García-Sastre, A. (2017). Subcellular Localizations of RIG-I, TRIM25, and MAVS Complexes. J Virol *91*.

Sanchez, J.G., Chiang, J.J., Sparrer, K.M.J., Alam, S.L., Chi, M., Roganowicz, M.D., Sankaran, B., Gack, M.U., and Pornillos, O. (2016). Mechanism of TRIM25 Catalytic Activation in the Antiviral RIG-I Pathway. Cell Rep *16*, 1315-1325.

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 USA *111*, 2494-2499.

Schaefer-Klein, J., Givol, I., Barsov, E.V., Whitcomb, J.M., VanBrocklin, M., Foster, D.N., Federspiel, M.J., and Hughes, S.H. (1998). The EV-O-derived cell line DF-1 supports the efficient replication of avian leukosis-sarcoma viruses and vectors. Virology *248*, 305-311.

Schafer, J.R., Kawaoka, Y., Bean, W.J., Suss, J., Senne, D., and Webster, R.G. (1993). Origin of the pandemic 1957 H2 influenza A virus and the persistence of its possible progenitors in the avian reservoir. Virology *194*, 781-788.

Schickli, J.H., Flandorfer, A., Nakaya, T., Martinez-Sobrido, L., García-Sastre, A., and Palese, P. (2001). Plasmid-only rescue of influenza A virus vaccine candidates. Philos Trans R Soc Lond, B, Biol Sci *356*, 1965-1973.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B.*, et al.* (2012). Fiji: an open-source platform for biological-image analysis. Nat Methods *9*, 676-682.

Schlee, M., and Hartmann, G. (2010). The Chase for the RIG-I Ligand—Recent Advances. Molecular Therapy *18*, 1254-1262.

Schmolke, M., Manicassamy, B., Pena, L., Sutton, T., Hai, R., Varga, Z.T., Hale, B.G., Steel, J., Pérez, D.R., and García-Sastre, A. (2011). Differential contribution of PB1-F2 to the virulence of highly pathogenic H5N1 influenza A virus in mammalian and avian species. PLoS Pathog *7*, e1002186.

Schoggins, J.W., Wilson, S.J., Panis, M., Murphy, M.Y., Jones, C.T., Bieniasz, P., and Rice, C.M. (2011). A diverse range of gene products are effectors of the type I interferon antiviral response. Nature *472*, 481-485.

Scholtissek, C., Rohde, W., Von Hoyningen, V., and Rott, R. (1978). On the origin of the human influenza virus subtypes H2N2 and H3N2. Virology *87*, 13-20.

Schrauwen, E.J., and Fouchier, R.A. (2014). Host adaptation and transmission of influenza A viruses in mammals. Emerg Microbes Infect *3*, e9.

Schultz, U., Kaspers, B., and Staeheli, P. (2004). The interferon system of non-mammalian vertebrates. Developmental & Comparative Immunology *28*, 499-508.

Selman, M., Dankar, S.K., Forbes, N.E., Jia, J.J., and Brown, E.G. (2012). Adaptive mutation in influenza A virus non-structural gene is linked to host switching and induces a novel protein by alternative splicing. Emerg Microbes Infect *1*, e42.

Seo, S.H., Hoffmann, E., and Webster, R.G. (2002). Lethal H5N1 influenza viruses escape host anti-viral cytokine responses. Nat Med *8*, 950-954.

Seth, R.B., Sun, L., Ea, C.K., and Chen, Z.J. (2005). Identification and characterization of MAVS, a mitochondrial antiviral signaling protein that activates NF-kappaB and IRF 3. Cell *122*, 669-682.

Shahangian, A., Chow, E.K., Tian, X., Kang, J.R., Ghaffari, A., Liu, S.Y., Belperio, J.A., Cheng, G., and Deng, J.C. (2009). Type I IFNs mediate development of postinfluenza bacterial pneumonia in mice. J Clin Invest *119*, 1910-1920.

Shapshak, P., Chiappelli, F., Somboonwit, C., and Sinnott, J. (2011). The influenza pandemic of 2009: lessons and implications. Mol Diagn Ther *15*, 63-81.

Shaw, M., and Palese, P. (2013). Orthomyxoviridae. In Fields virology, D.M. Knipe, and P. Howley, eds. (Philadelphia: Lippincott Williams & Wilkins), pp. 1151-1185.

Shi, Y., Yuan, B., Zhu, W., Zhang, R., Li, L., Hao, X., Chen, S., and Hou, F. (2017). Ube2D3 and Ube2N are essential for RIG-I-mediated MAVS aggregation in antiviral innate immunity. Nat Commun *8*, 1-14.

Shimizu, T., Takizawa, N., Watanabe, K., Nagata, K., and Kobayashi, N. (2011). Crucial role of the influenza virus NS2 (NEP) C-terminal domain in M1 binding and nuclear export of vRNP. FEBS Lett *585*, 41-46.

Short, K.R., Veldhuis Kroeze, E.J.B., Reperant, L.A., Richard, M., and Kuiken, T. (2014). Influenza virus and endothelial cells: a species specific relationship. Front Microbiol *5*.

Shu, L.L., Bean, W.J., and Webster, R.G. (1993). Analysis of the evolution and variation of the human influenza A virus nucleoprotein gene from 1933 to 1990. J Virol *67*, 2723-2729.

Sick, C., Schultz, U., Munster, U., Meier, J., Kaspers, B., and Staeheli, P. (1998). Promoter structures and differential responses to viral and nonviral inducers of chicken type I interferon genes. J Biol Chem *273*, 9749-9754.

Sieczkarski, S.B., and Whittaker, G.R. (2002). Influenza virus can enter and infect cells in the absence of clathrin-mediated endocytosis. J Virol *76*, 10455-10464.

Skehel, J.J., and Wiley, D.C. (2000). Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annu Rev Biochem *69*, 531-569.

