Stable knock-in of duck RIG-I into DF-1 chicken cells via CRISPR/Cas9 homology directed repair

by

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Abstract

Chickens and ducks differ in their susceptibility to avian influenza virus infection. Ducks are the primordial hosts and reservoir of avian influenza A viruses. Mallard ducks can asymptomatically harbour high-pathogenicity avian influenza (HPAI) and low-pathogenicity avian influenza (LPAI). Chickens, however, succumb to HPAI infection and die within 1-2 days. Ducks possess the innate immune RNA sensor retinoic acid-inducible gene I (RIG-I), whereas chickens do not. The lack of this key receptor is likely a major factor in the pronounced susceptibility of chickens to influenza A viruses. Here we insert the duck RIG-I gene into the chicken genome, using a CRISPR/Cas 9 approach in the chicken fibroblast cell line, DF-1. For a knock-in locus we chose a region on the chicken Z chromosome, homologous to where RIG-I is found in ducks. To achieve the desired knock-in we used a two-plasmid system. Firstly, a commercial cas9 expression plasmid was modified for use in avian cells. A homology directed repair plasmid was constructed to supply a knock-in template, consisting of duck RIG-I under the control of its own promoter and GFP under the control of the hCMV promoter. DF-1 cells were co-transfected with both plasmids, and successful transfectants were sorted via FACS. Sorted cells then underwent a negative selection process utilizing the human HSV thymidine kinase/ganciclovir inducible suicide gene system to kill cells with off-target knock-ins expressing HSV thymidine kinase. Genomic DNA was harvested from successfully selected cells and each resultant cell line was characterized by genomic PCR, rtPCR, and qPCR for the expression of duck RIG-I.

All cell lines recovered showed transgenic duck RIG-I and HSV thymidine kinase in genomic DNA suggesting a knock-in had occurred. However, no expression of duck

RIG-I or HSV thymidine kinase was observed by rtPCR or qPCR, and expression of GFP was silenced after ~7 days post transfection. We therefore cannot conclude whether the knock-in of duck RIG-I was on target. Despite the lack of transgene expression our work is a proof of the principle that large fragments of foreign DNA comprised of multiple protein coding genes each with a different promoter can be transfected into DF-1 cells. The production of a transgenic chicken cell line expressing duck RIG-I has the potential to be a vital tool in researching innate immunity in birds.

Preface

The experiments and data used to create Figure 3.8 were performed by Jordyn Pelechaty, an undergraduate student under my supervision.

The 500bp duck RIG-I promoter was obtained from Dr Yanna Xiao as published in 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 duck RIG-I mRNA coding sequence was obtained from Megan Barber as published in 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. 107: 5913–5918.

This thesis is an original work by Eric Friesen.

Dedication

I dedicate this work to my mother Dorothy and my father Don, who instilled in me a love and curiosity for the natural world and gave me the bravery to pursue knowledge of it.

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List of Abbreviations

ATP	Adenosine Triphosphate
bp	Base Pair
CARD	Caspase Activation and Recruitment Domain
cDNA	Complementary DNA
CRISPR	Clusters of Regularly Interspaced Palindromic Repeats
Cas9	CRISPR Associated protein
CTD	C- Terminal Domain
DAMPs	Damage Associated Molecular Patterns
DF-1	Douglas Foster 1 – Chicken Embryonic Fibroblast cell line
DNA	Deoxyribonucleic acid
DSB	Double Stranded Break
dsDNA	Double Stranded DNA
dsRNA	Double Stranded RNA
FACS	Fluorescence Activated Cell Sorting
GCV	Ganciclovir
GFP	Green Fluorescence Protein
GOI	Gene Of Interest
HA	Hemagglutinin
HBV	Hepatitis B Virus
HCC	Hepatocellular Carcinoma Cells
hCMV	Human Cytomegalovirus

HDR	Homology Directed Repair
HPAI	Highly Pathogenic Avian Influenza
HSV	Herpes Simplex Virus
IAV	Influenza A Virus
Indels	Insertions/deletions
ISG	Interferon Stimulated Gene
IFN	Interferon
kb	Kilobase
LGP2	Laboratory of Genetics and Physiology 2
LPAI	Low Pathogenicity Avian Influenza
LRR	Leucine Rich Repeat
MAVS	Mitochondrial Antiviral Signalling Protein
MDA5	Melanoma Differentiation Associated protein 5
mRNA	Messenger RNA
NA	Neuraminidase
NHEJ	Non-Homologous End Joining
NLR	NOD-Like Receptor
NLRP3	NLR family Pyrin domain containing 3
NOD2	Nucleotide-binding Oligomerization Domain-containing protein 2
nt	Nucleotide
PAMPs	Pathogen Associated Molecular Patterns
PBMCs	Peripheral Blood Mononuclear Cells
PCR	Polymerase Chain Reaction

PGC	Primordial Germ Cell
PRR	Pattern Recognition Receptor
qPCR	Quantitative PCR
RIG-I	Retinoic acid-Inducible Gene I
RLR	RIG-I-Like Receptor
RNA	Ribonucleic Acid
rtPCR	Reverse Transcriptase PCR
ssRNA	Single-Stranded RNA
sgRNA	Small Guide RNA
TK	Thymidine Kinase
TLR	Toll-Like Receptor
UTR	Untranslated Region
vRNA	Viral RNA
vRNP	Viral Ribonuclear Protein

Chapter 1 Introduction and Review of Literature.

1.1 A brief introduction to Influenza A viruses.

Influenza A viruses (IAV) are one of five genera that make up the *Orthomyxoviridae* family of viruses[1]. The IAV genome is divided into eight distinct segments composed of antisense single stranded ribonucleic acid (RNA) that encode 10-13 proteins of varying function[2,3,4] with additional accessory proteins dependent on strain[5]. Of these 10-13 proteins, two surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) comprise the major sites of antigenic recognition in an infected host. Both HA and NA have different isoforms in nature. There are currently 18 HA and 11 NA subtypes known[6]. H1-H16 and N1-N9 are found in wild birds, while H17N10 and H18N11 have more recently been identified in wild bats[7,8]. Differences in the isoforms of these antigenic glycoproteins are the basis for IAV classifications. The differences in HA and NA between viral subtypes allow for transmission and infection of different species, however almost all known virus subtypes circulate in wild aquatic birds[2]. IAV primarily affects mammals and birds, however recent evidence may suggest that amphibians can also host IAV[9]. Species that regularly interact with humans are of particular interest when studying IAV, as transmission between humans and domesticated or agricultural animals can often result in epidemic or pandemic outbreaks of influenza[10,11,12]. Along with differential hosts and patterns of transmission, different viral subtypes often diverge in the pathogenicity and severity of symptoms observed in infected hosts[2]. H5 and H7 viral subtypes are of particular concern as these are the two subtypes that can lead to infections resulting in high mortality rates of infected hosts, including humans, but especially high mortality rates in birds[2,3,13].

Although each subtype of IAV has different combinations of HA and NA isoforms, all eight segments of viral RNA (vRNA) contain complementary sequences of 12-13 nucleotides (nt) at their 3′ and 5′ ends which allow for the formation of a panhandle secondary structure[14]. The function of this RNA panhandle is to act as a promoter region for transcription of viral mRNA and replication of complementary vRNA[12,15,16]. These processes are necessary for the function of the viral "life cycle" and are therefore conserved structures in all viral subtypes. Furthermore, the conserved nature of the panhandle structure provides an ideal target for host immune defenses to recognize[13,14,17].

1.2 The reservoir host of influenza viruses and innate antiviral immunity.

Mallard ducks (*Anas platyrhynchos*) and other aquatic birds are the reservoir host to nearly all known circulating influenza strains[2]. Ducks are known to harbour 16 of all 18 HA subtypes and 9 of all 11 NA subtypes[2,3,5]. They can carry one, or a combination of different strains of IAV with little to no associated pathologies or illnesses[2]. However, if viral load becomes too high, or a particularly virulent strain of IAV is present, ducks can succumb to IAV infection[18,19]. The ability of ducks to asymptomatically harbour IAV, and their nature as migratory birds as well as agricultural stocks has allowed them to become major vectors for the transmission of many IAV strains to both humans and other agricultural livestock and this ability to transmit has earned them the nickname "Trojan horses" of influenza[18].

Avian influenza viruses are generally split into two categories, highly pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI)[2]. The HPAI classification is based on the ability of certain viral subtypes (H5, H7) to cause lethal

plague-like infections in chickens, while LPAI encompasses all other virulent subtypes that tend not to cause plague-like infections[2].The exact mechanism, or combination of mechanisms, that allow ducks to harbour both HPAI and LPAI remain elusive.

1.2.1 Toll-like receptors.

Toll-like receptors are one of the three key families of innate immune pattern recognition receptors. Toll-like receptors are an ancient family of innate immune sensors. TLRs are present in a wide variety of organisms from the fruit fly in which they were discovered, to modern humans[20]. The TLR family of receptors recognize a vast array of pathogen associated molecular patterns (PAMPs) from nearly all pathogenic organisms including viruses, bacteria, fungus, and eukaryotes[20]. TLRs are well established as initiators of both the innate and adaptive immune responses in most eukaryotes[20,21]. In ducks TLRs play a central role in the detection of pathogens[22,23]. In most vertebrates TLR7 and TLR 8 both recognize single stranded RNA (ssRNA) while TLR 3 recognizes double-stranded RNA (dsRNA), however TLR 8 is absent in ducks and chickens[24,25]. TLR7/8 are involved in detection of ssRNA viruses, including IAV, as they are endosomal resident PRRs and detect viral RNA after infection [23,25]. Both TRL7/8 signal through the adapter protein MyD88 which activates the NF-κB complex[26,27,28]. NF-κB is a ubiquitously expressed transcription factor that upregulates the production of inflammatory cytokines and upregulates interferon stimulated genes (ISGs) which function as innate and adaptive immune modulators of antiviral defences[20,29]. Some ISGs induced by the NF-κB transcription factor include *Mx-1* which inhibits viral transcription[30], *TRIM25* which activates retinoic acid-inducible gene I (RIG-I) via ubiquitination[31], and *IRF7* which stimulates further ISGs including type I interferons

(IFNs)[32]. Expression of these ISGs can also be induced through IRF signalling initiated by the RIG-I signalling pathway[93].

1.2.2 NOD-like receptors.

NOD-like receptors are the second crucial family of innate immune pattern recognition receptors. In addition to TLRs, the NOD-like receptor family of proteins are important players in innate antiviral defences[33]. Although NLRs are important in antiviral signalling and initiation of host inflammasome complexes, they can recognize a diverse array of PAMPs including bacterial flagellin, viral RNA, and fungal hyphae[34,35,36]. In addition to PAMPs, NLRs can also detect damage associated molecular patterns (DAMPs) produced by host cells in response to pathogen mediated or mechanical damage[37]. They can also trigger inflammation by the detection of environmental threats such as inorganic nanoparticles[38]. Nucleotide-binding oligomerization domain-containing protein 2 (NOD2) is a member of the NLR family. NOD2 has yet to be reported in the genome of *Anas platyrhynchos* or other species of duck, or in chickens[39]. NOD2 has been well characterized in mammals and has been shown to play an important role in innate immune signalling[33,40]. NOD2 is comprised of a C-terminal leucine rich repeat (LRR) domain, a central ATPase domain, and twin N-terminal CARD domains that function in signal transduction[33,41]. NOD2 is able to recognize and bind viral ssRNA through its LRR domain and activate IRF3 and IFN-β defences[33,42]. NOD2 does not directly signal the activation of type I interferons, but uses the adapter mitochondrial antiviral signalling protein (MAVS) to activate the NF-κB signalling pathway[42]. The tandem activation of both IRF3 and NF-κB by NOD2 allows for optimal production of a type I interferon response.

The most versatile NLR capable of detection of viral infection and initiation of host defences is NLR family pyrin domain containing protein 3 (NLRP3)[43]. There are two signals required for NLRP3 activation, an initial priming signal that upregulates NLRP3 production; and a second triggering signal that initiates inflammasome formation and release of cytokines[43,44,38]. The exact mechanism by which NLRP3 detects IAV remains unclear, however it has been shown that NLRP3 can be activated by M2, an IAV proton specific ion channel[45]. M2 is required for both acidification of virions as well as neutralization of the host cell *trans*-Golgi network during influenza infection[46]. The NLRP3 inflammasome can also be activated by the viral protein PB1-F2[47]. PB1-F2 is a non-structural protein identified as a virulence factor which disrupts mitochondrial function leading to cell death in infected cells[48]. In addition to detecting viral protein, NLRP3 can be activated by the detection of viral RNA[49]. In general, the activation of NLRP3 through viral RNA or viral protein both serve to initiate formation of the inflammasome complex and begin the process of inflammation to help defend against IAV infection.

1.3 Inflammation, cytokines, and the dangers of antiviral signalling.

Broadly speaking there are two kinds of inflammation, acute inflammation and chronic inflammation[50]. Acute inflammation is a process that is not specific to any one kind of insult or injury[51]. Rather it can be initiated through pathogen infection, mechanical injury, chemical exposure, or through inter/intra-cellular interactions[50]. Acute inflammation is characterized by increased vascular permeability facilitating increased movement of neutrophils, macrophages, and other peripheral blood mononuclear cells (PBMCs) and granulocytes to sites of insult or injury[52]. These cells then fulfill a dual

purpose, they seek out and destroy any invading pathogens they can recognize, and they release cytokines to signal surrounding cells and tissues of the perceived threat[52]. In addition to leakage of cellular components from circulation, plasma components including complement molecules, cytokines, and antibodies are also introduced into the site of insult, all with diverse protective functions[52].

At the molecular level acute inflammation usually involves a rapid and marked increase in cytokines and signalling molecules both in the extracellular milieu and within affected cells[52,53]. The combined effect of inflammation at the tissue, cell, and molecular level can sometimes be of detriment to the host. An excessive production of immune signalling cytokines and prolonged inflammation is thought to be a significant contributing factor to influenza morbidity in a wide range of susceptible species[54,55,56].

In the case of influenza and avian species, inflammation manifests differently between ducks and chickens depending on whether they are exposed to HPAI or LPAI[56]. Briefly, when ducks are infected with LPAI, weak immune gene upregulation, some inflammation, and minimal tissue damage is observed. Of the tissues affected, inflammation has been noted, although variably, in the lung, spleen, heart, and brain of most individuals; though the extent of inflammation and therefore tissue damage varies with species, viral strain, and individual genetics[57,58]. Ducks infected with HPAI can have a range of outcomes depending on the exact species of duck, and the exact strain of virus in each given infection[58,57]. Generally, ducks can survive and recover from HPAI infection though studies have shown they are not entirely impervious to IAV[57].

Chickens infected with HPAI display a more severe set of symptoms than ducks do. Briefly, chickens infected with HPAI typically die with 24 hours to 3 days post

infection[59]. Symptoms of HPAI viral infection can include: discolouration and sheen, change of visceral organs, haemorrhage and inflammation of perivascular blood vessels, myocarditis, necrosis and inflammation of the lung, liver, pancreas, and adrenal gland, depletion of splenic lymphocytes, thymic atrophy, necrosis and inflammation of skin, and necrosis and meningoencephalitis of the central nervous system[60], any number of which can be lethal.

Chickens infected with LPAI still display symptoms more prominently than ducks but to a lesser degree than when infected with HPAI. Briefly, when infected with LPAI chickens can display: congestion and infiltration of tracheal leukocytes, infiltration and congestion of pulmonary leukocytes followed by lesions in the lungs, swelling of kidney tubules and glomeruli, loss of body weight, decrease in egg production, bronchitis, tracheitis, sinusitis, proventricular haemorrhage, and secondary infections such as pneumonia[61,62]. The various symptoms of swelling, oedema, leukocyte infiltration, and necrosis can in large part be attributed to overly active inflammatory responses to viral infection[63]. Immune responses that are detrimental to the host such as these are referred to as cytokine storms, in which dysregulated immune responses culminate in increased morbidity from infection[64]. Innate immune signalling is therefore of chief importance in examining IAV and its associated effects on chickens and ducks. Of note in the difference in innate signalling between ducks and chickens is the RLR family of receptors.

1.4 RLRs and RIG-I.

Ducks employ a comprehensive suite of innate immune defences to address infection by IAV, chief among which is the RIG-I like receptor family (RLRs), in addition to TLRs and NLRs. The third class of innate immune PRRs found in ducks is the RLRs family, named for retinoic acid-inducible gene I (RIG-I) a key ssRNA sensor involved in anti-IAV signalling. The RLR family of PRRs is composed of three proteins, LGP2, MDA5, and RIG-I.

1.4.1 RIG-I.

Retinoic acid inducible gene I is an innate immune sensor of 5′ppp-ssRNA and the namesake receptor of the RLR family of proteins. The RIG-I protein consists of three domains, a C-terminal repressor domain (CTD), a central RNA-binding DExD/H box helicase domain and a pair of N-terminal tandem caspase activation and recruitment (CARD) domains[65]. Human and duck RIG-I share protein similarity, in humans RIG-I is 925 residues and a size of 106.62 kDa[66], and in ducks RIG-I is 933 residues and a predicted size of 106.60 kDa[67]. The CTD is involved in the recognition of both 5′OH-RNA and 5′-pppRNA, however the binding efficiency to 5′OH is significantly less than 5′-ppp[68]. Furthermore, it has been shown that the CTD of RIG-I is able to recognize a diverse range of substrates[68,69,70,71]. The binding of dsRNA, ssRNA, blunt-end RNA, OH-RNA, dsDNA, and ppp-RNA by the CTD of RIG-I explains in part the central role of RIG-I as critical receptor in the detection of not only IAVs but a wide range of viral pathogens. It has also been noted that the terminal modifications of RNA themselves are not the sole requirement for CTD binding or activation and that a “panhandle” structure is

necessary for full activation of RIG-I[70]. Panhandle structures of viral genomes generally arise from 12 and 13 nucleotide complementarities between the 3′ and 5′ ends of viral RNA resulting in the formation of a bulged panhandle structure[72]. This panhandle structure serves as a promoter for the initiation of viral RNA transcription[73]. The conservation and critical function of this panhandle makes for an ideal target for recognition by PRRs. More recent work has shown that recognition of the panhandle structure of influenza is indeed necessary for the full activation of RIG-I and induction of interferons as an antiviral defence[74].

RIG-I is a predominantly cytosolic receptor that is maintained at low levels of basal expression and upregulated when viral infection is detected[76]. The promoter and transcription binding sites of duck RIG-I have been recently characterized and suggest that RIG-I is upregulated via a positive-feedback loop[75]. The activation of transcription factors that modulate RIG-I is achieved by the earlier activation of RIG-I itself as well as by type I IFNs stimulated through MDA5 and MAVS signalling.

Recently it has been shown in human cells that a pool of RIG-I is maintained in the nucleus of host cells to detect viral RNA transcripts and viral ribonucleoproteins (vRNP) prior to their exiting the nucleus[76]. This nuclear pool of RIG-I appears to detect invading virus independently of the cytosolic receptor but works in concert with the cytosolic pool to contribute to a stronger induced antiviral signalling cascade[76]. Whether the two pools of RIG-I are a result of translocation of proteins between cellular compartments, or both are independently maintained sentinels remains unclear[76]. In either case the combined function of nuclear and cytoplasmic RIG-I detection of vRNA and

vRNP appears to allow for the detection of viral infection at multiple timepoints during infection.

In avian species RIG-I is thought to be activated and function in much the same way as it does in mammals. In ducks, RIG-I is upregulated following infection by both HPAI and LPAI[67,77]. It has also been shown that duck RIG-I can respond to infection by live virus as well as synthetic viral ligands[67,77]. Duck RIG-I has been shown to upregulate downstream effector genes similar to those genes upregulated by RIG-I in mice infected with RNA viruses[78,79]. The functional antiviral signalling of duck RIG-I has been reported in multiple species, mallard (*Anas platyrhynchos*)[67], and in Muscovy duck (*Cairina moschata*)[80]. Chickens, which lack RIG-I, have been shown to lack an IFN response to 5′-pppRNA, while initiating an IFN response to poly (I:C) suggesting that while they cannot signal through RIG-I they can signal through MDA5[67]. Furthermore, chicken cells made to express duck RIG-I have demonstrated an ability to signal through RIG-I similar to that observed in ducks[67].

1.4.2 MDA5.

The second RLR involved in innate antiviral signalling is MDA5 (melanoma differentiation-associated protein 5). MDA5 is a DExD/H box helicase protein composed of 3 domains, a C-terminal domain and DEAD box helicase domain, however MDA5 CTD does not appear to recognize ssRNA and binds only dsRNA[81,82]. The third domain is a tandem caspase activation and recruitment domain[82]. The CARD of MDA5 shares a similar function to most CARDs and facilitates protein-protein interactions required for protein activation or signalling cascades[82].

The helicase of MDA5 not only preferentially recognizes dsRNA over ssRNA or 5′ppp-ssRNA but it preferentially recognizes and binds dsRNA of a particular length[83]. MDA5 differentiates the length of invading dsRNA by binding to dsRNA and cooperatively forming MDA5 filaments[84]. The formation of MDA5 filaments along dsRNA is important for the adenosine triphosphate (ATP) hydrolysis function of MDA5, and further ATP hydrolysis impacts the stability and continuity of bound MDA5/dsRNA filaments[84]. ATP hydrolysis leads to efficient assembly and slower disassembly of MDA5/dsRNA filaments which may allow for more signalling interactions than MDA5/dsRNA filaments that rapidly dissociate[84]. The impact of length of dsRNA on MDA5 filament formation and ATP hydrolysis is a likely explanation for the property of dsRNA length dependent signalling of MDA5[84]. The formation of MDA5 filaments is also important for signal transduction. The oligomerization of MDA5 allows for MDA5 CARD domains to be brought into close proximity, however this is insufficient for CARD oligomerization alongside MDA5 filaments[85]. The ATP hydrolytic activity of MDA5 filaments allows for the formation of CARD oligomers attached to the core MDA5 filament by a ~100 amino acid linker sequence, which allows for the stochastic assembly of CARD oligomers of up to 11 units[85]. These CARD oligomers along with MDA5 filaments are then able to act on the CARD of MAVS and initiate signalling through the MAVS pathway[85]. The ability of MDA5 to sense and preferentially bind to dsRNAs of varying length and still activate MAVS is a key component to the versatility of MDA5 to sense and initiate immune responses to a variety of different viruses. Furthermore, the ability of MDA5 to sense dsRNA of a size that also activates duck RIG-I[84] allows for a small amount of redundancy in antiviral signalling.

In avian species MDA5 has been shown to function similarly to its mammalian counterpart. In both Muscovy and mallard ducks MDA5 is upregulated following infection with IAV[86,77]. In chicken cells MDA5 has been shown to respond to both HPAI and LPAI infection and initiate immune signalling in the absence of RIG-I[87,78]. Furthermore, chicken MDA5 can recognize both long and short form poly(I:C) and therefore initiate the MAVS signalling cascade in response to multiple ligands similarly to its role in mammalian signalling[88].

1.4.3 LGP2.

LGP2 (laboratory of genetics and physiology 2) is a DExD/H-box helicase of the RLR family of innate immune receptors that can bind to ssRNA with and dsRNA[89,90]. The LGP2 protein consists of two domains, a CTD that can bind dsRNA and 5′ppp-ssRNA[82], and a DEAD box helicase like domain that is likely involved in ATP recognition and hydrolysis, RNA binding, and recognition and binding of other similar helicase domains[89]. LGP2 is present in resting cells at a low basal level, yet can be induced and upregulated if a host cell senses infection by invading virus[91,92]. LGP2 is an IFN-stimulated gene, and as a result is activated by RIG-I and other IFN stimulating pathways, implying that LGP2 plays an active role in the feedback maintenance of anti-viral signalling pathways[92].

The exact role of LGP2 in innate immunity remains unclear, however LGP2 is known to be a modulator of the RIG-I and MDA5 signalling pathway. LGP2 can reportedly play a dual role in the control of RLR signalling depending on the context in which it is activated. When a host cell contains viral dsRNA or 5′ppp-ssRNA, LGP2 can

negatively regulate RIG-I activity by either competing for the available ligand and sequestering it, due to a slightly higher binding affinity than RIG-I[93]; or by directly inhibiting RIG-I signalling by directly binding to the RIG-I helicase domain[92]. It is worth noting that LGP2 has a higher affinity for dsRNA than for 5′ppp-ssRNA implying that RIG-I will be the majority PRR to recognize 5′ppp-ssRNA while LGP2 will compete incompletely for available ligand. In addition, MDA5 is not directly repressed by LGP2 as RIG-I is, even though MDA5 and RIG-I share similar helicase domains[92].

MDA5 is reportedly activated by LGP2 and does not share the same feedback inhibition as RIG-I. However, LGP2 has been shown to activate MDA5 at low concentrations but act as an inhibitor at high concentrations[94]. LGP2 has also been shown to bind available MDA5 ligand thereby potentiating MDA5 signalling, and may play a role in preventing MDA5 polymer formation[94,95,96]. It is unknown precisely when and by what exact mechanisms LGP2 will act to potentiate the downstream signalling events of RIG-I and MDA5, regardless LGP2 appears to play a central role in innate anti-viral signalling. This multifunctional role is evidenced by the ability of LGP2 to inhibit the Dicer complex in mammalian cells, thereby inhibiting RNAi antiviral activity[97]. It has been suggested that the inhibition of RNAi is useful in complex cell types to allow for optimal activity of interferon based antiviral mechanisms[98].

1.4.4 The RIG-I signalling pathway in brief.

Basally expressed RIG-I proteins in the cytoplasm detect and bind to the 5′-pppRNA panhandle structure of Influenza A genomic RNA. Through an ATP-dependent process monomers of bound RIG-I oligomerize[99]. The oligomerization of RNA bound

RIG-I facilitates a change in RIG-I structure. The conformation of unbound RIG-I is such that the 2CARD domain is held inactive by interactions with the central helicase domain[66]. The binding of vRNA is sufficient to release the 2CARD and allow the 2CARD domains of multiple RIG-I monomers to tetramerize and adopt a more active helical form[100]. The active tetramerized 2CARD structure can now be acted upon by TRIM25, an E3-ubiquitin ligase. TRIM25 is responsible for the polyubiquitination of tetramerized RIG-I 2CARD[31]. Ubiquitination of the tetramer serves to stabilize the complex and facilitate better downstream interactions with MAVS[100]. The ubiquitinated 2CARD of the RIG-I filament are then able to interact with the CARD domain of MAVS. The ubiquitination of the 2CARD tetramers is necessary as a regulatory check to prevent overactivation of MAVS and induction overactive signalling[101]. Once bound, the CARD of MAVS oligomerize in a "prion-like" fashion and culminate in multiple active MAVS aggregates, more specifically these aggregates form filaments from which signalling can be further potentiated[92]. It is at this point where the RIG-I and MDA5 signalling pathways share downstream outcomes. Aggregated MAVS form signalling platforms which are host to a wide array of signalling events[102]. It is also worth noting that the NLRP3 inflammasome, mentioned earlier, can be activated from MAVS aggregates[103,104]. Regarding duck RIG-I and antiviral signalling, among the events activated by MAVS aggregates including apoptosis and its inhibition, inflammation, mitochondrial transport functions, autophagy, and ubiquitination and phosphorylation[102], is the activation of IRF7 in birds. Interferon regulatory factor 7 (IRF7) is an evolutionarily conserved transcription factor in the IRF family and is present in all vertebrate lineages[105]. IRF7 is a key signalling molecule in the RIG-I pathway, upon its phosphorylation as a result of

activated MAVS, pIRF7 is translocated to the nucleus of the infected cell where it stimulates a range of ISGs[106]. The stimulated ISGs are then able to effect an antiviral response, in part by acting as paracrine signals to warn adjacent cells of infection, but also by acting in an autocrine fashion to further upregulate and stimulate cellular defences including the further upregulation of RIG-I among a vast activation of immune effectors[107,108].

1.4.5 Additional functions of RIG-I.

1.4.5.1 RIG-I and flaviviruses.

Aside from a central role in the detection of influenza, RIG-I is a PRR that can recognize multiple RNA viruses. RIG-I has been implicated in immune defence against flaviviruses such as Dengue, West Nile, and Zika[109,110]. *Flavivirus* is a genus in the family *Flaviviridae*[111] whose genome, in general, consists of one positive sense strand of RNA[112,113]. RIG-I detects these viruses in largely the same fashion as it does IAV, by recognizing the 5′ portion of viral transcripts, and the 5′ region of both Dengue and Zika viral genomes[109,110]. Recently a model for Dengue and Zika virus infection was developed using embryonic chickens[114]. Infection of chickens by either virus via mosquito, the common vector for both viruses, is rare however infection of chickens with West Nile virus by mosquitoes has been observed[115]. While chickens possess a protein, 2′-5′ oligoadenylate synthetase, that can inhibit West Nile virus replication[116], they are susceptible to Dengue and Zika virus infection[114].

1.4.5.2 RIG-I and cancer.

In addition to its role as a receptor of viral infection, RIG-I has also demonstrated an ability to control and regulate other cellular processes in mammals through its detection of RNAs and activation of interferon signalling. Recently RIG-I has been identified as a mediator of sensitivity and cell death to ionizing radiation in tumour cells[117]. Ionizing radiation leads to the formation of endogenous small nuclear RNAs (snRNAs) that can remain in the nucleus of irradiated cells but also translocate to the cytoplasm to more readily interact with RIG-I[117]. The binding of these small double-stranded snRNAs is sufficient to activate a RIG-I induced type I IFN response[117]. This IFN response is capable of recruiting immune cells to the location of tumour cells, thereby initiating an immune response to irradiated tumours secreting IFN[117], this response has also been observed in pancreatic cancer cells[118].

RIG-I has also been shown to play a role in IFN-α therapies in response to hepatocellular carcinoma (HCC)[119]. It has been proposed that in response to IFN-α in HCC cells, RIG-I binds to STAT1, preventing negative regulation of the JAK-STAT signalling pathway, leading to an increase in STAT1 activity and therefore an increase in ISG transcription[119]. STAT1 also impairs HCC proliferation and modulates HCC apoptosis through mechanisms involving p53 and cyclin E[120]. RIG-I has also been shown to modulate STAT1 signalling resulting in decreased growth in leukemia cells[121]. Furthermore, in an HCC mouse model RIG-I deficiency was associated with increased carcinogenesis[119]. RIG-I has also been shown to downregulate MMP9, a matrix metalloprotease involved in HCC tumour migration, via STAT1 which results in decreased HCC proliferation and migration[122].

In 1987 Kawaguchi and colleagues developed an immortal chicken cell line of hepatocellular carcinoma cells by chemical carcinogenesis to study oncogenesis in fowl[123]. The development of this cell line may have been aided by the lack of RIG-I in chickens, as RIG-I is apparently a key determinant of HCC propagation and migration[119,122]. Furthermore, hepatocellular carcinogenesis has been shown to correlate strongly with hepatitis B virus infection (HBV)[124,125], to which chickens are susceptible[126]. RIG-I has also been shown to be a key PRR for HBV[127] detection, by binding to the 5′ secondary structure of HBV pgRNA, as well as acting an effector to modulate HBV infection by blocking the interaction of HBV polymerase with 5′ε pgRNA[127]. Taken together this suggests that a lack of RIG-I may render chickens more susceptible not only to infection by a wide array of viruses but may also impart susceptibility to endogenous threats as well.

1.4.5.3 RIG-I and T-cells.

In addition to its role as a PRR, RIG-I may modulate immune functions beyond innate immunity. RIG-I is thought to influence the function of CD8+ T-cells during IAV infection[128]. Little is known about the interactions of RIG-I and T-cells in avian species, however in RIG-I$^{-/-}$ mice infected with wild type and recombinant IAVs, both CD4^{+} and CD8^{+} T-cells show decreased responsiveness to IAV infection[128]. Furthermore T-cells of RIG-I$^{-/-}$ mice show lower levels of activation and response to IAV antigen presentation than RIG-I$^{+/+}$ mouse T-cells[128]. In addition to altered T-cell signalling functions, it has been observed that RIG-I$^{-/-}$ mice show a lower absolute number of IAV specific CD8^{+} T-cells than RIG-I$^{+/+}$ mice[128]. Similar observations have been made in humans afflicted with dermatomyositis, an autoimmune disorder, where RIG-I mRNA and protein levels

have been correlated with decreased levels of peripheral T lymphocytes[129], although the exact mechanism of this correlation remains unclear. Dysregulated RIG-I signalling, and therefore dysregulated type I IFN signalling, appears to impact T-cell function and proliferation in mammals[128,129,130]. Whether T-cell function is influenced by RIG-I in avian species remains to be determined. However, the interaction between RIG-I signalling and T-cell function may serve as a critical linkage between innate and adaptive immunity to IAV infection.

1.4.5.4 RIG-I can bind incoming viral nucleocapsids.

Although RIG-I is a predominantly cytoplasmic, and to a lesser extent nuclear, PRR which binds and recognizes naked viral RNA, it also appears to function as a receptor capable of recognizing incoming viral nucleocapsids[131,132]. RIG-I has been shown to initiate antiviral signalling upon binding of viral nucleocapsids[131,132]. Binding of viral nucleocapsid suggests that RIG-I plays a key role in detecting viral infection immediately upon cell entry and not simply after viral replication has begun[131]. RIG-I binds to the 5′panhandle structure of viral nucleocapsids as it does with naked viral RNA[131]. Furthermore RIG-I has been shown to slow viral infection by binding nucleocapsids containing the avian isoform of PB2 with higher affinity than mammalian adapted PB2[132] . The ability of RIG-I to bind viral nucleocapsids of mammalian and avian adapted strains suggests that RIG-I is a critical PRR at all stages of viral infection.

1.4.6 A brief evolutionary context of RIG-I and RIG-I loss in chickens.

Although the exact evolutionary origins of RIG-I remain unknown, RIG-I and RIG-I homologs appear to be an ancient strategy for PAMP recognition and innate

immune signalling. RIG-I homologs have been reported in an array of organisms spanning evolutionary time. Recently a functional RIG-I homolog has been reported in the freshwater planarian *Dugesia japonica*[225]. It was first detected by transcriptomics and characterized by genomic sequence and protein homology compared to vertebrate RIG-I. In addition to RIG-I, homologs of the signalling proteins TRAF3 and TRAF6 were also reported, however there was no indication that a MAVS homolog was present in *D. japonica*[225]. This invertebrate RIG-I homolog was also found by qPCR to be upregulated following exposure of *D. japonica* to bacterial lipopolysaccharide and peptidoglycan, suggesting a role in invertebrate innate immunity[225]. The ability of this primitive RIG-I homolog to detect PAMPs that diverge significantly from vertebrate RIG-I PAMPs suggests that RIG-I may have evolved to detect different PAMPs.