Slemons, R.D., and Swayne, D.E. (1995). Tissue tropism and replicative properties of waterfowl-origin influenza viruses in chickens. Avian Dis *39*, 521-527.

Smith, G.J., Bahl, J., Vijaykrishna, D., Zhang, J., Poon, L.L., Chen, H., Webster, R.G., Peiris, J.S., and Guan, Y. (2009a). Dating the emergence of pandemic influenza viruses. Proc Natl Acad Sci U S A *106*, 11709-11712.

Smith, G.J., Vijaykrishna, D., Bahl, J., Lycett, S.J., Worobey, M., Pybus, O.G., Ma, S.K., Cheung, C.L., Raghwani, J., Bhatt, S.*, et al.* (2009b). Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature *459*, 1122-1125.

Smith, J., Smith, N., Yu, L., Paton, I.R., Gutowska, M.W., Forrest, H.L., Danner, A.F., Seiler, J.P., Digard, P., Webster, R.G.*, 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*.

Songserm, T., Jam-on, R., Sae-Heng, N., Meemak, N., Hulse-Post, D.J., Sturm-Ramirez, K.M., and Webster, R.G. (2006). Domestic ducks and H5N1 influenza epidemic, Thailand. Emerging Infect Dis *12*, 575-581.

Soubies, S.M., Hoffmann, T.W., Croville, G., Larcher, T., Ledevin, M., Soubieux, D., Quéré, P., Guérin, J.-L., Marc, D., and Volmer, R. (2013). Deletion of the C-terminal ESEV domain of NS1 does not affect the replication of a low-pathogenic avian influenza virus H7N1 in ducks and chickens. Journal of General Virology *94*, 50-58.

Soubies, S.M., Volmer, C., Croville, G., Loupias, J., Peralta, B., Costes, P., Lacroux, C., Guérin, J.-L., and Volmer, R. (2010a). Species-Specific Contribution of the Four C-Terminal Amino Acids of Influenza A Virus NS1 Protein to Virulence. J Virol *84*, 6733-6747.

Soubies, S.M., Volmer, C., Guérin, J.-L., and Volmer, R. (2010b). Truncation of the NS1 Protein Converts a Low Pathogenic Avian Influenza Virus into a Strong Interferon Inducer in Duck Cells. Avian Diseases *54*, 527-531.

Spesock, A., Malur, M., Hossain, M.J., Chen, L.M., Njaa, B.L., Davis, C.T., Lipatov, A.S., York, I.A., Krug, R.M., and Donis, R.O. (2011). The virulence of 1997 H5N1 influenza viruses in the mouse model is increased by correcting a defect in their NS1 proteins. J Virol *85*, 7048-7058.

Stallknecht, D.E., Kearney, M.T., Shane, S.M., and Zwank, P.J. (1990). Effects of pH, Temperature, and Salinity on Persistence of Avian Influenza Viruses in Water. Avian Diseases *34*, 412-418.

Steidle, S., Martínez-Sobrido, L., Mordstein, M., Lienenklaus, S., García-Sastre, A., Stäheli, P., and Kochs, G. (2010). Glycine 184 in nonstructural protein NS1 determines the virulence of influenza A virus strain PR8 without affecting the host interferon response. J Virol *84*, 12761-12770.

Stieneke-Gröber, A., Vey, M., Angliker, H., Shaw, E., Thomas, G., Roberts, C., Klenk, H.D., and Garten, W. (1992). Influenza virus hemagglutinin with multibasic cleavage site is activated by furin, a subtilisin-like endoprotease. EMBO J *11*, 2407-2414.

Studahl, M. (2003). Influenza virus and CNS manifestations. J Clin Virol *28*, 225-232.

Sturm-Ramirez, K.M., Hulse-Post, D.J., Govorkova, E.A., Humberd, J., Seiler, P., Puthavathana, P., Buranathai, C., Nguyen, T.D., Chaisingh, A., Long, H.T.*, et al.* (2005). Are ducks contributing to the endemicity of highly pathogenic H5N1 influenza virus in Asia? J Virol *79*, 11269-11279.

Su, S., Fu, X., Li, G., Kerlin, F., and Veit, M. (2017). Novel Influenza D virus: Epidemiology, pathology, evolution and biological characteristics. Virulence *8*, 1580-1591.

Subbarao, E.K., London, W., and Murphy, B.R. (1993). A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. J Virol *67*, 1761-1764.

Sugita, S., Yoshioka, Y., Itamura, S., Kanegae, Y., Oguchi, K., Gojobori, T., Nerome, K., and Oya, A. (1991). Molecular evolution of hemagglutinin genes of H1N1 swine and human influenza A viruses. J Mol Evol *32*, 16-23.

Sun, H., Jiao, P., Jia, B., Xu, C., Wei, L., Shan, F., Luo, K., Xin, C., Zhang, K., and Liao, M. (2011). Pathogenicity in quails and mice of H5N1 highly pathogenic avian influenza viruses isolated from ducks. Vet Microbiol *152*, 258-265.

Tate, M.D., Ong, J.D.H., Dowling, J.K., McAuley, J.L., Robertson, A.B., Latz, E., Drummond, G.R., Cooper, M.A., Hertzog, P.J., and Mansell, A. (2016). Reassessing the role of the NLRP3 inflammasome during pathogenic influenza A virus infection via temporal inhibition. Sci Rep *6*, 27912.

te Velthuis, A.J.W., Long, J.C., Bauer, D.L.V., Fan, R.L.Y., Yen, H.-L., Sharps, J., Siegers, J.Y., Killip, M.J., French, H., Oliva-Martín, M.J.*, et al.* (2018). Mini viral RNAs act as innate immune agonists during influenza virus infection. Nature Microbiology *3*, 1234.

Teijaro, John R., Walsh, Kevin B., Cahalan, S., Fremgen, Daniel M., Roberts, E., Scott, F., Martinborough, E., Peach, R., Oldstone, Michael B.A., and Rosen, H. (2011). Endothelial Cells Are Central Orchestrators of Cytokine Amplification during Influenza Virus Infection. Cell *146*, 980-991.

Teijaro, J.R., Walsh, K.B., Rice, S., Rosen, H., and Oldstone, M.B.A. (2014). Mapping the innate signaling cascade essential for cytokine storm during influenza virus infection. PNAS *111*, 3799-3804.

Tetrault, D.G. (2015). Characterizing the effects of differentially adapted influenza PB2 proteins on duck MAVS-mediated interferon beta signaling. In Department of Biological Sciences (University of Alberta).

Thomas, M., Kranjec, C., Nagasaka, K., Matlashewski, G., and Banks, L. (2011). Analysis of the PDZ binding specificities of Influenza A Virus NS1 proteins. Virol J *8*, 25.

Thomas, P.G., Dash, P., Aldridge, J.R., Ellebedy, A.H., Reynolds, C., Funk, A.J., Martin, W.J., Lamkanfi, M., Webby, R.J., Boyd, K.L.*, 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*, 566-575.

Tong, S., Zhu, X., Li, Y., Shi, M., Zhang, J., Bourgeois, M., Yang, H., Chen, X., Recuenco, S., Gomez, J.*, et al.* (2013). New world bats harbor diverse influenza A viruses. PLoS Pathog *9*, e1003657.

Treanor, J.J., Snyder, M.H., London, W.T., and Murphy, B.R. (1989). The B allele of the NS gene of avian influenza viruses, but not the A allele, attenuates a human influenza A virus for squirrel monkeys. Virology *171*, 1-9.

Trinchieri, G. (2010). Type I interferon: friend or foe? Journal of Experimental Medicine *207*, 2053-2063.

Tschopp, J., and Schroder, K. (2010). NLRP3 inflammasome activation: the convergence of multiple signalling pathways on ROS production? Nature Reviews Immunology *10*, 210-215.

Tumpey, T.M., Szretter, K.J., Van Hoeven, N., Katz, J.M., Kochs, G., Haller, O., García-Sastre, A., and Staeheli, P. (2007). The Mx1 gene protects mice against the pandemic 1918 and highly lethal human H5N1 influenza viruses. J Virol *81*, 10818-10821.

Turnbull, M.L., Wise, H.M., Nicol, M.Q., Smith, N., Dunfee, R.L., Beard, P.M., Jagger, B.W., Ligertwood, Y., Hardisty, G.R., Xiao, H.*, et al.* (2016). Role of the B Allele of Influenza A Virus Segment 8 in Setting Mammalian Host Range and Pathogenicity. J Virol *90*, 9263-9284.

Uyeki, T.M. (2009). Human Infection with Highly Pathogenic Avian Influenza A (H5N1) Virus: Review of Clinical Issues. Clin Infect Dis *49*, 279-290.

Vanderven, H.A., Petkau, K., Ryan-Jean, K.E.E., Aldridge, J.R., Webster, R.G., and Magor, K.E. (2012). Avian influenza rapidly induces antiviral genes in duck lung and intestine. Mol Immunol *51*, 316-324.

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*, 8359-8366.

Varga, Z.T., and Palese, P. (2011). The influenza A virus protein PB1-F2: killing two birds with one stone? Virulence *2*, 542-546.

Varga, Z.T., Ramos, I., Hai, R., Schmolke, M., Garcia-Sastre, A., Fernandez-Sesma, A., and Palese, P. (2011). The influenza virus protein PB1-F2 inhibits the induction of type I interferon at the level of the MAVS adaptor protein. PLoS Pathog *7*, e1002067.

Vasin, A.V., Petrova-Brodskaya, A.V., Plotnikova, M.A., Tsvetkov, V.B., and Klotchenko, S.A. (2017). Evolutionary dynamics of structural and functional domains of influenza A virus NS1 protein. Problems of Virology *62*, 246-258.

Vasin, A.V., Petrova, A.V., Egorov, V.V., Plotnikova, M.A., Klotchenko, S.A., Karpenko, M.N., and Kiselev, O.I. (2016). The influenza A virus NS genome segment displays lineage-specific patterns in predicted RNA secondary structure. BMC Res Notes *9*, 279.

Volmer, C., Soubies, S.M., Grenier, B., Guérin, J.-L., and Volmer, R. (2011). Immune response in the duck intestine following infection with low-pathogenic avian influenza viruses or stimulation with a Toll-like receptor 7 agonist administered orally. Journal of General Virology *92*, 534-543.

Vreede, F.T., and Brownlee, G.G. (2007). Influenza virion-derived viral ribonucleoproteins synthesize both mRNA and cRNA in vitro. J Virol *81*, 2196-2204.

Wagner, R., Matrosovich, M., and Klenk, H.D. (2002). Functional balance between haemagglutinin and neuraminidase in influenza virus infections. Rev Med Virol *12*, 159-166.

Wallensten, A., Munster, V.J., Karlsson, M., Lundkvist, A., Brytting, M., Stervander, M., Osterhaus, A.D., Fouchier, R.A., and Olsen, B. (2006). High prevalence of influenza A virus in ducks caught during spring migration through Sweden. Vaccine *24*, 6734-6735.

Wang, W., Riedel, K., Lynch, P., Chien, C.Y., Montelione, G.T., and Krug, R.M. (1999). RNA binding by the novel helical domain of the influenza virus NS1 protein requires its dimer structure and a small number of specific basic amino acids. Rna *5*, 195-205.

Ware, L.B., and Matthay, M.A. (2000). The Acute Respiratory Distress Syndrome. New England Journal of Medicine *342*, 1334-1349.

Weber-Gerlach, M., and Weber, F. (2016). Standing on three legs: antiviral activities of RIG-I against influenza viruses. Curr Opin Immunol *42*, 71-75.