A RIG-I homolog has also been reported in another invertebrate species, the Pacific oyster *Crassostrea gigas*[226]. In addition to RIG-I, Huang et al. also describe the first isolation and cloning of an invertebrate MAVS homolog. Both CgRIG-I and CgMAVS were cloned from cDNA and found to be functional in roles homologous to avian and human RIG-I and MAVS. CgRIG-I was found to bind poly(I:C), and CgMAVS was found to play a role in immune signalling[226]. CgRIG-I and CgMAVS were also found by qRT-PCR to be upregulated following stimulation by poly(I:C) and infection by OsHV-1, an oyster herpes virus[226]. In addition to RIG-I and MAVS, oyster homologs to TRAF2, TRAF3, TRAF6, IRF2, and IRF8 were also reported. The findings of functional RIG-I homologs in invertebrates suggests that RIG-I or RIG-I like genes have played a role in innate immunity from early on in evolution. Furthermore, these findings suggest that RIG-I homologs may have diverged in the specific PAMP that they

recognize[225,226], but not in their ability to recognize a pathogen. This suggests that the most primitive form of RIG-I may have established and maintained its role as an innate immune PRR throughout evolution.

RIG-I or a RIG-I-like homolog seems to have persisted through evolution having been found in invertebrates, fish, birds, and mammals[65,67,225,226,227]. Interestingly RIG-I seems to be missing in certain species including chickens[67], and the Chinese tree-shrew[228]. The absence of RIG-I in chickens is likely not a result of domestication. Most genome assemblies for *Gallus gallus* are not assembled from reads of domesticated agricultural lines of chicken, but more often are assembled from reads of the genome of red junglefowl, the ancestor of domesticated chickens. To date, RIG-I has not been found in the genome of red junglefowl and therefore appears to have been lost prior to domestication. The divergence of chickens from ducks happened approximately 90mya[229]. The domestication of chickens appears to have taken place during the Holocene period in south-east Asia ~9500 years ago from the red junglefowl species *Gallus gallus spadiceus*[230]. The absence of RIG-I in the chicken genome has been demonstrated by a lack of detectable RIG-I transcripts, lack of evidence of RIG-I sequences in the chicken genome, and a functional inability of chicken cells to detect RIG-I specific ligands[67,193]. RIG-I is not the sole innate immune gene missing in chickens, Riplet/RNF135 an E3 ubiquitin ligase that modifies RIG-I, the cytokine IRF3, and TLR8 all appear missing in chickens[193].

1.5 Chicken innate immunity and the consequences of a lack of RIG-I.

The different inflammatory responses to HPAI and LPAI in chickens and ducks, despite both animals being from a common evolutionary lineage, have some notable distinctions in their ability to succumb to, fight off, and resolve or survive IAV infections. The differences in the response to viral infection between these two species leads to the question, what is the difference in innate immunity between these two seemingly closely related species? And if there is a significant difference in innate immunity can we take advantage of that difference in an effort to reduce chicken susceptibility to IAV and improve infection outcomes in chickens. The most notable of these differences to examine is the lack of RIG-I in the genome of *Gallus gallus*. Chickens have been shown to lack both RIG-I and NOD2, however the downstream signalling machinery utilized by these PRRs remains in place. RIG-I shares a nearly identical signalling pathway with MDA5 and therefore it seems that a major missing puzzle piece to chicken susceptibility to IAV may be RIG-I, as RIG-I is a key PRR involved in the detection of RNA viruses[133,134].

As MDA5 and RIG-I share convergent downstream signalling machinery through their respective interaction with MAVS, the upregulation of one detector often impacts the regulation of the other due to their shared signalling adapters and transcription factors[107]. This may partially be why the sole reliance on MDA5 in chickens can lead to the observed increase in mortality from IAV infection. Given the importance of RIG-I as a detector of many different ligands in other species, it follows that a lack of this key receptor renders chickens more susceptible to establishment of many viral infections, including avian influenza. As a result, the lack of RIG-I in chickens represents a

considerable weakness in the defence against influenza, and likely a contributor to the susceptibility of chickens to IAV. Chickens are susceptible not only to influenza viruses but are susceptible to other single strand RNA viruses. Newcastle disease virus, infectious bronchitis virus sometimes called avian coronavirus, Flaviviruses such as Dengue and Zika, as well as to infections by the families *Picornaviridae, Calcivirdae,* and *Retroviridae*[114,231,232,233,234].

1.6 A brief introduction to CRISPR/Cas9.

CRISPR/ Cas9 (clustered regularly interspaced short palindromic repeats / CRISPR associated protein 9) is a bacterial defence system in which the genetic material of invading pathogens is targeted[135]. In bacteria, resident endonucleases cleave invading DNA[136]. The resulting small fragments may be adapted into the CRISPR locus and transcribed as RNA and these RNAs can then complex with Cas proteins and provide targeting instructions to attack DNA homologous to the acquired targeting RNA[136]. The last step of the process, the precise targeting of DNA by an RNA/endonuclease pair has, in the last decade, been adapted for use as a tool in genetic modification[137,138,139]. Recently a variety of CRISPR/Cas9 systems have been shown to be effective for the silencing, knock-in, and knock-out of genes of interest in a wide array of model systems and organisms[140,141,142]. Of the different forms this system has taken recently, I am interested in methods of gene knock-ins using CRISPR/Cas9 that use more traditional molecular techniques like a two-plasmid system for homology directed repair. In a two-plasmid CRISPR/Cas 9 system a small guide RNA (sgRNA) containing pre-programmed targeting instructions for the desired locus of interest, is expressed from a plasmid. This sgRNA is part of a larger RNA scaffold which once transcribed can be bound by Cas9

protein expressed from the same plasmid. This Cas9-sgRNA complex is then free to hybridize to and cleave both strands of host genomic DNA at the locus of interest[138]. The resultant double-stranded break (DSB) can then be repaired.

The second of the two plasmids contains the sequence of the gene of interest to be knocked into the target locus. The gene of interest sequence is flanked on either side by regions that are homologous to the flanks of the DSB in the target locus. This homology to the target locus helps the gene of interest sequence hybridize over the DSB in order to act as repair instructions for use in normal templated DNA repair[143]. Normal cellular DNA repair machinery is then free to copy the gene of interest sequence into the DSB and thereby integrate the gene of interest stably into the target locus of the host genome[143] (Figure 1.1). This process is known as homology directed repair (HDR) and can be highly precise but is often inefficient for the knock-in of large genes of interest[144]. Still, in our case when trying to knock a lost gene back into an organism in the spot from which it was lost, precision of knock-in took priority in an effort to avoid disrupting other vital gene functions.

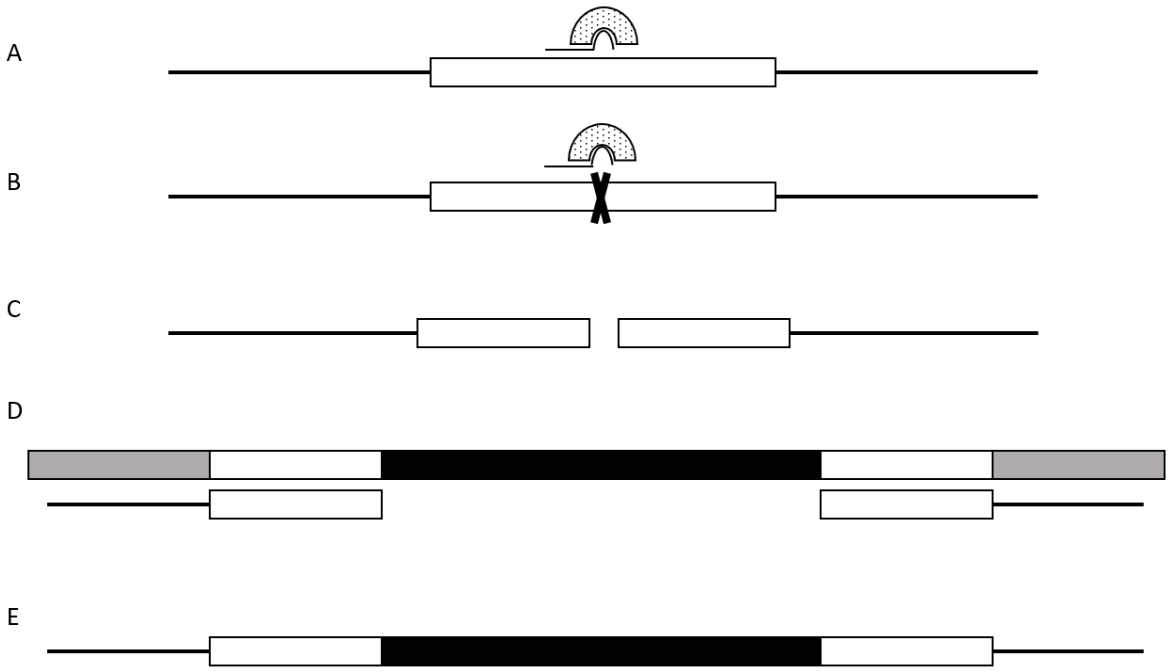

Figure 1.1. Schematic of CRISPR/Cas9 two-plasmid mediated homology directed repair. A) Cas9 and the sgRNA bind together and hybridize to the locus of interest. B-C) Cas9 then cleaves both strands of host gDNA resulting in a double stranded break. D) The HDR repair template plasmid hybridizes to the region surrounding the DSB guided by homology arms upstream and downstream of the gene of interest. E) Normal cellular templated DNA repair utilizes the HDR template as an instruction to repair the DSB and the gene of interest is knocked into the host genome.

1.7 Herpes Simplex 1 Thymidine Kinase and inducible suicide genes.

Herpes simplex 1 virus (HSV) encodes a thymidine kinase (TK) that has been used as a therapeutic drug-inducible negative selection mechanism[154]. This type of mechanism is known as an inducible "suicide gene" as cells expressing HSV TK will die when given a drug, while cells without TK will survive in the presence of the drug[152,154,155]. By including HSV-TK in the HDR template plasmid I hope to be able to take advantage of this selection system to kill any potential off-target knock-ins.

Thymidine kinase (TK) is one of four types of salvage enzyme that catalyze the phosphorylation of deoxynucleoside precursors into deoxyribonucleotides that can be integrated into elongating DNA structures[145,146]. TK is unique among the nucleoside kinases in that its activity is tied to the cell cycle[147]. However, its function is of vital importance to healthy cell division. Nucleoside kinases are not unique to humans and some DNA viruses have been found to encode their own nucleoside kinases to facilitate viral DNA synthesis; as is the case with the human herpes simplex-1 virus (HSV) where it has been implicated as virulence factor[148,149].

HSV TKs ability to phosphorylate nucleosides or their analogues has been utilized as a therapeutic antiviral strategy since the 1970's [149]. The TK encoded by HSV can preferentially phosphorylate acyclic nucleoside analogues rather than normal nucleosides[150,151]. The differential binding and phosphorylation of analogues by HSV TK has been employed as a system to kill cells that have been infected with HSV or cells that otherwise express HSV TK[152]. In such a system any cell expressing HSV TK will be able to phosphorylate a given suite of nucleosides[150,151]. If those nucleosides are able to be integrated into replicating DNA, they may disrupt the processing or assembly of new cellular DNA and the host cell may die as a result of dysregulated DNA replication[153]. TK, when expressed in a cell, is harmless without the presence of particular nucleoside analogues including ganciclovir (GCV)[154]. Ganciclovir is an acyclic guanosine analogue that lacks a phosphate and can be monophosphorylated by HSV TK[155]. Once GCV is phosphorylated it can be further phosphorylated eventually becoming GCV-triphosphate (GCV-TP), a close analogue to guanosine[155,152]. GCV-TP can then be utilized by DNA transcription machinery in place of guanosine and is preferentially integrated into

elongating DNA backbones[153,152]. GCV and GCV-TP have an acyclic sugar moiety instead of a deoxyribose moiety[151]. This lack of a cyclic sugar backbone prevents further elongation of a DNA backbone during transcription, this disrupts DNA replication leading to the death of the cell in which both TK and GCV are present[155,152].

Due to the TK cassette being located "outside" the sequence flanked by the homology arms it is not intended to be knocked into transfected cells. The HSV-TK cassette would not be located in the space of the DSB and should not be included as DSB repair instructions (Figure 1.1, Figure 3.2). In the event that a knock-in occurs off-target or in a location other than the intended DSB, the homology arms should not provide any stabilization by hybridizing to the host genome and therefore any portion or the entirety of the HDR template may be integrated into the host genome. If this is the case, then TK may be expressed and can act on GCV supplied to transfected cells. If a cell undergoes an off-target knock-in and TK is expressed in the presence of GCV that off-target cell should succumb to selection. However, if TK is expressed in the absence of GCV or likewise GCV is applied to cells without TK expression the cell would survive selection. It is entirely possible that some combination of these events may occur and the selection system may not kill all off-target cells.

1.8 CRISPR in domestic chickens.

1.8.1 CRISPR in a chicken system.

Since 2015 a number of reports using CRISPR approaches to modify the genome of chickens and chicken cells have been reported[156]. One of the earliest of these reports utilized a Cas9 non-homologous end-joining (NHEJ) approach with multiple sgRNAs to

knock-out the transcription factor PAX7 in chicken primordial germ cells (PGCs) in ovo[156]. By supplying a Cas9 plasmid with no additional template or repair instructions for the induced DSB, the endogenous error prone NHEJ pathway of DNA repair is utilized and as a result insertions or deletions (indels) often accompany repair of the DSB. These indels may manifest as early stop codons, frameshift mutations, substitution mutations resulting in non-functional proteins resulting in disruption of the coding sequence of the target gene where the DSB was made, in the case above the gene PAX7.

Early CRISPR work in chickens employed this NHEJ knock-out strategy,[156,157,158] however more recent works have expanded beyond NHEJ or HDR mediated knock-outs and have moved to include NHEJ, HDR, or similar mechanisms to mediate knock-in of genes into chicken cells or chicken embryos[159,160,161,162]. Recent approaches attempting to knock-in functional genes into chicken cells have utilized a variety of DNA repair mechanisms to achieve successful knock-in[159,160,161,162]. To date most genes of interest (GOIs) knocked into chicken cells via CRISPR have been relatively small genes[160], or fluorophores[161,162]. An attempt to knock-in a protein coding gene and promoter larger than ~1kb has yet to be reported. However, given the recent increase in reports of CRISPR knock-ins in chicken cells and the diversity of knock-in strategies a successful knock-in of larger or more complex GOIs, similar to our approach, may not be far away.

1.8.2 Recent similar publications and interest in CRISPR knock-ins in chickens.

Since 2015 CRISPR/Cas9 been used in chickens in almost any available form of the system, however in 2019 two instances of targeted gene insertion were reported, one in the OVA locus[161] and one in the Z chromosome[162]. In 2019 Lee *et al* used a two plasmid CRIPSR approach to knock-in green fluorescent protein (GFP) under the control

of human cytomegalovirus (hCMV) into the Z chromosome of chicken primordial germ cells. The target site chosen by Lee *et al* to knock-in GFP is near the purported breakage point on the Z chromosome, however the site of insertion was ~370kb downstream of *ACO1*. The cells modified by Lee *et al* were then placed into chicken embryos and allowed to hatch. There are similarities between the work published in 2019 and our approach but there are also some important differences. In their work the authors opted for an NHEJ mediated knock-in rather than HDR, their cassette of interest to knock-in was ~4 kb where our genes of interest were 5.3 kb, however both contained GFP[162]. While their work did not include innate immune genes, the similarities in approach between our work and theirs, supports the idea of using a two-plasmid CRISPR-mediated approach to knock-in functional genes into chicken cells and chickens.

1.9 A brief look at CRISPR modification of cell lines.

Adaptation of the CRISPR/Cas9 system to function as a precise gene editing tool has allowed for new approaches to the modification and development of cell lines. CRISPR editing has been applied to a wide range of animal cell lines including fish, human, mouse, rat, quail, and chicken[163,164,165,166,167,168]. In many of these early reports CRIPSR was utilized to knock-out a gene and thereby create a new stably modified cell line[163,164,169,166,167,168]. Most of these approaches employed a Cas9 enzyme to induce either DSBs or single or double nicks in host cell DNA to disrupt gene function. CRISPR had also been utilized to remove a large fragment (30 Mb) of chromosomal DNA from the human cell line HAP1 resulting in a fully haploid human cell line[164].

However, CRISPR mediated knockouts were not the only initial uses of the system to modify cell lines. The use of CRISPR to knock-in a genetic element for the

creation of a new cell line had also been reported. In 2014 Park *et al* used CRISPR to attach an inducible destabilizing domain to the critically important TCOF-1 protein in human cells[170]. Shortly thereafter CRISPR had been utilized to knock-in genetic elements and protein coding genes. In one case the fluorescent reporter Venus, and a gene involved in embryonic neural development, *Sox-1,* were both knocked into haploid mouse embryonic stem cells[165]. In general, CRISPR had therefore demonstrated a practical utility for the precise knock-in or knockout of genes of interest in a wide range of cell types.

The chicken embryonic fibroblast line DF-1 has been widely used as a model system in which to investigate avian cell function since its first use in 1998[171,172]. Briefly, DF-1 cells are a non-transformed, spontaneously immortalized cell line of embryonic chicken fibroblasts derived from East Lansing Line chickens[173]. DF-1 cells were initially intended for the propagation and harvesting of viruses and viral proteins[173]. However, DF-1 cells have been used to study cell biology in a range of contexts including parasitology[174,175,176], toxicology[177,178,179], genetic engineering[180,181,182,182], and in particular virology and immunology[67,78,183,180,184]. The widespread use of DF-1 cells has generated a considerable knowledge base concerning the culturing and maintenance of the cells as well as their general characteristics and functions. In addition to their wide applicability to diverse research areas, the DF-1 cell line my be of particular interest in the study of influenza. Influenza infection in humans, ducks, and chickens occurs initially in epithelial tissues of the respiratory and gastrointestinal tract[3,235,236]. Currently there are no chicken epithelial cell lines, however DF-1 cells being fibroblasts may be a reasonable facsimile as epithelial cells and fibroblasts are known to interact following lung injury[237].