Weber, F. (2015). The catcher in the RIG-I. Cytokine *76*, 38-41.

Weber, F., Wagner, V., Rasmussen, S.B., Hartmann, R., and Paludan, S.R. (2006). Double-Stranded RNA Is Produced by Positive-Strand RNA Viruses and DNA Viruses but Not in Detectable Amounts by Negative-Strand RNA Viruses.

Weber, M., Gawanbacht, A., Habjan, M., Rang, A., Borner, C., Schmidt, Anna M., Veitinger, S., Jacob, R., Devignot, S., Kochs, G.*, et al.* (2013). Incoming RNA Virus Nucleocapsids Containing a 5′-Triphosphorylated Genome Activate RIG-I and Antiviral Signaling. Cell Host Microbe *13*, 336-346.

Weber, M., Sediri, H., Felgenhauer, U., Binzen, I., Bänfer, S., Jacob, R., Brunotte, L., García-Sastre, A., Schmid-Burgk, Jonathan L., Schmidt, T.*, et al.* (2015). Influenza Virus Adaptation PB2-627K Modulates Nucleocapsid Inhibition by the Pathogen Sensor RIG-I. Cell Host Microbe *17*, 309-319.

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*, 152-179.

Webster, R.G., Yakhno, M., Hinshaw, V.S., Bean, W.J., and Copal Murti, K. (1978). Intestinal influenza: Replication and characterization of influenza viruses in ducks. Virology *84*, 268-278.

Wei, L., Cui, J., Song, Y., Zhang, S., Han, F., Yuan, R., Gong, L., Jiao, P., and Liao, M. (2014). Duck MDA5 functions in innate immunity against H5N1 highly pathogenic avian influenza virus infections. Vet Res *45*, 66.

Wei, L., Jiao, P., Song, Y., Cao, L., Yuan, R., Gong, L., Cui, J., Zhang, S., Qi, W., Yang, S.*, et al.* (2013). Host immune responses of ducks infected with H5N1 highly pathogenic avian influenza viruses of different pathogenicities. Vet Microbiol *166*, 386-393.

Wharton, S.A., Belshe, R.B., Skehel, J.J., and Hay, A.J. (1994). Role of virion M2 protein in influenza virus uncoating: specific reduction in the rate of membrane fusion between virus and liposomes by amantadine. J Gen Virol *75 ( Pt 4)*, 945-948.

WHO (World Health Organization) (2018). Influenza (Avian and other zoonotic). <https://www.who.int/news-room/fact-sheets/detail/influenza-(avian-and-other-zoonotic)> Accessed on January 20, 2020.

Wille, M., Bröjer, C., Lundkvist, A., and Järhult, J.D. (2018) Alternate routes of influenza A virus infection in Mallard (*Anas platyrhynchos*). Vet Res *49*, 110.

Wise, H.M., Foeglein, A., Sun, J., Dalton, R.M., Patel, S., Howard, W., Anderson, E.C., Barclay, W.S., and Digard, P. (2009). A complicated message: Identification of a novel PB1-related protein translated from influenza A virus segment 2 mRNA. J Virol *83*, 8021-8031.

Wise, H.M., Hutchinson, E.C., Jagger, B.W., Stuart, A.D., Kang, Z.H., Robb, N., Schwartzman, L.M., Kash, J.C., Fodor, E., Firth, A.E.*, et al.* (2012). Identification of a novel splice variant form of the influenza A virus M2 ion channel with an antigenically distinct ectodomain. PLoS Pathog *8*, e1002998.

Wisskirchen, C., Ludersdorfer, T.H., Müller, D.A., Moritz, E., and Pavlovic, J. (2011). The Cellular RNA Helicase UAP56 Is Required for Prevention of Double-Stranded RNA Formation during Influenza A Virus Infection.

Woo, H.M., Lee, J.M., Kim, C.J., Lee, J.S., and Jeong, Y.J. (2019). Recovery of TRIM25-Mediated RIG-I Ubiquitination through Suppression of NS1 by RNA Aptamers. Mol Cells *42*, 721-728.

Wu, B., Peisley, A., Tetrault, D., Li, Z., Egelman, E.H., Magor, K.E., Walz, T., Penczek, P.A., and Hur, S. (2014). Molecular imprinting as a signal-activation mechanism of the viral RNA sensor RIG-I. Mol Cell *55*, 511-523.

Wu, W., Zhang, W., Booth, J.L., and Metcalf, J.P. (2012). Influenza A(H1N1)pdm09 Virus Suppresses RIG-I Initiated Innate Antiviral Responses in the Human Lung. PLoS ONE *7*, e49856.

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.

Wu, X., Yang, J., Na, T., Zhang, K., Davidoff, A.M., Yuan, B.Z., and Wang, Y. (2017). RIG-I and IL-6 are negative-feedback regulators of STING induced by double-stranded DNA. PLoS ONE *12*, e0182961.

Wyde, P.R., Wilson, M.R., and Cate, T.R. (1982). Interferon production by leukocytes infiltrating the lungs of mice during primary influenza virus infection. Infect Immun *38*, 1249-1255.

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*, 156-165.

Xu, J., Zhong, H.A., Madrahimov, A., Helikar, T., and Lu, G. (2014). Molecular phylogeny and evolutionary dynamics of influenza A nonstructural (NS) gene. Infect Genet Evol *22*, 192-200.

Xu, K., Klenk, C., Liu, B., Keiner, B., Cheng, J., Zheng, B.J., Li, L., Han, Q., Wang, C., Li, T.*, et al.* (2011). Modification of nonstructural protein 1 of influenza A virus by SUMO1. J Virol *85*, 1086-1098.

Xu, L.G., Wang, Y.Y., Han, K.J., Li, L.Y., Zhai, Z., and Shu, H.B. (2005). VISA is an adapter protein required for virus-triggered IFN-beta signaling. Mol Cell *19*, 727-740.

Yamada, S., Shinya, K., Takada, A., Ito, T., Suzuki, T., Suzuki, Y., Le, Q.M., Ebina, M., Kasai, N., Kida, H.*, et al.* (2012). Adaptation of a Duck Influenza A Virus in Quail. J Virol *86*, 1411-1420.

Ye, Z., Liu, T., Offringa, D.P., McInnis, J., and Levandowski, R.A. (1999). Association of influenza virus matrix protein with ribonucleoproteins. J Virol *73*, 7467-7473.

Yen, H.L., Liang, C.H., Wu, C.Y., Forrest, H.L., Ferguson, A., Choy, K.T., Jones, J., Wong, D.D., Cheung, P.P., Hsu, C.H.*, et al.* (2011). Hemagglutinin-neuraminidase balance confers respiratory-droplet transmissibility of the pandemic H1N1 influenza virus in ferrets. Proc Natl Acad Sci U S A *108*, 14264-14269.

Yoon, S.-W., Webby, R.J., and Webster, R.G. (2014). Evolution and ecology of influenza A viruses. Curr Top Microbiol Immunol *385*, 359-375.

Yoshizumi, T., Ichinohe, T., Sasaki, O., Otera, H., Kawabata, S., Mihara, K., and Koshiba, T. (2014). Influenza A virus protein PB1-F2 translocates into mitochondria via Tom40 channels and impairs innate immunity. Nat Commun *5*, 4713.

Yuen, K.Y., and Wong, S.S.Y. (2005). Human infection by avian influenza A H5N1. Hong Kong Med J *11*, 189-199.

Zell, R., Krumbholz, A., Eitner, A., Krieg, R., Halbhuber, K.J., and Wutzler, P. (2007). Prevalence of PB1-F2 of influenza A viruses. J Gen Virol *88*, 536-546.

Zeng, H., Goldsmith, C., Thawatsupha, P., Chittaganpitch, M., Waicharoen, S., Zaki, S., Tumpey, T.M., and Katz, J.M. (2007). Highly Pathogenic Avian Influenza H5N1 Viruses Elicit an Attenuated Type I Interferon Response in Polarized Human Bronchial Epithelial Cells. J Virol *81*, 12439-12449.

Zeng, W., Sun, L., Jiang, X., Chen, X., Hou, F., Adhikari, A., Xu, M., and Chen, Z.J. (2010). Reconstitution of the RIG-I Pathway Reveals a Pivotal Role of Unanchored Polyubiquitin Chains in Innate Immunity. Cell *141*, 315-330.

Zheng, L., Baumann, U., and Reymond, J.L. (2004). An efficient one-step site-directed and site-saturation mutagenesis protocol. Nucleic Acids Res *32*, e115.

Zhou, H., Jin, M., Chen, H., Huag, Q., and Yu, Z. (2006). Genome-sequence analysis of the pathogenic H5N1 avian influenza A virus isolated in China in 2004. Virus Genes *32*, 85-95.

Zhou, H., Zhu, J., Tu, J., Zou, W., Hu, Y., Yu, Z., Yin, W., Li, Y., Zhang, A., Wu, Y., *et al.* (2010). Effect on virulence and pathogenicity of H5N1 influenza A virus through truncations of NS1 eIF4GI binding domain. J Infect Dis *202*, 1338-1346.

Zhu, Q., Yang, H., Chen, W., Cao, W., Zhong, G., Jiao, P., Deng, G., Yu, K., Yang, C., Bu, Z., *et al.* (2008). A Naturally Occurring Deletion in Its NS Gene Contributes to the Attenuation of an H5N1 Swine Influenza Virus in Chickens. J Virol *82*, 220-228.

Zhu, W., Li, L., Yan, Z., Gan, T., Li, L., Chen, R., Chen, R., Zheng, Z., Hong, W., Wang, J., *et al.* (2015). Dual E627K and D701N mutations in the PB2 protein of A(H7N9) influenza virus increased its virulence in mammalian models. Sci Rep *5*, 14170.

Zielecki, F., Semmler, I., Kalthoff, D., Voss, D., Mauel, S., Gruber, A.D., Beer, M., and Wolff, T. (2010). Virulence determinants of avian H5N1 influenza A virus in mammalian and avian hosts: role of the C-terminal ESEV motif in the viral NS1 protein. J Virol *84*, 10708-10718.

Zohari, S., Gyarmati, P., Ejdersund, A., Berglof, U., Thoren, P., Ehrenberg, M., Czifra, G., Belak, S., Waldenstrom, J., Olsen, B., *et al.* (2008). Phylogenetic analysis of the non-structural (NS) gene of influenza A viruses isolated from mallards in Northern Europe in 2005. J Virol *5*, 147.

Zohari, S., Munir, M., Metreveli, G., Belak, S., and Berg, M. (2010). Differences in the ability to suppress interferon beta production between allele A and allele B NS1 proteins from H10 influenza A viruses. J Virol *7*, 376.