The proximity of fibroblasts to epithelial tissue and their role in connective tissue structure may also be relevant for investigating the expression of duck RIG-I in chickens. The expression of an innate immune receptor in cells that would likely be the first to encounter influenza suggests DF-1 cells may be a suitable model system. It is worth noting however that DF-1 cells are not without drawbacks, especially in a genetic modification context. DF-1 cells are a spontaneously immortalized cell line, and as such might be subject to genetic aberrations that may facilitate perpetual growth and division[245]. This is partially evidenced by DF-1 cells having a ~22% polyploidy rate, as reported by the manufacturer. The presence of extra chromosomes may present a compounding challenge in the CRISPR modification of DF-1 cells. However, the advantages of using DF-1 cells likely outweigh the drawbacks.

Furthermore, our group in particular has demonstrated that duck RIG-I could be expressed and activate cell signalling pathways that lead to the induction of interferon reporter activation in DF-1 cells[67]. Furthermore, given the origin of DF-1 cells in studying immunity and infection, and the history of DF-1 use by our group I chose to use DF-1s as the progenitors into which I would knock duck RIG-I.

With the growing prevalence and availability of CRISPR technology a number of cell line modifications and developments have been reported. Most strategies remain focused on the use of knockouts for modification[185,186,187], as CRISPR based NHEJ strategies use fewer constructs and foreign DNAs in general allowing for streamlined experimental design. CRISPR modifications have therefore been reported in a wide range of host cell types including chicken cell lines. Recent uses of CRISPR in chicken systems to achieve both knockouts and knock-ins have been reported in two chicken cell types,

primordial germ cells (PGCs)[184,188,189] and the embryonic fibroblast line DF-1[180,181,190]. These approaches have generally used the CRISPR/Cas9 platform to induce DSBs in host immune genes and relied on the error inducing NHEJ repair mechanism to knockout target genes and examine resultant phenotypes[157,180,181,189]. Furthermore, a recent report has used CRISPR to explore the role of chicken MDA5 in innate signalling by knocking out MDA5 in DF-1 cells[180]. The results of this MDA5 knockout corroborate the central role of MDA5 in the induction of IFN signalling in response to poly(I:C)[180]. In addition to this recent report CRISPR/Cas9 has been used to knock in GFP into multiple loci in both DF-1s and PGCs[180,191]. Although recent, these reports corroborate that the embryonic fibroblast line DF-1 is well suited for CRISPR based approaches.

1.10 Thesis project Aims.

Previous work by our group suggests that transient over-expression of dRIG-I in chicken fibroblast cells is sufficient to augment innate immune response against influenza infection[67,78]. In that work, DF-1 cells were transfected with either an empty expression vector or with an expression vector for dRIG-I. Those cells that expressed dRIG-I were shown to have lower viral titre than cells without dRIG-I[67]. Additional experiments showed that expression of duck RIG-I and viral infection was sufficient to upregulate an array of innate immune genes[78]. Further research has shown that the core promoter responsible for driving RIG-I expression in ducks is sufficient to drive gene expression in chicken cells[75]. From these findings we conclude that the stable knock-in and expression of duck RIG-I in the genome of domestic chickens will result in a functional transgene which may improve innate immune defence against IAV, and restore vital innate immune signalling function in a chicken model.

This project is comprised of three aims all with the goal of creating a model cell line to study host immunity to avian influenza. The first aim of the project was to construct a homology directed repair template plasmid suitable to direct the knock-in of duck RIG-I and eGFP into the genome of chicken cells. The second aim was to modify a commercial Cas9 plasmid for use in an avian system that would target the precise locus where I intended to insert duck RIG-I. The third aim was to use these plasmids in tandem to knock duck RIG-I into a particular locus in the Z chromosome of chicken cells.

Research into understanding disease dynamics and molecular mechanisms of pathogenesis is crucial in maintaining healthy agricultural livestock and preventing zoonotic disease outbreaks. Here I knock duck RIG-I into the genome of chicken fibroblasts (DF-1) by employing a CRISPR/Cas9 two-plasmid homology directed repair approach. This work hopes to contribute to the body of knowledge by investigating the creation of a model cell line for understanding how potential dRIG-I transgenic chickens or other similar immune-modified organisms may react to, or effect, host-pathogen disease dynamics.

Chapter 2 Materials and Methods.

2.1 Plasmid propagation.

All plasmids were grown in DH5-α chemically competent *E. coli* (New England Biolabs) on LB media. Plasmid DNA was extracted from transformed *E. coli* via column purification kit (GeneAid). Plasmids phRG-TK and pAL119-TK were kindly gifted by Dr Brad Magor's group (University of Alberta).

2.2 DNA isolations and HDR plasmid construction.

To facilitate the construction of a 14 kb HDR donor plasmid and modification of a Cas9 expression plasmid, all necessary components were cloned via polymerase chain reaction (PCR) as individual cassettes. All PCR reactions in this work were done using Phusion High-Fidelity DNA polymerase (New England Biolabs). All PCR primers used were designed by hand (Table 2.1). PCR amplicons were purified by gel electrophoresis and column purification (Luna-Nanotech) (Table 2.1). Where required, chicken genomic DNA was collected and purified by column purification (Qiagen). Following PCR amplification, DNA fragments were spliced via overlap extension PCR[192] (Table 2.2). All fragments were also cloned into a pCR2.1-TOPO storage vector (Invitrogen) and validated by Sanger sequencing upon isolation and splicing via primer walking (Table 2.3) Overlap extensions spliced fragments were joined together to form the completed circular HDR plasmid (Figure 3.2) by Gibson Assembly using a Gibson assembly kit (New England Biolabs). All Gibson assembly primers were designed by hand and validated with NEBuilder (Table 2.4).

2.3 dRIG-I promoter reporter vector construction.

In order to corroborate the ability of the 500 bp duck RIG-I promoter to drive gene expression at an appreciable level in DF-1 cells a dRIG-I promoter reporter vector was constructed. The reporter consisted of a pcDNA3.1(+) backbone, from which the MCS and CMV promoter were removed by restriction digest. To the resultant backbone fragment we inserted a cassette with the mCherry fluorophore under the control of the 500 bp dRIG-I promoter, with an SV40 poly-A signal, by Gibson assembly (Table 2.4).

2.4 Cell culture.

Wild type DF-1 cells, an immortalized embryonic chicken fibroblast line[172] (UMNSAH/DF-1 ATCC®CRL-12203) were maintained in culture at 39°C and 5% CO_2 in growth media consisting of Dulbecco's modified eagle medium (DMEM) (Gibco) + 10% FBS (Gibco) and 1% Penicillin/Streptomycin (Gibco). Cells were passaged by trypsin digestion every 4 days with a 1:4 split.

2.5 Cell transfection.

All transfections were done using Lipofectamine 2000 according to manufacturers instructions at a ratio of 2.5 μL per μg of plasmid DNA delivered. Cells were then imaged via confocal microscopy.

2.6 Confocal microscopy.

Cells that were transiently transfected with HDR plasmid were imaged via confocal microscopy on a Leica TCS SP5. Prior to imaging cells were fixed with 1% paraformaldehyde. Cells were stained with Hoechst 33342 nuclear stain (Life

technologies) and mounted to a glass coverslip. Stained cells were imaged with excitation wavelengths of 350 nm for Hoechst, 587 nm for mCherry, and 488 nm for eGFP.

2.7 Fluorescence activated cell sorting and negative selection.

Twenty-four hours after transfection with both Cas9 and HDR plasmids, cells were trypsinized and pelleted at 4000x g at 4°C. Cells were then resuspended at ~$1.0x10^6$ cells/mL in a cell sorting buffer (1% PBS + 1% FBS + 0.5 mM EDTA). Cells were sorted on a BD FACS ARIA II into pure FBS (Gibco). Positively sorted cells were then pelleted and resuspended in selection media consisting of growth media with the addition of 5 μM ganciclovir and allowed to recover for 10 days. Cells were then trypsinized and seeded at a density of 1 cell/well in a 96-well plate and allowed to expand to confluency under selection for 3 weeks with media changes every 5 days. All confluent wells were trypsinized and cells were seeded in 24-well plates; cells were again allowed to expand to confluency under selection for ~3 weeks. Cells were maintained in 24-well plate culture for 1 week.

2.8 Cell screening.

All surviving cell lines were screened via PCR for genomic presence of duck RIG-I. Cells were trypsinized and pelleted before genomic DNA was isolated by column purification (Qiagen). Genomic DNA was used as a PCR template for amplification of the duck RIG-I cassette: from the promoter to the 3′UTR (Table 2.1). Genomic DNA was also used as a PCR template for amplification of chicken GAPDH, and HSV TK (Table 2.1).

2.9 Western Blot.

DF-1 cells were seeded in 6 well plates and transiently transfected with 2 μg of Cas9 plasmid as above. Cells were lysed in place in lysis buffer (150 mM NaCl +1.0% Triton X-100 + 50 mM TRIS pH 8.0 + cOmplete proteinase inhibitor [Sigma]). Whole cell lysate was boiled in 8x Laemmli buffer prior to electrophoresis. Whole cell lysate was run on 12% SDS-PAGE resolving gel for ~2 hr followed by transfer to nitrocellulose membrane. The membrane was then blotted with anti-Cas9 antibody (AbCam #ab191468) overnight followed by blotting with goat-anti-mouse secondary antibody for 1 hr. This process was repeated exactly with anti-chicken-β-actin primary antibody. Blots were imaged on a ChemiDoc imager (BioRad) with 20 min exposure.

2.10 HSV TK/GCV cell death assay.

Wild type DF-1 cells, DF-1 cells transiently transfected with 10 μg HDR template plasmid for 24 hr, and one representative CRISPR modified clone (P2D5) from the 12 surviving lines of dRIG-I+ cells, were seeded at $5.0x10^5$ cells/mL. Cells were then immediately exposed to a range of (0-20 μM) ganciclovir concentrations for 72 hrs. growth media supernatant and adherent cells were collected and assessed for viability using trypan blue (Gibco) dye exclusion assay. Cells were incubated in equal volume of trypan blue (0.4%) for 3 min prior to enumeration by haemocytometer.

2.11 RNA extraction, cDNA synthesis, rtPCR and qPCR.

To investigate transcription of transgenes, dRIG-I positive cells were stimulated with 500 ng poly (I:C) and 250 ng RIG-I ligand (Invivogen) for 24 hr. Cells were then pelleted, and RNA was isolated from pellets by column purification (Luna Nanotech).

RNA was treated with TurboDNase (Invitrogen) as per manufacturer's instructions to eliminate gDNA contamination. Complementary DNA (cDNA) was synthesized from RNA using oligo DT (IDT) and Superscript III (Invitrogen). cDNA was then used as template for reverse transcriptase PCR (rtPCR) and was diluted 1:10 for use as quantitative PCR (qPCR) template. rtPCR was done to amplify transcripts of HSV TK from all 12 clonal cell lines (Table 2.1). A set of cDNA was prepared without reverse transcriptase as a control for gDNA contamination. qPCR was done using a Quantstudio 3 (Applied Biosystems), using a primer-probe strategy amplifying for chicken GAPDH, duck RIG-I, and chicken Mx-1 (Table 2.5).

Table 2.1. List of primers used for PCR amplification of DNA fragments.

DNA fragment	Direction	Sequence
500 bp duck RIG-I promoter	Fwd	GAGCGGCGGAGACAAAGTGCCA
	Rev	GGCTGGGCTCTGCCGGCC
Duck RIG-I coding sequence	Fwd	CGGCCGGCAGAGCCC
	Rev	GCCATTGAGGTACCTAGCACACAC
Duck RIG-I 3′ UTR	Fwd	GTGTGTGTGCTAGGTACCTCAATGGCA
	Rev	GAAGTCTGGGATCTAGGTGAGGAGAAGGG
5′ homology arm	Fwd	TGGAATGATTCCCCATGTCCGTAGA
	Rev	TTCTTAAAAGAGTGCCTGCATTTAATATATA
3′ homology arm	Fwd	AAAATGCAATGAAAACAGGTGGACAACA
	Rev	TGCCAGGCTGCTGTGTTAGCT
eGFP cassette	Fwd	CGTTACATAACTTACGGTAAATGGCCCG
	Rev	CGCGTTAAGATACATTGATGAGTTTGG
HSV TK promoter	Fwd	CGGGCTCGAGATCTAAATGAGTCTT
	Rev	CAGTACCGGAATGCCAAGCTTTTAAG
HSV TK coding sequence	Fwd	ATCCCGACATGGCTTCGTACC
	Rev	GGTCGGGGAGAGGAGTGTTA
Chicken U6.3 promoter	Fwd	CAGACAGACGTCAGGCTTTCTAAG
	Rev	GACTAAGAGCATCGAGACTGCGG
mCherry coding sequence	Fwd	ATGGTGAGCAAGGGCGAG
	Rev	TTACTTGTACAGCTCGTCCATGCC

Table 2.2. List of primers used for overlap extension PCR splicing of DNA fragments.

DNA fragments	Direction	Sequence
mCherry + pcDNA	Fwd	GCGGCCGGCAGAGCCCAGCCATGGTGAGCAAGGGCGAGGA
dRIG-I coding sequence + dRIG-I promoter	Fwd	GCCATTGAGGTACCTAGCACACAC
dRIG-I coding sequence + dRIG-I 3′ UTR	Fwd	GTGTGTGTGCTAGGTACCTCAATGGCA
eGFP + 3′ Homology Arm	Rev	GCTCGACATGTTCTCCTTTTACGTTAC
HSV TK promoter + HSV	Rev	GTACGAAGCCATGTCGGGATCAGTACCGGAATGCCAAGC

Table 2.3. List of primers used for Sanger sequencing reactions of each of the HDR template, avian adapted eSpCas9(1.1), and dRIG-I-mCherry pcDNA plasmids.

Template	Binding Location	Primer Sequence
HDR – dRIG-I	Start	ATGACGGCGGAGGAGAA
	Internal	GCATCGCGTCCAGCATCCCTCGGA
	Internal	ACTGGAAGAGCTTGTCTGCATCCTG
	Internal	CCCATCAAAACACCTGGCTTTATGT
	Internal	ACAGGTATGACCCTCCCAAGCCAG
	5′ UTR / Start	CGGCCGGCAGAGCCCAGCC
	Stop / 3′ UTR	GTGTAGGAGAGTAATAGATGCACTA
	3′ UTR internal	GCCATTGAGGTACCTAGCACACAC
	Internal	CTACCTTGACATACAGGCCATATCC
	dRIG-I Prom. internal	GGCTGGGCTCTGCCGGCC
	dRIG-I Prom.	GAGCGGCGGAGACAAAGTGCCA
	dRIG-I Prom. internal	CGGCCGGCAGAGCCC
	dRIG-I Prom. / Start	CGGCCGGCAGAGCCCAGCCAT
	Internal	GCCAGTATGACATATTCTG
	Internal	ATCAGGCATGTGCTTATATAG
	Internal	AGTTCTCAATTGCTTCTGCCA
HDR - 5′ homology arm	5′	TGGAATGATTCCCCATGTCCGTAGA
	Internal	GGCTCATTTCCTGTCCATGTTGT
	3′	TTCTTAAAAGAGTGCCTGCATTTAATATATA
	Internal	CTCAACAGAACAGCTAACTG
HDR – eGFP	hCMV Prom. 5′	CGTTACATAACTTACGGTAAATGGCCCG
	Internal	CTTGTACAGCTCGTCCATGCCGA
	Stop	CGCGTTAAGATACATTGATGAGTTTGG
	Internal	GACACGCTGAACTTGTGG
HDR - 3′ homology arm	3′	TGCCAGGCTGCTGTGTTAGCT
	Internal	ACTCTCTCTTCCTGTTTCTAAGATGC
	5′	AAAATGCAATGAAAACAGGTGGACAACA
	Internal	GATAGACAACAAAAGCAGTG
HDR – HSV TK	Prom. 5′	CGGGCTCGAGATCTAAATGAGTCTT
	Prom. internal	CAGTACCGGAATGCCAAGCTTTTAAG
	Prom. / Start	ATCCCGACATGGCTTCGTACC
	3′ / Stop	GGATCCTCAGTTAGCCTCCC
	Internal	CGAATGAGAGTGTTTCGTTC
	Internal	GGTCCACTTCGCATATTAAG

Table 2.3 Continued.