# Appendix

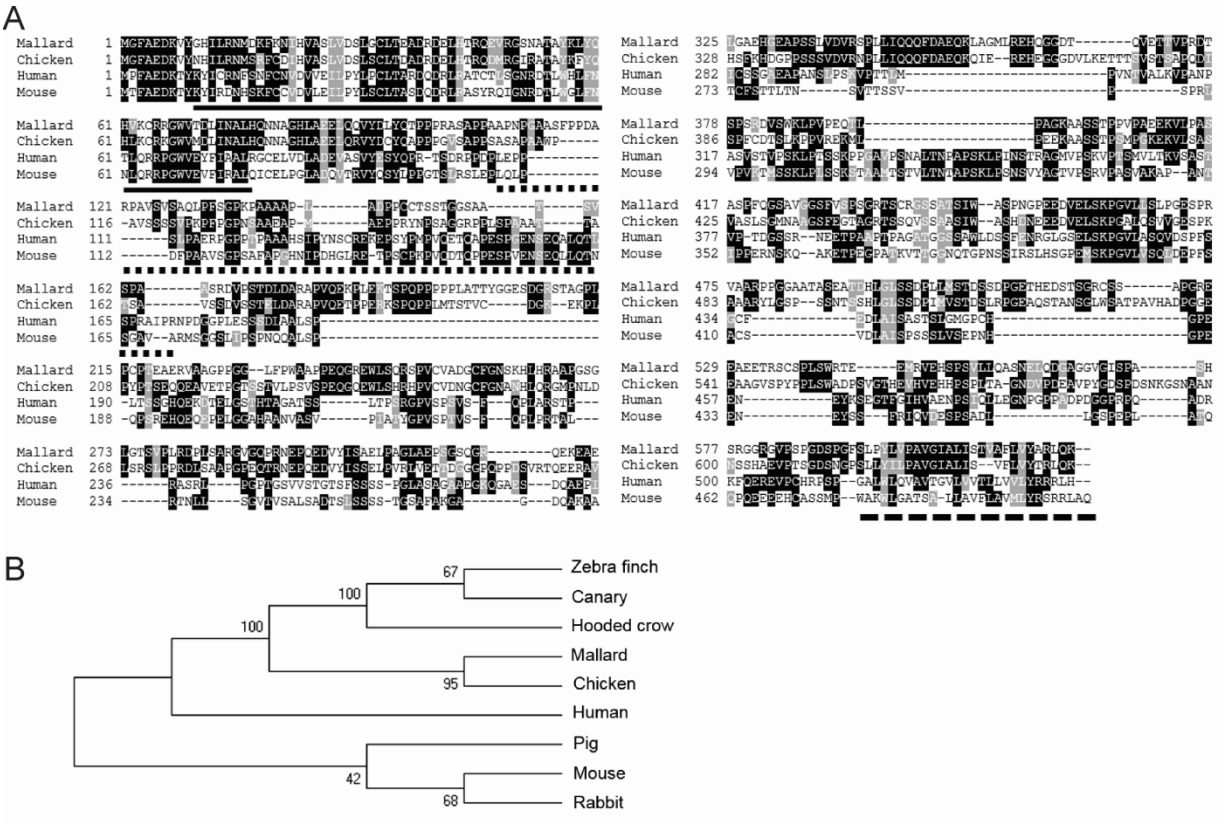



**Figure S1. MAVS sequences are poorly conserved between species.**
(A) Amino acid alignment of duck, chicken, human, and mouse MAVS proteins, made with Clustal Omega and edited with Boxshade. Black and grey shading indicates identical and similar amino acids (50% threshold). The solid line, dotted line, and dashed line indicate the CARD, proline-rich region, and transmembrane domains, respectively. (B) Phylogenetic analysis of MAVS proteins by the Neighbor-Joining method using MEGA7. The bootstrap consensus tree was inferred using the Neighbor-Joining method from 1000 replicates. *This figure was made by Dr. Yanna Xiao.*

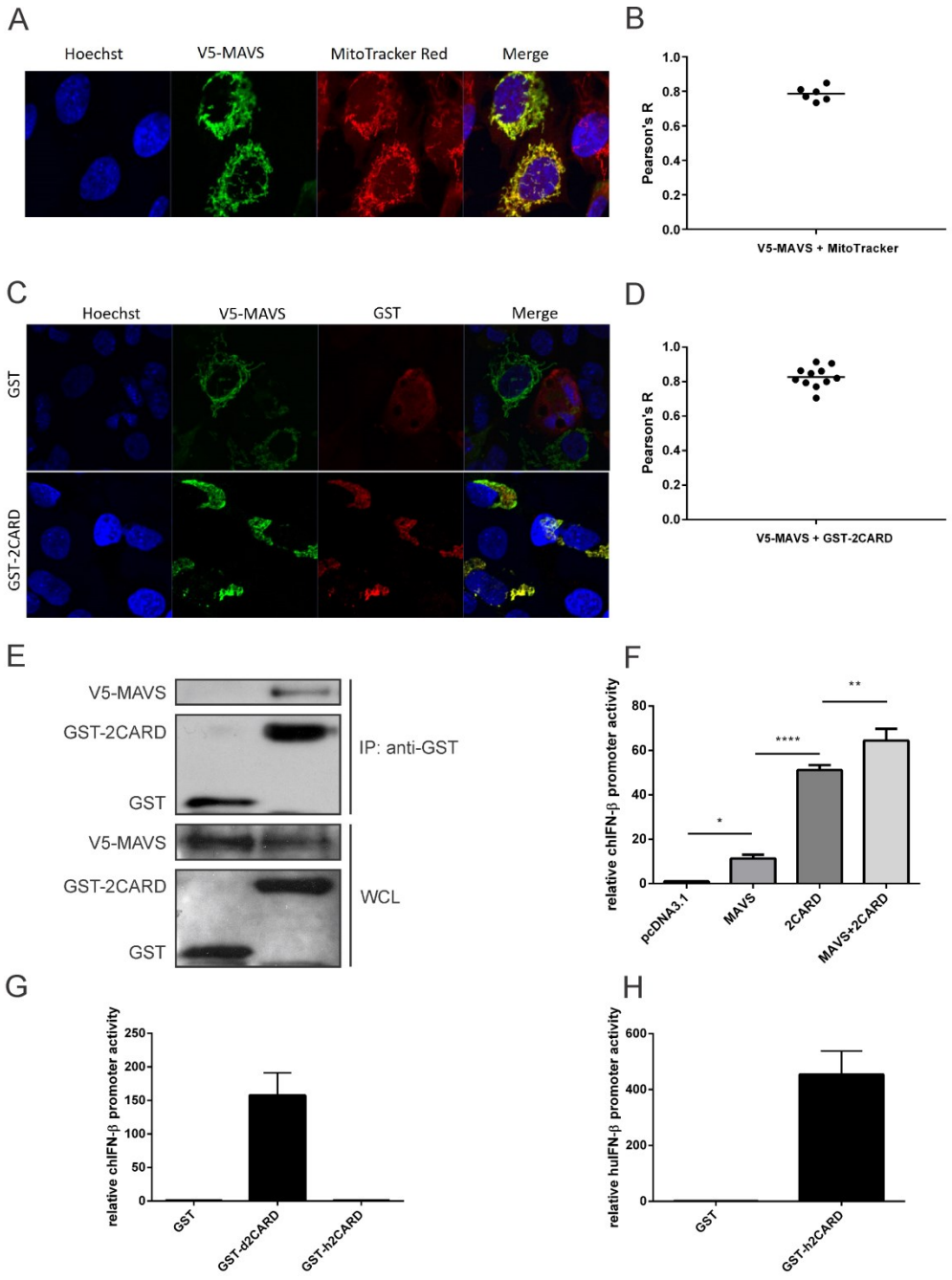
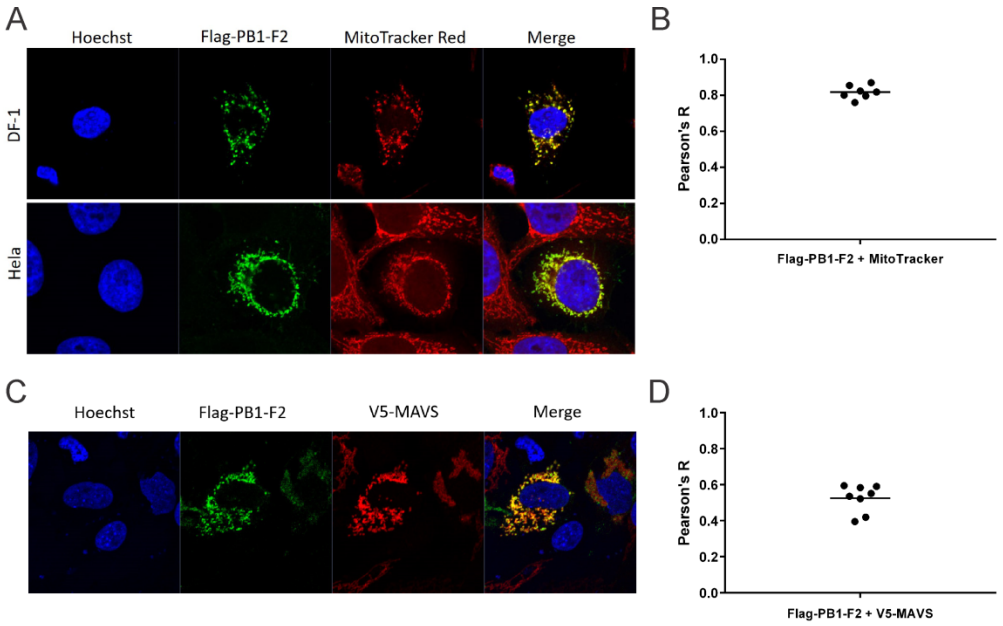



**Figure S2. Duck MAVS localizes to mitochondria and interacts with duck RIG-I CARD domains in DF-1 cells.**

(A) DF-1 cells overexpressing duck MAVS (V5-MAVS, green) were also stained to show mitochondria (MitoTracker Red) and nuclei (blue). (B) Co-localization of V5-MAVS with mitochondria was quantified using Pearson's correlation coefficient (Pearson's R). Bar shows mean value from 6 analyzed cells. (C) DF-1 cells overexpressing duck MAVS and GST-tagged duck RIG-I CARD domains (GST-2CARD) or GST alone were stained for GST (red) and V5-MAVS (green). (D) Co-localization of V5-MAVS with GST-2CARD was quantified using

Pearson's correlation coefficient (Pearson's R). Bar shows mean value from 11 analyzed cells. (E) DF-1 cells overexpressing V5-MAVS and GST-2CARD were lysed. Clarified whole cell lysates (WCL) were subjected to GST pulldown (anti-GST) and blotted with anti-GST and anti-V5 antibodies. (F) Duck MAVS and RIG-I-CARD overexpression promotes chicken IFN-β promoter activity in DF-1 cells as measured by dual luciferase assay. Graph bars show mean ± standard deviation of three independent experiments (n=3). Means were compared using one-way ANOVA with a Tukey's multiple comparisons post-hoc test (*, $p \leq 0.05$; **, $P \leq 0.01$; ***, $P \leq 0.001$). (G) In chicken DF-1 cells, duck RIG-I CARD domains (GST-d2CARD) but not human RIG-I CARD domains (GST-h2CARD) stimulate chIFN-β activity as measured by dual luciferase assay. (H) GST-h2CARD is functional in human AD293T cells and promoter activity of the huIFN-β reporter vector was measured by dual luciferase assay as above. *This figure was made by Dr. Yanna Xiao.*