Template	Binding Location	Primer Sequence
HDR – HSV TK	Internal	CACTCTTGCAGCGTTAGCAG
	Internal	CTGCTAACGCTGCAAGAGTG
HDR – pCRTOPO2.1	Internal	GTAAAACGACGGCCAG
	Internal	CAGGAAACAGCTATGAC
	Internal	CTGGCCGTCGTTTTACAACG
	Internal	CATGATTGAACAAGATGGATTGCAC
	Internal	GAGTATGAGTATTCAACATTTCCGTG
	Internal	AGCTGTTTCCTGTGTGAAATTG
	Internal	GAAGATCCTTTGATCTTTTCTACG
	Internal	CATACTCTTCCTTTTTCAATTCAG
	Internal	CGAAACGATCCTCATCCTGTC
	Internal	GGCTCCATACCGACGATCTGCG
	Internal	TGGTAACTGTCAGACCAAGTTTAC
eSpCas9(1.1)	Internal	CTGTTTCCCCTGGCCAGAG
	Internal	CTGGCAGACAGGATGGCC
	Internal	CAGGTGCTCCAGGTAATTAAAC
	Internal	CTGTCAGACCAAGTTTACTCA
	AmpR. Start	ATGAGTATTCAACATTTCCGTG
	Internal	ACGCGCCCTGTAGCGGC
	Internal	CATCGAGCAGATCAGCGAG
	Internal	GAGTTCGTGTACGGCGAC
	Internal	GCACGAGCACATTGCCAATCT
	Internal	GATCGAGAAGATCCTGACCT
	Chic. β-Actin Prom. Start	GAACGTGGGGCTCACCTCGA
	Internal	CCTCGACCATGGTAATA
	Internal	ATGTAACGGGTACCTCTA
	Internal	CATGGTAATAGCGATGACTAATAC
	Internal	GCCTATGGAAAAACGCCAG
dRIG-I-mCherry pcDNA	Internal	CAGTCGAGGCTGATCAGCG
	Internal	TGCGCTGCTTCGCGATG
	mCherry Start	ATGGTGAGCAAGGGCGAG
	mCherry Stop	TTACTTGTACAGCTCGTCCATGCC

Table 2.4. List of primers used to attach regions of complementarity by PCR for Gibson assembly.

DNA fragment	Direction	Primer sequence
HDR - 5′ homology arm	Fwd	ATTACGCCAAGCTTGGTACCGAGCTAGAAAATAAATGAGTACTTTTGG
	Rev	GTCTCCGCCGTCCTTCTTAAAAGAGTGCCTGC
HDR - dRIG-I	Fwd	GATCCCAGACTTCCGTTACATAACTTACGGTAAATGGC
	Rev	GATCTCGAGCCCGCAGGGTTCCCTGGGGTAG
HDR - eGFP	Fwd	GATCCCAGACTTCCGTTACATAACTTACGGTAAATGGC
	Rev	GATCTCGAGCCCGCAGGGTTCCCTGGGGTAG
HDR - HSV TK	Fwd	CCAGGGAACCCTGCGGGCTCGAGATCTAAATG
	Rev	CGGCCGTTACTAGTGGATCCGAGCTGGTCGGGGAGAGGAGTGTTAG
eSpCas9(1.1)	Fwd	GCCTTTTGCTGGCCTTTTGCTCACATGTTTACATGTCAGACAGACGTC
	Rev	CCGTAAGTTATGTAACGGGTACCTCTAGAGCCATTTGTCTGCAG
dRIG-I-mCherry pcDNA	Fwd	GATATACGCGTTGACATTGATTATTGAGAGCGGCGGAGACAAAGTGCCAGG
	Rev	CTGCAGAATTCCACCACACTGGATTACTTGTACAGCTCGTCCATGCCGCC

Table 2.5. Summary of primers and probe sequences used for qPCR amplification of chicken Mx-1, chicken GAPDH, and duck RIG-I.

Gene	Direction	Sequence
cMx-1	Fwd	GAGAGAATCGTATACTGCCAAGATAACA
	Rev	TGAAACGGATGCAAAACTGATC
	Probe	FAM-CGCTCTACC/Zen/AAGGCAGAAATACTCGGCA-3IABkFQ
cGAPDH	Fwd	GGTGCTAAGCGTGTTATCATCTCA
	Rev	CATGGTTGACACCCATCACAA
	Probe	FAM-CTCCCTCAG/Zen/CTGATGCCCCCATG-3IABkFQ
dRIG-I	Fwd	GGAGAGCAGGATATGTAGAG
	Rev	GGTCAGGTAGGATAAAGCATC
	Probe	FAM-TCCGCAGGT/Zen/GTTCAGTGCAAATGAAA-31ABKFQ

Chapter 3 Results.

3.1 Identification of a target locus for the knock-in of duck RIG-I.

During the evolution of birds, the gene encoding RIG-I was lost in chickens and remains missing[193]. To determine where the RIG-I gene was lost in chickens I compared the Z chromosome of ducks to the Z chromosome of chickens. When those two regions are compared, the identity and position of the flanking genes suggests a locus from which RIG-I was lost in chickens (Figure 3.1). When each of the Z chromosomes are aligned it appears that there was a recombination event resulting in the reversal of a section of the chromosome encompassing approximately 12 genes, or blocks of genes. At the 5′end of this section in the chicken, RIG-I appears to have been lost, which would suggest a point of chromosomal breakage near the sequence of RIG-I (Figure 3.1). The order of genes of both chromosomes shows a pattern that suggests a double stranded break and chromosomal inversion event took place at the locus of *DDX58* and *LOX* in chickens (Figure 3.1).

To identify a region of the Z chromosome suitable for the knock-in of RIG-I a unique region of genomic DNA was needed. 25 kb of DNA was examined in 2.5 kb segments for a section with no known genes, no large repetitive sequences, and only one instance of that segment in the chicken genome. The site chosen for the knock-in of RIG-I is the centre of chromosomal position 71,285,522 bp - 71,286,122 bp which would place RIG-I at the 3′ end of the “MROH-like block” in between *ACO1* and *PRR16*. This position was chosen to mimic the current position of RIG-I in the duck genome as it seemed logical to put a “lost” gene back where it was “lost” from. Furthermore, the

region of the Z chromosome surrounding *ACO1* should be euchromatic as *ACO1* (aconitase 1) is a key enzyme of the Krebs' cycle and therefore should be under continuous transcription[194,195]. An open chromatin conformation is essential to facilitate the knock-in of RIG-I. To achieve such a knock-in we chose to use a CRISPR/Cas9 system.

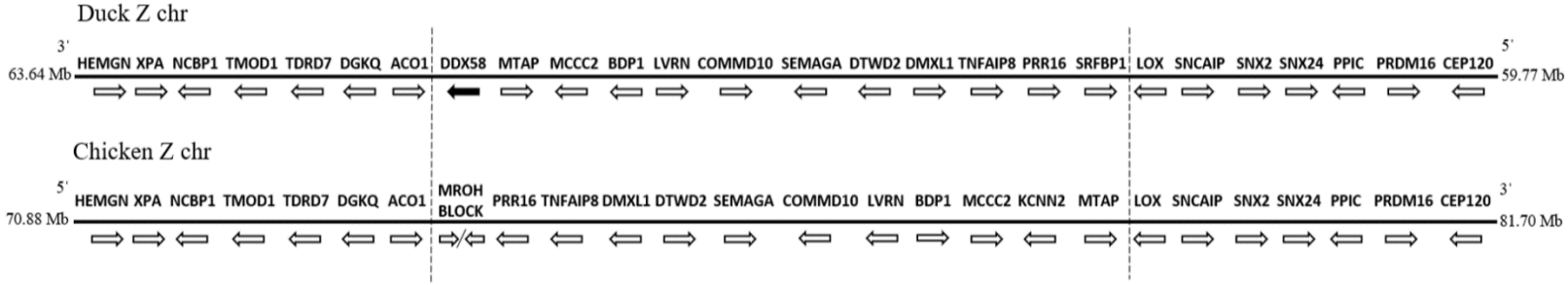

Figure 3.1. Schematic map of genes surrounding the *DDX58* locus in *Anas platyrhynchos* (top) and the corresponding locus in *Gallus gallus* (bottom). Compiled using NCBI gene annotations: *Gallus gallus* NCBI annotation release 104, *Anas platyrhynchos* annotation release 103.

3.2 Plasmid design and construction.

3.2.1 HDR template plasmid design rationale.

To knock duck RIG-I into a specific locus in the chicken genome I constructed an HDR template plasmid. In addition to the HDR template plasmid, a Cas9 and sgRNA expression plasmid was also required. Of the wide range of Cas9 expression plasmids commercially available I chose eSpCas9(1.1) (Addgene # 71814) for its simplicity and ease of availability, however this plasmid required modification for use in an avian context. Replacement of eSpCas9(1.1) mammalian U6 with the avian derived U6.3 along with an sgRNA sequence targeted to the chicken Z chromosome was achieved with no observed mutations as verified by Sanger sequencing.

To test the ability of the RIG-I promoter to drive expression of a fluorescent gene we generated a construct dRIG-I/mCherry construct. Replacement of pcDNA3.1 hCMV promoter and MCS with mCherry under the control of the 500 bp duck RIG-I promoter was done with no observed mutations as verified by Sanger sequencing.

The third plasmid, which required *de novo* assembly, was the duck RIG-I HDR repair template plasmid. This plasmid was constructed by a combination of overlap extension PCR and Gibson assembly, with the only observed mutation being a 203 bp truncation of the CMV enhancer upstream of the CMV promoter driving eGFP expression (Figure 3.4). This truncation can likely be explained by a polymerase jump due to the presence of a repeat of 9 nucleotides (ACGGTAAAT) in the enhancer sequence (Figure 3.4). This mutation did not affect CMV promoter function or eGFP expression.

3.2.2 HDR template and Cas9 expression plasmid construction.

To enable easy propagation and selection of the HDR plasmid in bacteria I chose to base the HDR plasmid on the commercial pCR2.1-TOPO cloning vector (Invitrogen). This backbone contains features which make the cloning, growth, and propagation of the plasmid in *E. coli* cells straightforward. pCR2.1-TOPO contains two antibiotic resistance genes allowing for selection with either ampicillin or kanamycin when grown in bacterial cells. The backbone also contains a cloning site that interrupts a bacterial lac operon. This operon interruption system allows for detection of successful transformants by a change in colour observed on a treated agar plate. Together these features provide the HDR plasmid with tools to make the propagation of the plasmid more streamlined in the laboratory. The pCR2.1-TOPO backbone was obtained by restriction enzyme digest using EcoRI to remove the MCS, followed by T4 ligation to re-circularize the vector. Removal of the MCS was necessary to maintain the colour-based screening mechanism present in pCR2.1-TOPO that allows for visual identification of successfully transformed *E. coli* during cloning. The resultant "blank" pCR21.TOPO vector was prepared for Gibson assembly by SacI restriction enzyme digest.

To guide duck RIG-I to the DSB in the chicken genome I cloned two homology arms to flank the gene of interest from the immortal chicken embryonic fibroblast cell line DF-1. Homology arms were created by cloning the endogenous genomic sequences either 5′ or 3′ of the intended knock-in locus. The upstream and downstream homology arms were each 1.25 kb in length as longer homology arms tend to yield more efficient knock-ins[196] and the total amount of homology, up to around 2 kb, is purportedly better suited for large knock-ins[197,198,199]. Each homology arm was placed up or downstream of

the gene of interest on the plasmid to mimic the orientation of the genomic sequence to which they should hybridize (Figure 3.2).

The genomic sequence of RIG-I in mallard ducks is approximately 22 kb, a sequence far too large to be knocked in with any degree of certainty. Therefore, I decided to use the 2.8 kb coding sequence of duck RIG-I, originally obtained from mRNA, which we previously demonstrated to have activity in chicken cells[67,78]. To further mimic the conditions in which RIG-I is normally found in the duck genome I decided not to use a promoter that might overexpress duck RIG-I but to use the recently characterized endogenous promoter of RIG-I in ducks[75]. Of the reported lengths of duck RIG-I promoter that showed activity, the 250 bp form was reported to be the most active[75]. I therefore chose the 500 bp form in an effort to reduce the possibility of duck RIG-I expression being too high in chicken cells that normally lack the receptor. The 500 bp form of the promoter was placed downstream of the 5′ homology arm and upstream of the coding sequence of RIG-I (Figure 3.2).

In addition to duck RIG-I under the control of its own promoter, I included a fluorophore to be expressed with duck RIG-I to allow the use of fluorescence activated cell sorting (FACS) as a high-throughput screening method. I chose green fluorescent protein (GFP) to take advantage of the number of established techniques using this fluorophore. To control GFP I chose the *Human betaherpesvirus 5* "human cytomegalovirus" (HCMV or CMV) virus promoter, which confers high levels of activity to whichever gene it controls and thereby maximizes GFP expression in successfully transfected cells. Due to the size of the HDR template, transfection efficiencies were expected to be low, so a highly active fluorophore would in theory, maximize the FACS

yield of successfully transfected cells. The CMV/GFP cassette was placed downstream of the duck RIG-I promoter and coding sequence, but upstream of the 3′ homology arm to ensure knock-in along with duck RIG-I (Figure 3.2).

Downstream of the 3′ homology arm, outside the window of genes to be knocked-in, is a negative selection mechanism comprised of the *Human alphaherpesvirus 1* (HSV) thymidine kinase gene (TK) and its endogenous promoter (Figure 3.2). The HSV TK cassette was placed upstream of the pCR2.1-TOPO backbone resulting in a circular vector (Figure 3.2).

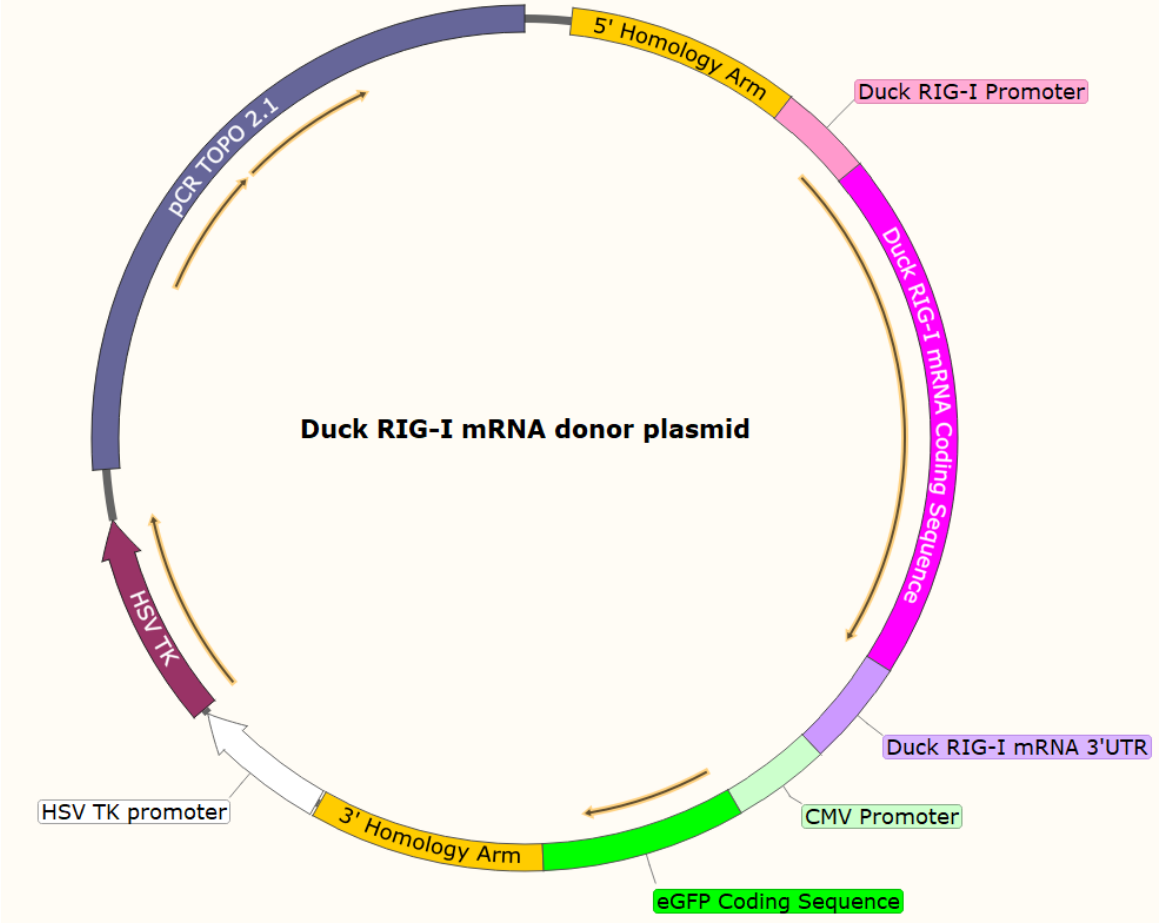

Figure 3.2. Schematic map of HDR template plasmid. Duck RIG-I is driven by the 500 bp form of its native promoter. eGFP is driven by the human cytomegalovirus promoter. Herpes virus thymidine kinase is driven by its native promoter.

3.2.3 Modifying eSpCas9(1.1) for use in an avian model.

To facilitate expression of a Cas9 enzyme in a chicken system we modified a commercially available Cas9 plasmid. Our group has observed a tendency of some mammalian promoters to not effectively drive gene expression in avian systems. In keeping with this, the plasmid from which eSpCas9(1.1) and its sgRNA would be expressed required modification. First to install the sgRNA sequence of choice, and second to replace the mammalian U6 promoter driving sgRNA expression with a chicken homolog.

In order to install the desired sgRNA sequence into the gRNA scaffold I attempted to use PCR and T4 ligation. Due to a 44 bp repeat in the commercial plasmid, immediately downstream of the sgRNA locus, PCR amplification was unsuccessful. The distance between each of the 44 bp repeat motifs was 38 bp, a distance small enough to make PCR amplification impractical as any polymerase used would fail to distinguish one motif from the other. This lack of fidelity resulted in amplicons which were duplicated, truncated, or absent completely. Furthermore, the 44 bp motif made excision of that particular sequence by restriction enzyme digestion impractical. A restriction enzyme whose target sequence lies in the 44 bp repeat motif would only recognize one site but not both or would not recognize either site at all. The 44 bp motif therefore prevented the modification of the sgRNA scaffold and made the installation of targeting sgRNA sequences impractical. I therefore decided to synthesize the sequence which coded for both the target sgRNA and the RNA scaffold required for Cas9 binding and insert the synthesized “targeting cassette” downstream of the U6 promoter (Figure 3.3).

To replace the mammalian U6 promoter which drives sgRNA and scaffold expression I chose to use a chicken derived homolog of U6. The use of avian U6 promoters for the purpose of expressing CRISPR sgRNA has been recently utilized for a CRISPR mediated knock-out in chicken DF-1 cells[200]. Of the 4 different chicken U6 promoters I chose promoter U6.3 as it has an ability to drive functional small RNAs in chicken cells[201]. The U6.3 promoter was placed upstream of the sgRNA and scaffold (Figure 3.3).

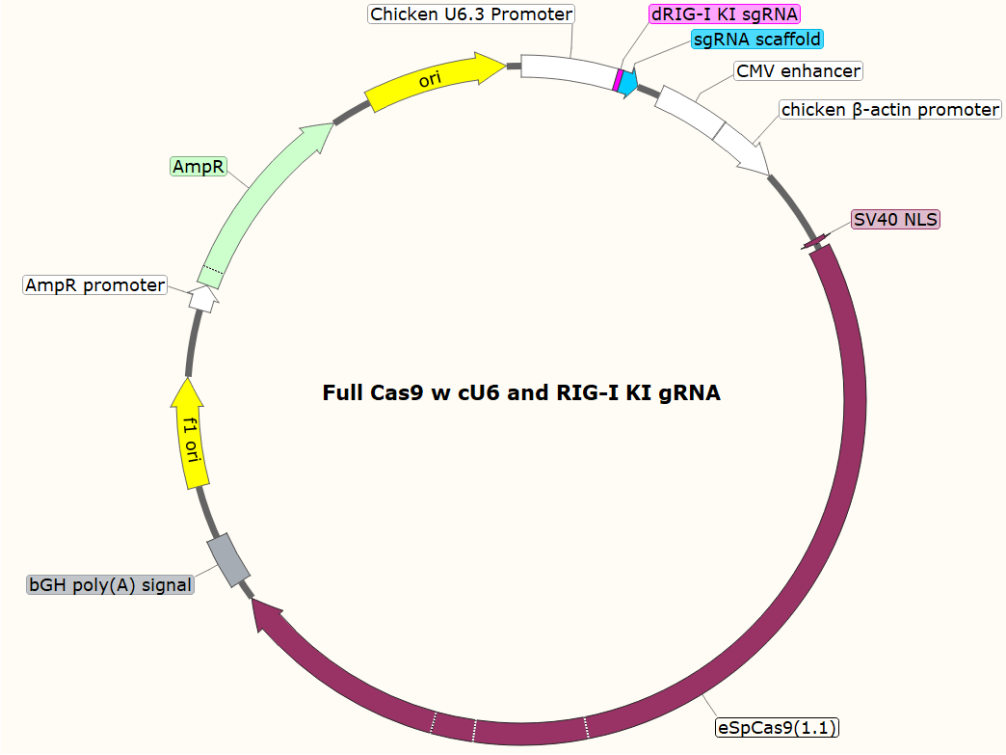

Figure 3.3. Schematic map of Cas9 and sgRNA expression plasmid. A commercially available eSpCas9(1.1) vector was modified to include a chicken derived U6.3 promoter and an sgRNA sequence targeted for the chicken Z chromosome.

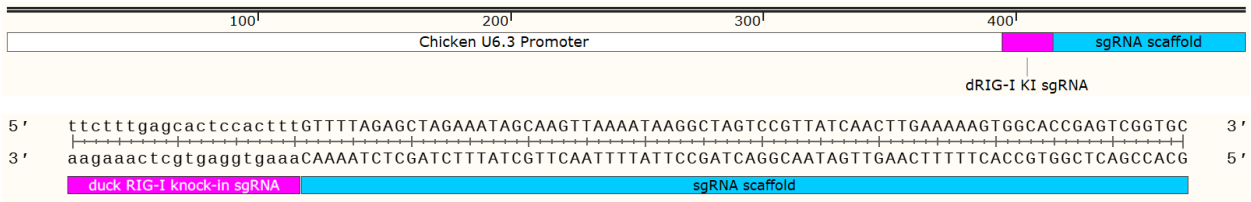

Figure 3.4. Schematic map and sequence of duck RIG-I knock-in sgRNA and sgRNA scaffold. The chicken U6.3 promoter (394 bp) drives expression of the duck RIG-I knock-in sgRNA (20 bp) and sgRNA scaffold (76 bp) complex, as found in the avian adapted eSpCas9(1.1) plasmid.

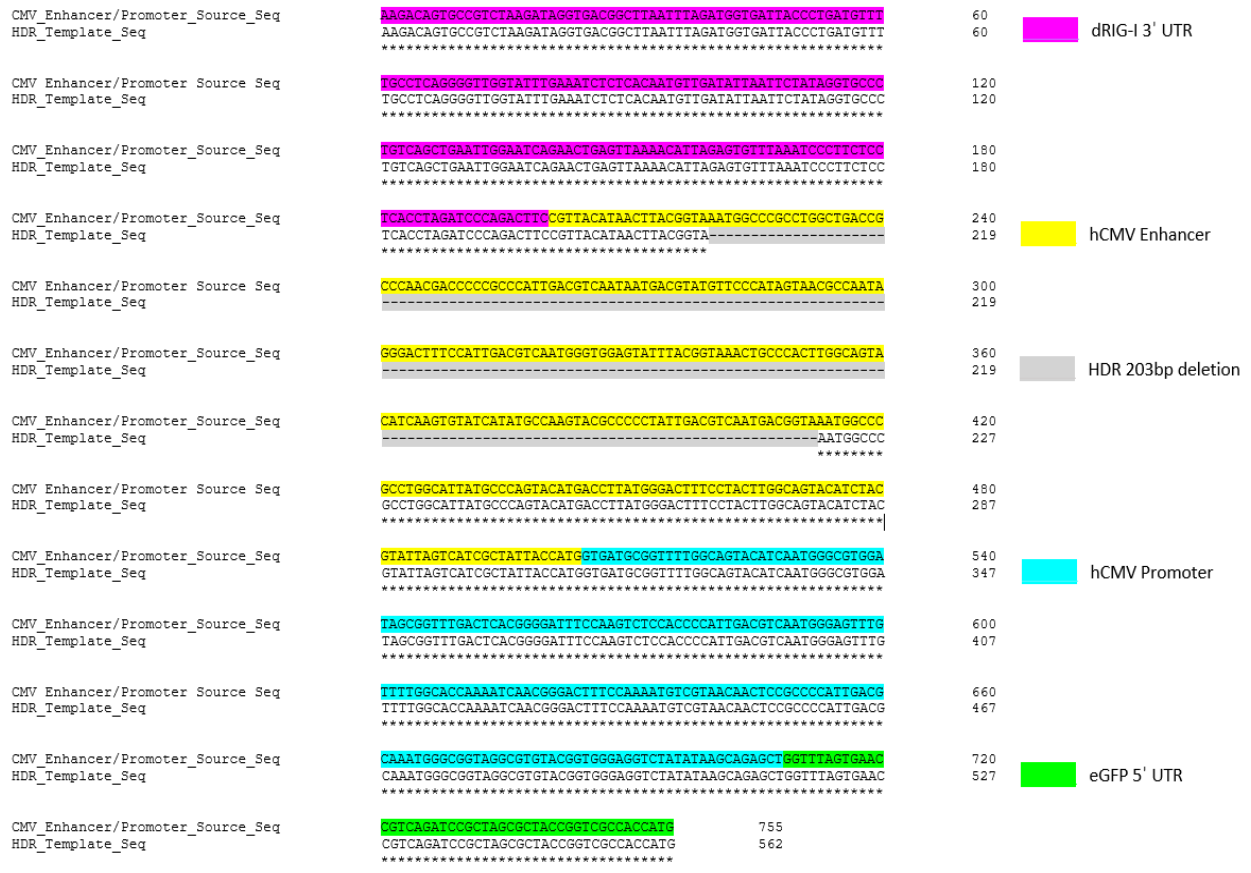

Figure 3.5. Sequence alignment of wild type hCMV enhancer/promoter and dRIG-I HDR template shows a 203 bp truncation of hCMV enhancer. HDR template construction resulted in the deletion of 203 bp from the hMCV enhancer control element while the hCMV promoter remained intact. Sequences were generated using Sanger sequencing and primer walking. Contiguous sequences were constructed using DNAstar Lasergene software and aligned using the web based Clustal Omega tool.

3.3 Transient plasmid transfections

3.3.1 HDR template plasmid transient transfection shows plasmid uptake and GFP expression in wild-type DF-1 cells.

To test whether the HDR template plasmid could be taken up by cells in the first place, I transiently transfected wild type DF-1 cells with 0.5 µg, 1.0 µg, 2.0 µg, and 5.0 µg of plasmid via lipofection. After 24 hr the cells were washed, fixed, stained, and imaged via confocal microscopy (Figure 3.5). Cells expressing GFP were seen at all concentrations of template plasmid showing that the plasmid was able to be transfected into DF-1 cells and genetic elements from the plasmid are expressed.

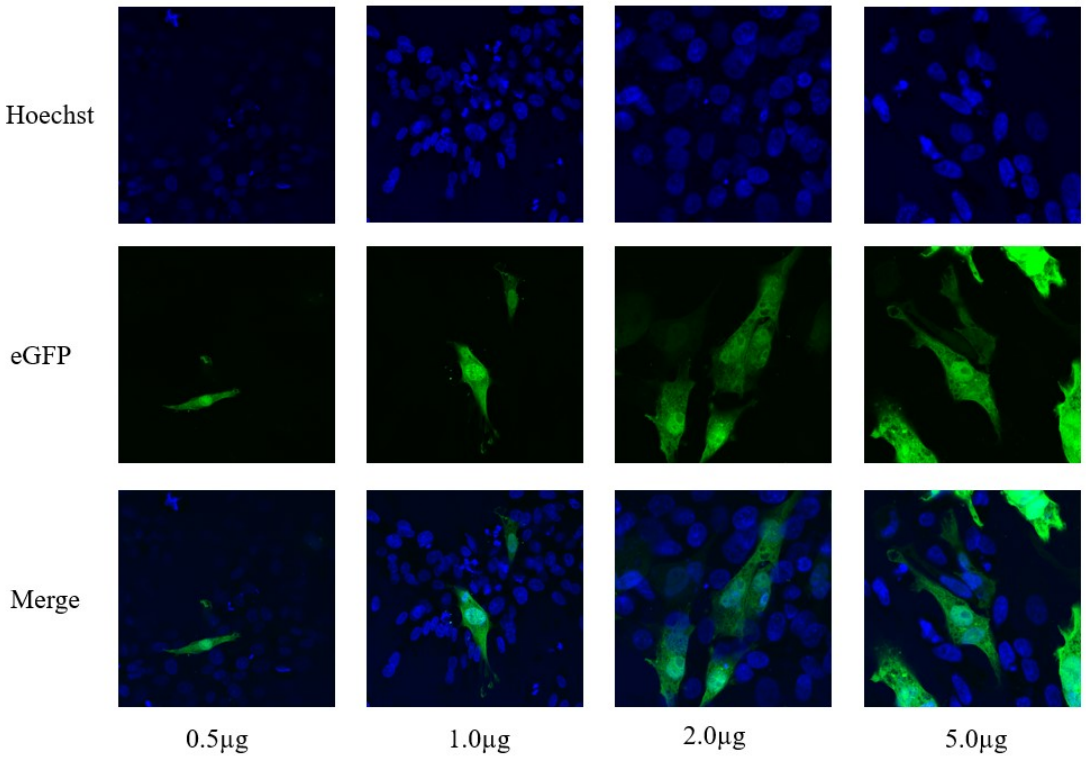

Figure 3.6. DF-1 cells transiently transfected with HDR template plasmid show expression of eGFP. Cells were seeded at a density of $1.0x10^5$ cells/mL and transfected with 2.5 µL Lipofectamine 2000/µg DNA. Cells were fixed and stained 24 hrs post transfection and imaged 48 hrs post transfection. Cells were fixed and stained with Hoechst nuclear stain 24 hrs post transfection. Cells were imaged under 1000x magnification, GFP fluorescence was observed at λ=509 nm.

3.3.2 Activation dependent expression of 500bp duck RIG-I promoter in transiently transfected DF-1 cells.

To corroborate our groups earlier findings that a 500 bp form of the duck RIG-I promoter was sufficient to drive gene expression in DF-1 cells[75] I, with the help of an undergraduate student, constructed a plasmid with the fluorophore mCherry under the control of the 500 bp duck RIG-I promoter (Figure 3.6). We then transfected 1.0 µg of this plasmid into DF-1 wild type cells via lipofection. No expression of the mCherry fluorophore was observed. We then repeated the transfection, however after 24 hours cells were transfected again with 1.0 µg of high molecular weight poly (I:C), a synthetic dsRNA agonist of MDA5, to stimulate an interferon response and consequently the duck RIG-I promoter. 24 hours after stimulation, transfected cells were fixed and stained and imaged as previously described. Expression of the mCherry fluorophore was observed following stimulation of cells with poly (I:C) (Figure 3.7).

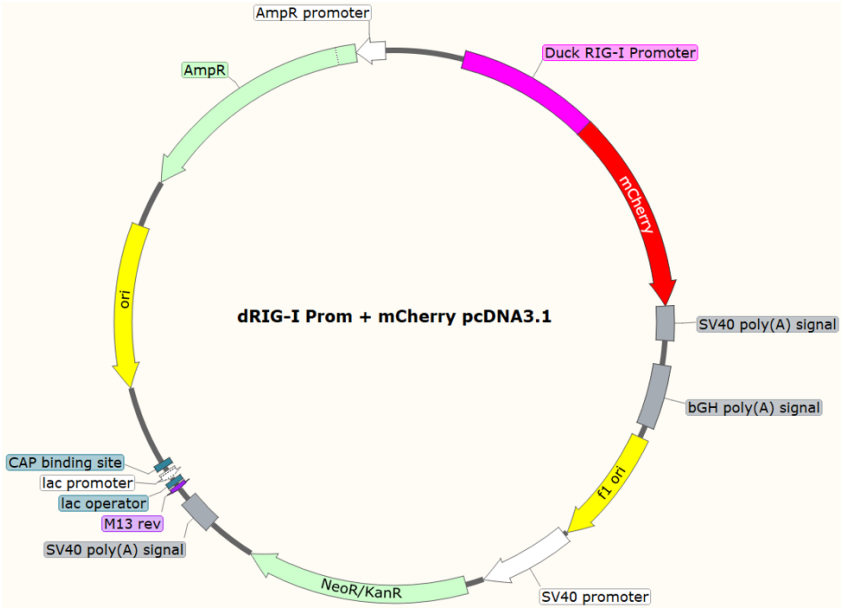

Figure 3.7. Schematic map of mCherry/duck RIG-I promoter expression plasmid. The CMV promoter and MCS of pcDNA3.1 was excised and replaced with a cassette containing the mCherry fluorophore under the control of the 500 bp duck RIG-I promoter. This plasmid was completed by Jordyn Pelechaty under my supervision.

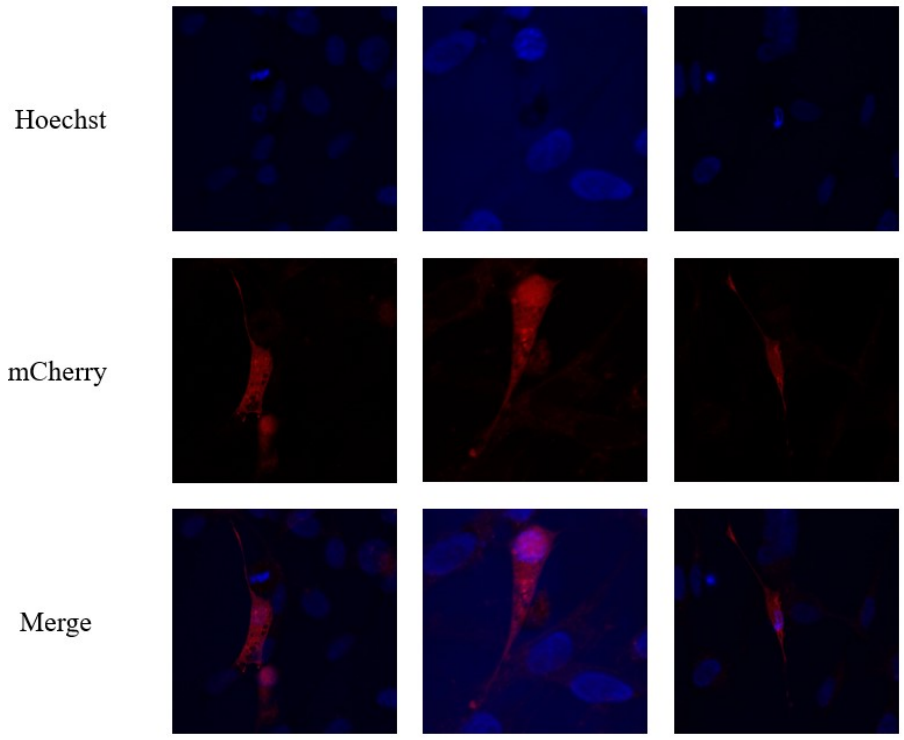

Figure 3.8. DF-1 cells transiently transfected with duck RIG-I promoter reporter plasmid show expression of mCherry. Wild type DF-1 cells were transfected with 1.0 µg of reporter plasmid. 24 hrs post transfection cells were stimulated with 1.0 µg poly (I:C) 24 hrs prior to fixing and staining with Hoechst nuclear stain. Cells were imaged under 1000x magnification, mCherry fluorescence was observed at λ=587 nm. This work was done by undergraduate student Jordyn Pelechaty under my supervision.