A
Hoechst
Flag-PB1-F2
MitoTracker Red
Merge
DF-1
Hela
B
Pearson's R
1.0
0.8
0.6
0.4
0.2
0.0
Flag-PB1-F2 + MitoTracker
C
Hoechst
Flag-PB1-F2
V5-MAVS
Merge
D
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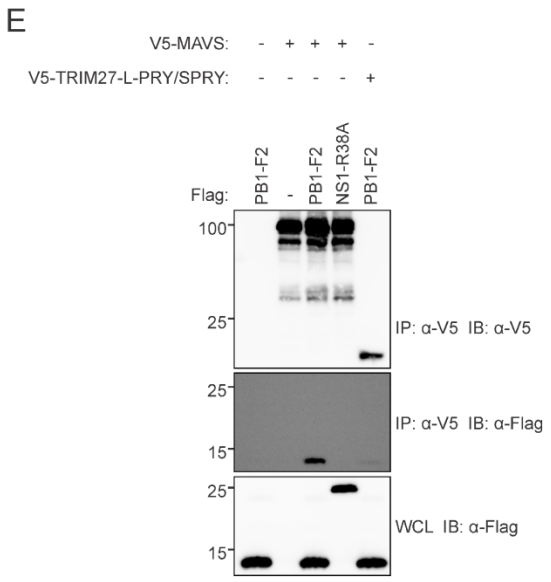
0.8
0.6
0.4
0.2
0.0
Flag-PB1-F2 + V5-MAVS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V5-MAVS: - + + + -
V5-TRIM27-L-PRY/SPRY: - - - - +
Flag: PB1-F2 - PB1-F2 NS1-R38A PB1-F2
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IP: α-V5 IB: α-V5
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IP: α-V5 IB: α-Flag
25
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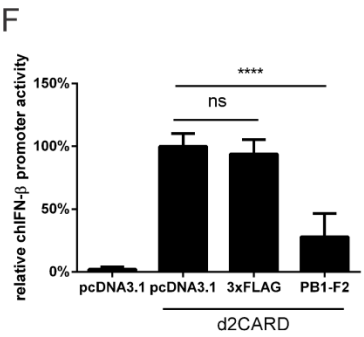
WCL IB: α-Flag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relative chIFN-β promoter activity
150%
100%
50%
0%
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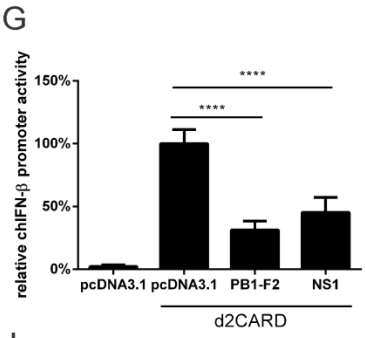
pcDNA3.1 pcDNA3.1 3xFLAG PB1-F2
d2CARD


G
relative chIFN-β promoter activity
150%
100%
50%
0%
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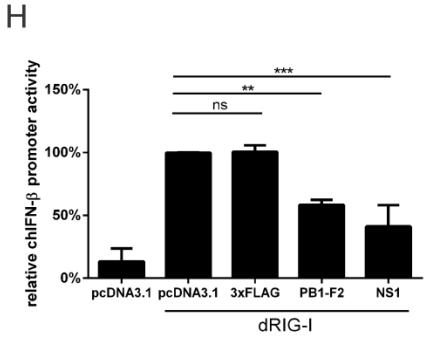
pcDNA3.1 pcDNA3.1 PB1-F2 NS1
d2CARD


H
relative chIFN-β promoter activity
150%
100%
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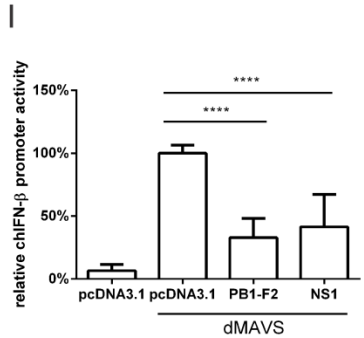
ns
pcDNA3.1 pcDNA3.1 3xFLAG PB1-F2 NS1
dRIG-I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pcDNA3.1 pcDNA3.1 PB1-F2 NS1
dMAVS

**Figure S3. PR8 PB1-F2 co-localizes with duck MAVS and inhibits innate signaling activity in DF-1 cells.**

(A) Mitochondrial localization of PB1-F2 in DF-1 and Hela cells overexpressing Flag-PB1-F2 (anti-Flag, green) and stained with MitoTracker Red. (B) Co-localization of PB1-F2 with MitoTracker in DF-1 cells was quantified using Pearson's correlation coefficient (Pearson's R). Bar shows mean value from seven analyzed cells. (C) DF-1 cells overexpressing Flag-PB1-F2 and duck MAVS (V5-MAVS). (D) Co-localization of V5-MAVS with Flag-PB1-F2 was quantified using Pearson's correlation coefficient (Pearson's R). Bar shows mean value from eight analyzed cells. (E) DF-1 cells were transfected with V5-MAVS or a V5-tagged fragment of duck TRIM27-L (V5-TRIM27-L-PRY/SPRY), along with Flag-tagged PB1-F2 or Flag-tagged R38A-mutant PR8 NS1 (NS1-R38A), as indicated. Clarified whole cell lysates (WCL) were subjected to anti-V5 immunoprecipitation (IP), followed by immunoblotting (IB) with anti-V5 and anti-Flag antibodies. (F and G) PB1-F2, but not empty vector (pcDNA3.1) or the Flag tag (3xFlag), inhibits chicken IFN-β promoter activity downstream of duck RIG-I CARD domains (2CARD) in DF-1 cells, by dual luciferase assay. (H) PB1-F2, but not empty vector (pcDNA3.1) or the Flag tag (3xFlag), inhibits chicken IFN-β promoter activity downstream of full-length duck RIG-I (dRIG-I) in DF-1 cells, by dual luciferase assay. (I) PB1-F2 inhibits chicken IFN-β promoter activity downstream of duck MAVS (dMAVS) in DF-1 cells, by dual luciferase assay. Graph bars show mean ± standard deviation of three independent experiments (n=3). Means were compared using one-way ANOVA with a Tukey's multiple comparisons post-hoc test (**, $P \leq 0.01$; ***, $P \leq 0.001$; ****, $P \leq 0.0001$; ns, non-significant). Total transfected DNA quantity was always kept constant with empty vector. *Panel (E) in this figure was made by me, the rest were made by Dr. Yanna Xiao and Adam Moghrabi.*