3.3.3 Transient eSpCas9(1.1) plasmid transfection shows expression of Cas9 protein in DF-1 cells.

To examine whether the avian adapted eSpCas9(1.1) plasmid would work in DF-1 cells I transiently transfected 2.0 μg of the Cas9 plasmid into DF-1 cells via lipofection. Wild type cells were also transfected with empty pcDNA3.1+ as a transfection control. After 24 hours cells were lysed and whole cell lysate was collected and prepared for a polyacrylamide gel and Western blot. Whole cell lysate was blotted with an α-chicken actin primary antibody as well as an α-Cas9 primary antibody, both were followed by incubation with goat-α-mouse secondary antibody. Wild type DF-1 cells showed no Cas9 protein whereas transfected cells were positive for Cas9 protein at the predicted size of 160 kDa (Figure 3.8). Both cell lysates displayed chicken β-actin protein expression (Figure 3.8).

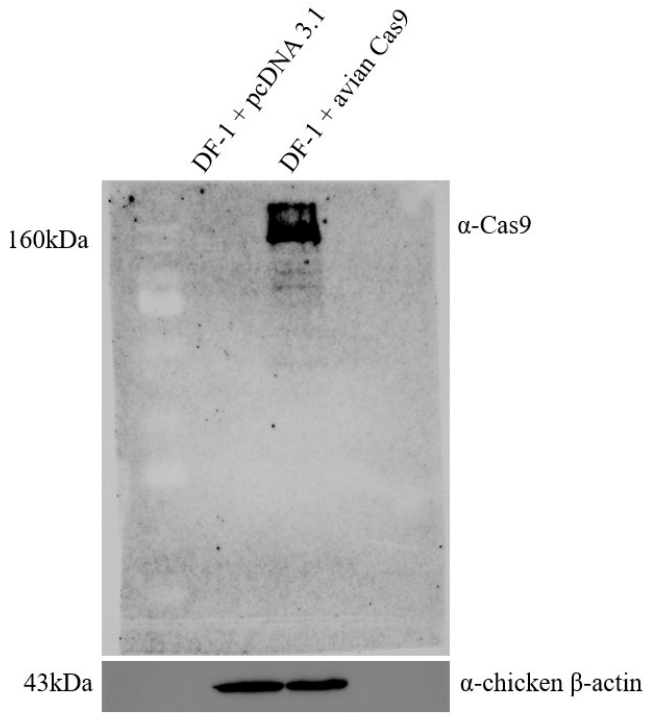

Figure 3.9. Wild type DF-1 cells transiently transfected with avian adapted eSpCas9(1.1) show expression of Cas9 protein. Whole cell lysate collected 24 hr post transfection shows expression of Cas9 protein detected by western blot.

3.4 Fluorescence activated cell sorting and collection of GFP positive cells from DF-1 cells transfected with both HDR and avian eSpCas9(1.1).

To generate stably transfected DF-1 cells expressing both dRIG-I and GFP DF-1 cells were transfected with both the HDR template plasmid and the Cas9 plasmid simultaneously. Inclusion of GFP in the HDR template plasmid was expected to result in the expression of GFP at levels sufficient for sorting via FACS. DF-1 cells were transfected with 2.0 µg each of HDR template and avian eSpCas9(1.1) plasmids. Wild type DF-1 cells were also transfected with 2.0 µg of avian eSpCas9(1.1) alone as a transfection and autofluorescence control. 24 hours post-transfection cells were trypsinized and collected for FACS. HDR+/Cas9+ cells were sorted for GFP expression relative to autofluorescence of Cas9+ cells. 8000 cells were collected and returned to culture conditions for selection and propagation. Success of this transfection, as measured by the number of GFP+ cells in a sample, was 2.1% (Figure 3.9) however transfection success has been observed in other identical transfections ranging from 0.9% to 17.1% (Data not shown). These identical transfections were done to obtain a population of stably transfected cells which would survive and propagate. However, in each attempt (n=7), following FACS each set of cells died within 7-14 days post sort. The death of a sorted cell population resulted in the need to repeat the transfection, sort, and recovery protocol until a population of sorted cells were able to survive sorting and propagate to form a bulk transfected population.

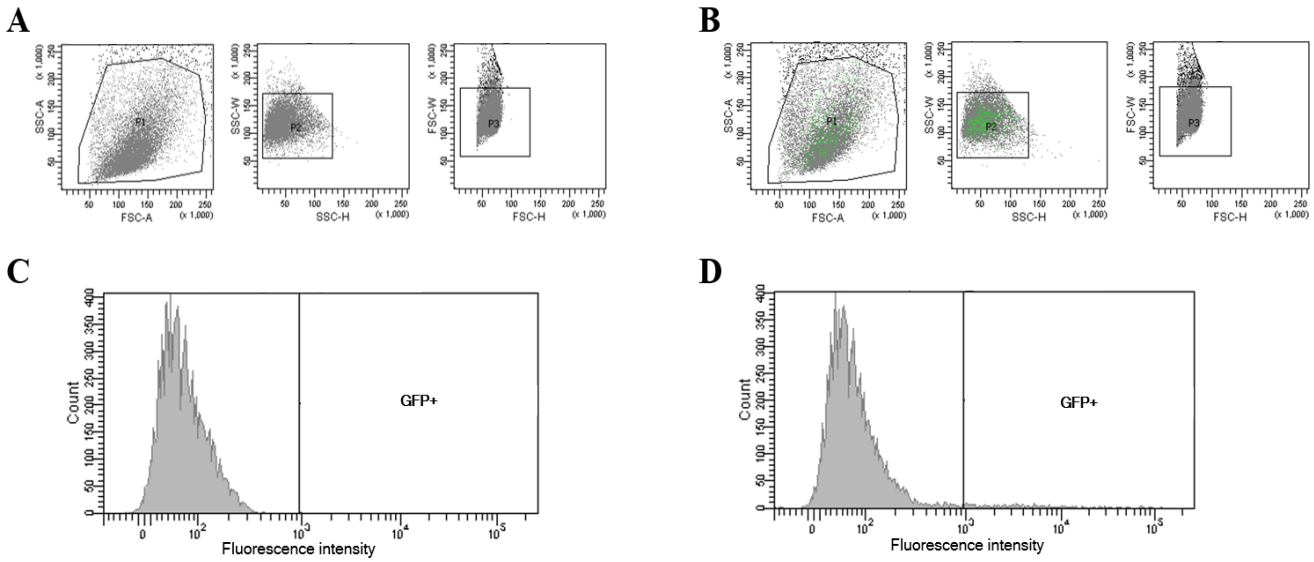

Figure 3.10. DF-1 cells transfected with both HDR and Cas9 plasmids display GFP expression (B, D) while cells transfected with Cas9 plasmid alone do not (A, C). Scatter plots of parent groups from which cells were sorted (A, B). GFP+ cells appear in all three parent clusters of HDR+/Cas9+ cells but not Cas9+ (A, B). Histogram plots depict distribution of cells counted according to fluorescence intensity (C, D). Cas9+ cells were set as autofluorescence control (C). GFP fluorescence above Cas9+ control was seen in HDR+/Cas9+ cells (D). Distribution data was collected from a sample of 12,372 count-events with 261 registering as GFP+, suggesting a transfection success rate of 2.1%. 8,000 GFP+ cells in total were collected and returned to culture conditions.

3.5 PCR of isolated clonal cell lines reveals exogenous DNA.

To determine whether each isolated clonal cell line had integrated DNA from the HDR plasmid, PCR was done on genomic DNA extracted from each cell line following isolation and propagation by limiting dilution. The presence of exogenous DNA in DF-1 cells would suggest an integration of HDR template DNA into the chicken genome.

To isolate individual transfected cells and grow clonal cell lines I resuspended cells to 5 cells/mL and aliquoted into 96-well plates in growth media containing 5 µM GCV and allowed to propagate for ~3 weeks. During this 3-week incubation period all cell cultures ceased expressing GFP. After 3 weeks of growth under selection, 56 cell lines survived and were propagated to confluency. These 56 cell lines were transferred to

24-well plates under the same selection pressure and allowed to propagate to confluency for ~3 weeks. Of the 56 lines, 34 survived selection after 3 weeks.

To examine clonal cell lines for presence of exogenous DNA, we isolated genomic DNA from an aliquot of each clone. Duck RIG-I was amplified from the genomic DNA of 14 of the 34 cell lines. From each of the 34 cell lines chicken GAPDH was amplified. HSV TK was amplified from 32 cell lines. Of these 14, 12 survived subsequent passaging (Figure 3.10). Identity of amplified dRIG-I and HSV TK was confirmed by Sanger sequencing of PCR amplicons.

Gel electrophoresis of PCR amplicons may suggest that the amount of integration of HDR template DNA may not have been equal between all cell lines (Figure 3.10. Relative to each other, some cell lines (P2D5, P2A5, P1B4, P1B6) (Figure 3.10) show increased amplification of duck RIG-I DNA while others (P2D5, P2A1, P2C2, P2C4, P2A5, P1A2) (Figure 3.10) show increased amplification of HSV TK DNA. Whether this is the result of on/off target integrations, different copy numbers of integrated genes, or differences in polymerase amplification of transgene DNA are not yet discernable. However, it was observed that cell lines P2C5 and P2B1 showed no amplification of HSV TK DNA by PCR (Figure 3.10). Positive controls for PCR amplification of each target gene were included. The HDR plasmid was used as a positive control PCR template for the amplification of duck RIG-I and HSV TK. An aliquot of wild type DF-1 cell genomic DNA was used as a positive control PCR template for the amplification of chicken GAPDH. A PCR negative control was also included.

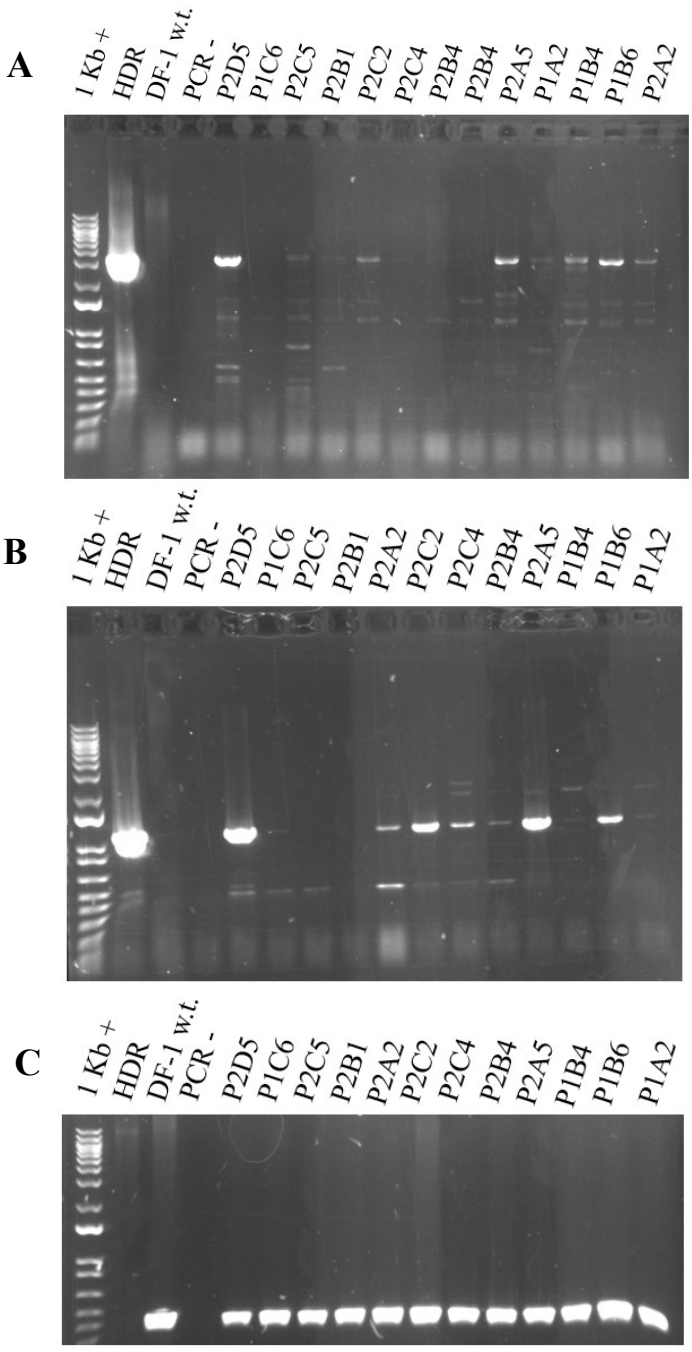

Figure 3.11. Isolated clonal cell lines show presence of chicken duck RIG-I (A), HSV TK (B) and chicken GAPDH (C) in genomic DNA via PCR. Each cell line survived GCV selection for ~6 weeks prior to gDNA extraction.

3.6 rtPCR and qPCR of transgene expression in CRISPR cell lines.

Cell lines were stimulated with 500 ng poly (I:C) + 250 ng RIG-I ligand via lipofection for 24 hrs after which, cells were collected, RNA was harvested, and cDNA was synthesized. qPCR showed amplification of chicken GAPDH from all 14 cell lines examined (Table 3.1). Chicken Mx-1 was not detected by qPCR in any cell lines (Table 3.1). Duck RIG-I was detected above background levels in DF-1 cells transfected with a pcDNA3.1 duck RIG-I overexpression promoter[67] but not detected above background in isolated clonal cell lines or wild type DF-1 (Table 3.1). HSV TK was not detected by rtPCR in any of the 12 isolated clonal cell lines (Data not shown).

Table 3.1 qPCR quantitation values (triplicate) for chicken GAPDH, duck RIG-I, and chicken Mx-1 amplified from cDNA synthesized from total cellular mRNA. Mean C_T values (n=3) of each clonally isolated cell, wild type DF-1, and dRIG-I transfected DF-1 cells following qPCR amplification for chicken GAPDH, duck RIG-I, and chicken Mx-1.

	cGAPDH		dRIG-I		cMx-1	
Cell Line	**C_TMean**	**StDev**	**C_TMean**	**StDev**	**C_TMean**	**StDev**
P2C2	21.958	0.050	35.521	0.561	Undetected	N/A
P1B6	18.927	0.142	Undetected	N/A	Undetected	N/A
P1C6	20.878	0.496	Undetected	N/A	Undetected	N/A
P2B1	17.943	0.080	31.972	0.649	Undetected	N/A
P1A2	25.519	0.646	35.480	1.856	Undetected	N/A
P2A2	18.858	0.152	Undetected	N/A	Undetected	N/A
P2C5	19.733	0.190	36.262	0.839	Undetected	N/A
P2A5	18.930	0.067	Undetected	N/A	Undetected	N/A
P1B4	20.366	0.200	Undetected	N/A	Undetected	N/A
P2D5	19.187	0.160	Undetected	N/A	Undetected	N/A
P2B4	20.817	0.142	34.999	1.160	Undetected	N/A
P2C4	18.357	0.066	36.052	0.227	Undetected	N/A
DF-1 w.t.	19.511	0.248	34.901	1.36	Undetected	N/A
DF-1 + pcDNA_RIG-I	16.847	0.476	17.544	1.71	Undetected	N/A

3.7 HSV TK and ganciclovir toxicity in DF-1 cell death assay.

To examine the efficacy of HSV TK in DF-1 cells in the presence of ganciclovir wild type DF-1 were transfected with 10µg HDR template plasmid 24 hrs prior to seeding. Wild type DF-1, HDR transfected DF-1, and CRISPR cell line P2D5 were seeded in 24 well flasks at $5.0x10^5$ cells/mL and allowed to grow for 72 hrs. Growth media supernatant and adherent cells were collected for cell counting by trypan blue staining. Wild type DF-1 and P2D5 cells showed similar toxicity profiles with gradual decline in proportion of living cells up to 10 µM ganciclovir, with sharp declines from 10 µM to 20 µM ganciclovir, there was no significant difference between the two (Figure 3.11). DF-1 transiently transfected with HDR plasmid showed significant decline in proportion of live cells beginning at 0.1 µM ganciclovir (Figure 3.11). The largest difference between DF-1 and DF-1+HDR was observed at 10 µM where DF-1 cells showed 63% survival whereas DF-1+HDR showed 20% (Figure 3.11).

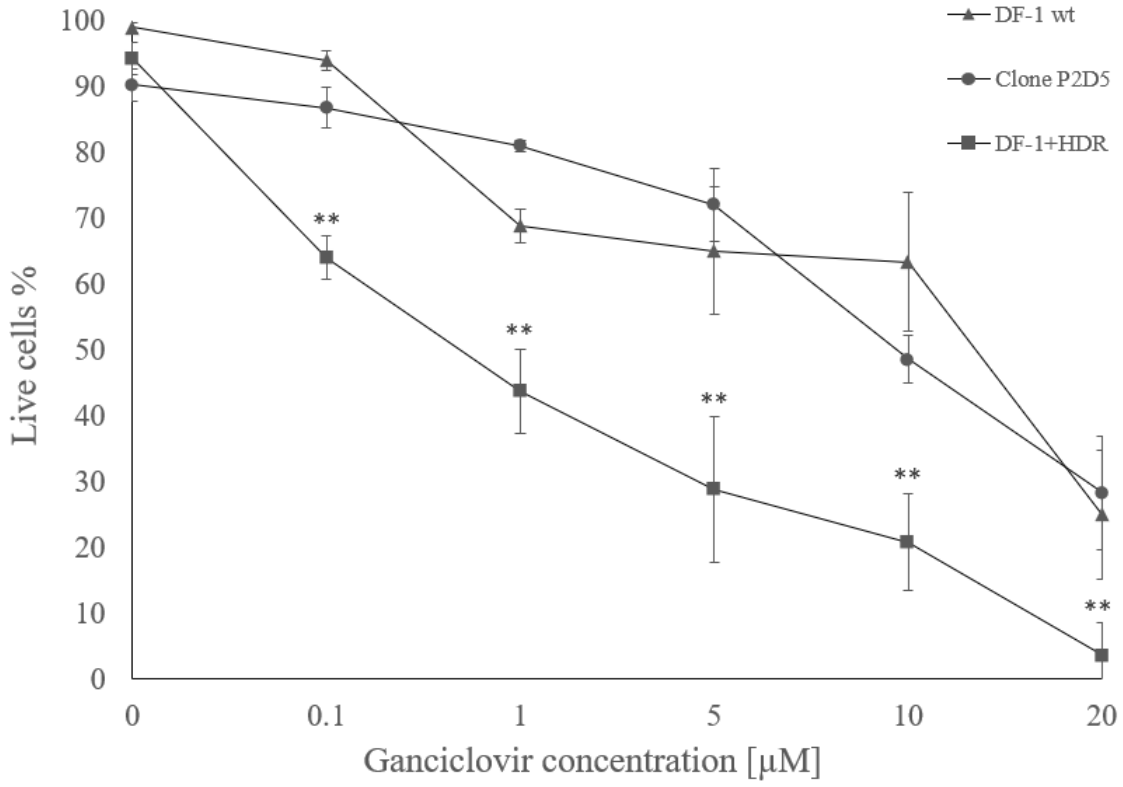

Figure 3.12. DF-1 cells transfected with HDR template plasmid appear more sensitive to ganciclovir than CRIPSR modified DF-1 or wild type DF-1 cells. DF-1 cells transiently transfected with HDR template plasmid show increased sensitivity to ganciclovir compared to wild type DF-1 cells or a representative CRISPR modified cell line, while a CRISPR modified cell line shows no difference in sensitivity from wild type DF-1 cells. DF-1 cells were transfected with 10 μg HDR template plasmid 24 hrs prior to seeding. Cells were seeded at $5.0x10^5$ cells/mL in growth media supplemented with varying concentrations of ganciclovir and allowed to grow for 72 hrs. Cells were collected and stained with 0.4% trypan blue prior to enumeration by haemocytometer. Data presented are mean values of triplicates, error bars represent one standard deviation about the mean. Asterisks indicate statistically significant difference as determined by Tukey's HSD ($p < 0.01$).

Chapter 4 Discussion.

The first task of this work was to identify a locus on the chicken Z chromosome into which to knock duck RIG-I. This was done by examining where on the duck Z chromosome *DDX58* was found. Due to the lack of comprehensive annotation of the duck genome the two closest genes to RIG-I, *ACO1* and *DGKQ* were used as "landmarks" to identify the corresponding fragment on the chicken Z chromosome. From here, 2.5 kb sections of the Z chromosome were analyzed for sequence similarity to genes annotated in the NCBI genbank database using their BLAST search tool. Our goal was to identify a region of genomic DNA with no known genetic elements. Roughly one year after we identified a suitable 2.5 kb locus the chicken genome annotation was updated, and the region of DNA we had selected for knock-in was shown to be located in a cluster of genes of the MROH family. The MROH (maestro heat-like repeat) family is composed of a handful of genes with varying, and largely unknown, functions[202]. However, one member of the family, *MROH4* has a domain that suggests mediation of protein-protein interactions and is involved in spermatogenesis in mice[203]. Although the proposed knock-in locus lies in a cluster of MROH and MROH-like genes the 2.5 kb knock-in locus still showed no genetic elements of interest, and was for all intents and purposes suitable for the knock-in of a large DNA fragment. However, as genome annotations are continually updated and reorganized it may be that a reannotated or reorganized chicken Z chromosome may uncover some fragment of a RIG-I gene or homolog. This may be especially true in the case of the chicken Z chromosome as chromosomal breakpoints and rearrangements may make sequence validation and annotation more difficult and may allow for genetic elements to remain undetected or go unnoticed.

Chromosomal recombination and breakage events appear to be more common in avian species than in mammals[238]. The frequency of chromosomal recombination in birds suggests that chromosomal breakage and recombination events may be responsible for some factors impacting avian evolution[238,239,240,241]. Indeed, investigations by cytogenic mapping have determined several regions of chromosomal rearrangements when comparing ducks and chickens[242]. Furthermore, it was reported that chromosomal rearrangement events were present in chickens on chromosomes 1,2,4,7,8, and Z [242].

The precise mechanism responsible for the apparent loss of RIG-I in chickens remains unclear. However, it may be likely that RIG-I was lost in chickens rather than a failing to be acquired during evolution. This may be partially explained by the synteny of the chicken and duck Z chromosomes (Figure 3.1) and the predisposition for chromosomal breakage and recombination events in birds. A lack of detectable RIG-I in the genome of red junglefowl, the progenitor to domesticated chickens, as well as frequent recombination events in birds suggests that RIG-I was lost from chickens during the 90-million-years of divergence from ducks.

The second objective of this work was to design and construct two plasmids suitable for the knock-in of duck RIG-I into the genome of chicken cells by homology directed repair. The isolation of the HDR plasmid components was done using Phusion hi-fidelity polymerase to reduce the chances of polymerase induced mutations. The only mutation observed in the construction of the HDR plasmid was a 203 bp deletion in the hCMV enhancer sequence upstream of eGFP. This sequence was not critical in the function of eGFP as the hCMV promoter was sufficient to drive eGFP expression. The deletion of 203 bp may likely have been caused by polymerase slippage, a process

whereby short or long repeats of DNA can form hairpin secondary structures that may be inaccessible to polymerase enzymes that cannot dissociate template DNA strands from one another during replication[204]. Phusion DNA polymerase, common in many Gibson assembly kits as well as being the hi-fidelity enzyme of choice used in my experiments, does not display strand displacement activity and therefore can be susceptible to polymerase slippage[204].

Transient transfection of all three plasmids (HDR, Cas9, and mCherry) used in this work showed that each plasmid could be transfected into DF-1 cells via lipofection. My results further show that protein coding genes from these plasmids can be expressed in transfected cells. Furthermore, the expression of GFP in transfected cells was sufficient to differentiate and sort successfully transfected cells from non-transfected cells by FACS. As determined by FACS, the transfection efficiency of the HDR template plasmid varied from 0.9% to 17.1%.

Although transiently transfected cells express GFP at levels amenable for FACS these cells ceased expression of GFP ~ 5 days into selection post-sort. Some loss of GFP expression in the selected population was to be expected as the plasmids delivered were relatively large and the transfection efficiencies were low. It was hypothesized that ~1% of transfected cells would have stably integrated GFP into their genome and would therefore continue to express it, while the majority of cells would have been only transiently transfected and therefore would lose GFP expression after ~7 days post transfection, as had been previously observed. However, what was not expected was that all cells post-sort would lose GFP expression. Of the several plausible explanations for the observed loss of fluorescence in transfected cells post-sort, one may be that the CMV

promoter is reportedly prone to silencing by methylation and is prevented from activating gene transcription both *in vitro* and *in vivo*[205,206]. Methylation of promoters, and in particular the CMV promoter, is reported to be a contributing factor to transgene silencing or decreased transgene transcription as early as 24 hours post transfection in some systems[207]. A second explanation of decreased GFP expression may be that due to the 203 bp deletion observed in the HDR plasmid (Figure 3.4) the CMV enhancer was truncated and therefore rendered non-viable. The truncation of the CMV enhancer may have reduced the potential for stronger GFP activation as the CMV enhancer increases CMV driven gene expression without being strictly necessary[208,209].

The delayed silencing of GFP expression was not the only instance of a lack of transgene expression observed in transfected cells. Indeed, in successfully transfected and cultured cells I observed no expression of HSV TK by rtPCR, and no expression of dRIG-I by qPCR. A plausible explanation for a lack of expression of these genes may be methylation of transgene DNA.

The methylation status of the target knock-in region of the Z chromosome was not determined experimentally. The state of chromatin density was inferred from the genes adjacent to the target site, namely ACO1. ACO1 is an aconitase involved in the Krebs' cycle, a central cellular metabolic pathway[194,195], and as such the chromatin around it should be open and readily accessed by transcription machinery. However, the state of neighbouring chromatin is no guarantee of the state of chromatin at the target knock-in locus. It would be beneficial to establish the methylation status of that particular region by bisulfite genome sequencing[243]. If it were determined that DNA methylation may affect transgene expression the inclusion of insulator sequences in the HDR template may

alleviate methylation related problems. Insulator sequences are DNA sequences that are reported to protect transgenes from silencing by DNA methylation or histone deacetylation[244]. The inclusion of insulator sequences and the determination of knock-in locus methylation status may be worth consideration if the methylation of transgenes was determined to be a cause of transgene silencing.

However an equally likely explanation for transgene silencing is that there may have been multiple copies of DNA from the HDR template knocked into DF-1 genomic DNA resulting in the formation of concatemers or a head-to-tail insertion rendering the knocked-in DNA silenced or transcriptionally non-active. The formation of concatemers, repetitive segments of genetic material, has been known to be a driver of transgene silencing in many species[210,211,212]. Concatemer formation may be a likely explanation for the silencing of multiple transgenes reported here. This may be supported by an observed difference in the amount of PCR amplicons generated during gDNA PCR, as the same number of PCR cycles was used for each reaction. It is also unlikely that the apparent variation in the amount of amplified DNA is the result of extrachromosomal DNA. Genomic DNA was harvested from clonally expanded cell lines after a total of approximately 5 weeks in culture after transfection with HDR and Cas9 plasmids. This long period of propagation included ~9 rounds of passaging to maintain healthy cell populations. It is unlikely that extrachromosomal copies of transgene DNA would persist after numerous cell passages.

Lack of expression by concatemer is supported by the presence of HSV TK in genomic DNA from transfected cells, as HSV TK DNA should only be present in cells that have undergone off-target DNA integration events; in which case the DNA from the

HDR template plasmid may not have been influenced by homology arm binding and therefore could have integrated into DF-1 genomic DNA in any location any number of times resulting in the formation of concatemers or head-to-tail knock-ins. However, due to a lack of whole genome sequence data and/or Southern blot data of transfected cells these explanations cannot be corroborated and would require further investigation.

The lack of expression of transgenes from stably transfected DF-1 cells does not necessarily imply that the individual components of the HDR template plasmid were non-functional in an avian system. Here I have demonstrated the components that comprise the HDR plasmid are fully functional in an avian system in a transient context. Furthermore, the presence of exogenous DNA in extracted genomic DNA from transfected cells suggests a successful knock-in of dRIG-I into DF-1 cells, albeit not as precisely as was desired.

The lack of transgene expression was demonstrated by qPCR analysis of duck RIG-I and chicken Mx-1 expression. CRISPR modified cell lines showed no expression of duck RIG-I above background by qPCR. The apparent amplification of some duck RIG-I transcript in any cells examined was likely a result of non-specific polymerase binding and amplification as amplicons that appear above the C_T threshold of 30-35 cycles cannot be counted in good faith as reliable on target amplicons. This is corroborated by the clearly detectable RIG-I signature in DF-1 cells transiently transfected with a pcDNA3.1 duck RIG-I expression vector, in which duck RIG-I is expressed under the control of the hCMV promoter. The hCMV promoter has been shown to be highly active in DF-1 cells in a transient expression context[67]. The activity of the hCMV promoter was corroborated when DF-1 cells were transiently transfected with

HDR template plasmid, and GFP expression was observed. Furthermore, chicken GAPDH was detected by qPCR in all cell lines as expected.

The expression of duck RIG-I may have also been impeded by a lack of an intron in the mRNA derived coding sequence in the HDR template plasmid. A lack of an intron in the coding sequence of dRIG-I may have led to errors in transgene expression. The presence of an intron in genomic DNA necessitates the splicing of pre-mRNA to form correctly translated mRNA[213]. This process is the same for every endogenously expressed gene in a cell. However, by excluding an intron from the dRIG-I coding sequence the need for correct splicing may have been negated. Failure to force transgene pre-mRNA through cellular splicing machinery may have resulted in lowered or non-existent transgene expression as introns in transgenes have been noted to increase transgene expression in several organisms[214,215,216,217,218]. Furthermore, the failure of transgene transcripts to be spliced may have rendered the host cell unable to detect transgene mRNA. Pre-mRNA that has been properly spliced in the nucleus will be accompanied by factors that help facilitate nuclear export[219,220,221]. In addition is has been reported that proper intron splicing is required for efficient nuclear export of intron containing mRNAs[222]. Therefore the lack of an intron in the coding sequence of dRIG-I may have prevented transgene expression at two levels, the lack of an intron itself and the lack of an intron to initiate pre-mRNA splicing and maturation may have inhibited transgene mRNA recognition and/or nuclear export.

Chicken Mx-1 was not detected in any cell line. This result may be explained by a failure of poly(I:C) or a RIG-I agonist to activate IFN signalling in transfected cells. The lack of expression of duck RIG-I in CRISPR modified cell lines as an explanation for no

observed Mx-1 expression may also be discounted as the expression of duck RIG-I in pcDNA3.1 RIG-I transfected cells made no difference in the detection of Mx-1 when exposed to the same poly(I:C) and RIG-I agonist by lipofection. This suggests that the lack of detection of Mx-1 may have been a result of experimental error, either through amplification or detection failure.

Chapter 5 Conclusions and Future Directions.

In this proof of principle work, I report the initial successes and areas for refinement in the use of CRISPR/Cas9 two-plasmid homology directed repair for the knock-in of duck RIG-I into the genome of DF-1 cells at a specific target locus. Here I have demonstrated that an assembly of a large plasmid containing multiple genes under the control of multiple different promoters can be transfected into, and transiently expressed in DF-1 cells. Furthermore, I have shown that the individual components of an HDR template plasmid: 500 bp duck RIG-I promoter, eGFP, HSV TK, and HSV TK promoter are functional in DF-1 cells, and that eSpCas9 can be expressed in DF-1 cells in a transient context.

Transient transfections are not the intended purpose of the plasmids created here however. The purpose of the HDR template and avian adapted Cas9 plasmids were to facilitate the knock-in of two genes, dRIG-I and eGFP, into the genome of DF-1 cells at a precise locus. Here I have shown that the two-plasmid system employed is sufficient to knock multiple genes into the genome of chicken cells, demonstrating a potential utility of this approach for the generation of cell lines in a laboratory. However, when examined in the context of whole organism genome modification the shortcomings of this approach render it non-viable. The relatively low transfection efficiency, high rate of off-target knock-in, long selection period, and unstable expression of knocked-in transgenes suggests that co-delivery of a Cas9 and HDR template plasmid by lipofection is not appropriate for the generation of transgenic organisms. However, the materials and techniques presented here show promise for use in a different approach. The HSV TK and GCV inducible suicide system was shown to work in DF-1 cells, the expression of

GFP in DF-1 cells is amenable to FACS, the expression of Cas9 protein suggests a viable approach to genome editing in DF-1 cells, and the ability to knock-in large pieces of foreign DNA (> ~5kb) has been initially investigated in DF-1 cells.

To more fully understand the mechanics of the genome modifications presented here there are two further experiments which would fill in the remaining knowledge gaps, those being a Southern blot and whole genome sequencing. The exact number of copies of transgenes present in stably transfected DF-1 cells is currently unknown. The use of Southern blot to determine copy number and relative genomic position of knock-in would show which cells underwent concatemeric knock-in. Whole genome sequencing of transfected cells would allow for the absolute genomic position and sequence of transgenes to be studied and inferences regarding the efficiency of the HDR knock-in process in DF-1 cells could be made.

Furthermore, a change in the basic strategy of plasmid delivery may prove to be a more efficient and reliable option. The technologies for lentiviral transfections for the delivery of transgenes and CRISPR machinery have been growing in ease of use, breadth of compatible organisms, and market availability[223,224]. The use of a lentiviral vector may improve on the shortcomings of a lipofection based transfection strategy, as lentiviral transfection already encompasses the machinery needed for stable knock-ins.

Additionally, different CRISPR approaches may be investigated for their appropriateness. For instance, rather than blunt ended DSBs a Cas9 double nickase may be employed to lower the possibility of aberrant DSB repair by spacing out breaks in host genomic DNA. The length of homology arms could also be adjusted to facilitate better fidelity of DNA directing the knock-in of large fragments of DNA. Optimization of

homology arm length would allow for the design of HDR template plasmids with minimally sized genes of interest. In keeping with this the size of the template DNA fragment could be lowered by perhaps using a multiple knock-in strategy. A multiple knock-in strategy could be used to simultaneously deliver both duck RIG-I and GFP from separate HDR templates or cassettes. Thereby decreasing the size of transgene DNA integrated into the genome at each particular locus.

In addition to altering the delivery method of template DNA, changing the form of Cas9 host genome cleavage, the use of multiple sgRNAs could be implemented. This would be a rather involved approach as the use of multiple sgRNAs and therefore multiple DSB or double nick sites would necessitate the use of multiple pairs of homology arms to facilitate accurate HDR. However, the use of multiple sgRNAs may provide for more chances to correctly knock duck RIG-I into the chicken genome.

The work presented here is a proof of principle exploration into the knock-in of large DNA fragments containing multiple genes into a precise locus in the chicken Z chromosome. The information gathered here will hopefully guide further research into chicken transgenics along a more effective and informed path.

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