A study of the chicken IFN lambda system

by

Andreas Rohringer
B.Sc., M.Sc.

Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

School of Medicine
Deakin University

August, 2016

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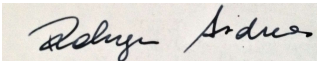
Signed: 

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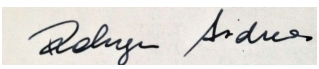
Signed: 

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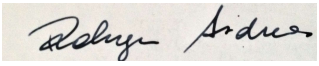
Acknowledgements:

Firstly, I would like to express my sincere gratitude to my advisors Prof. Alister Ward, and Dr. Andrew Bean for the continuous support of my Ph.D. study and related research, for their patience, motivation, and immense knowledge. Their guidance helped me in throughout the research and writing of this thesis. I could not have imagined having better advisors and mentors for my Ph.D. study.

My sincere thanks also goes to Tamara Gough, Dr. Jeffrey Butler, Dr. David Williams and Dr. Celine Deffrasnes who provided me with research support, guidance and an open ear whenever needed. Without their enduing support, it would not be possible to conduct this research.

Last but not the least, I would like to thank my family: my mother and sister as well as Dr. Amy Burroughs for supporting me throughout the writing of this thesis and in our amazing life journey so far.



Funding support:

This work was supported by the Deakin University International Postgraduate Research Scholarship, the CSIRO Top up scholarship and the Poultry CRC.

1 Abstract

High Pathogenic Avian Influenza (HPAI) viruses, such as H7N9 and H5N1 HPAI, have a very large effect on poultry production and there is a need for new strategies to deal with these infections. The characterization of antiviral immune pathways is essential for understanding host-pathogen mechanisms to underpin improved therapies for both human and livestock use. The interferon (IFN) pathway stimulates the expression of a myriad of interferon stimulated genes (ISGs) that impact viral replication. This study aimed to characterize the chicken type III IFN response, its receptor complex and its downstream mediator.

The induction of type III IFN in comparison to type I IFNs was examined in chicken splenocytes stimulated with TLR agonists and chicken IFNs. The induction of type I and type III IFNs by PIC and LPS was similar but IFN and ISG induction kinetics differed in response to IFNα and IFNλ with IFNα inducing a more rapid induction. Furthermore, age and sex dependent differences in the induction of type I and type III IFNs were identified, while females had higher levels of type I IFNs as immature and mature birds, immature males had a higher IFNλ response.

The IFNλR complex is composed of two receptor chains, the IFNλ receptor 1 (IFNλR1) and Interleukin 10 receptor 2 (IL-10R2), which utilizes the Janus kinase (JAK) and Signal transducer and activator of transcription (STAT) pathway for signaling. A chicken IFNλR1 gene was identified, which showed conserved sequence and synteny with its human counterpart. Selective siRNA mediated knockdown of either chain reduced IFNλ mediated ISG expression and STAT activation, while selective JAK1 inhibition blocked the IFNλ mediated ISG upregulation. This points towards a conserved IFNλR/JAK/STAT/ISG pathway in chickens.

The interferon-induced protein with tetratricopeptide (IFIT) repeats 5 (IFIT5) is an ISG that has been shown to be important in the antiviral response in mammals. A single IFIT was identified in chicken which showed synteny with the human IFIT genes and encoded a 470 amino acid protein with strong conservation to mammalian IFITs especially IFIT5. It was induced in chicken splenocytes by IFNα, IFNλ and TLR ligands *ex vivo*, in the lungs of chickens following infection with a highly pathogenic avian influenza virus (HPAI). This highlighted the conserved role of chicken IFIT5 in the response to viral infections like HPAI.

2 List of Abbreviations

AA	amino acids
aa/s	amino acid substitutions per site
AI	avian Influenza
ANOVA	analysis of variance
APC	antigen presenting cells
bp	base pair
CHD	cytokine receptor homology domain
CHO	Chine Hamster ovary cells
CO_2	carbon dioxide
CRF2	class II cytokine receptor family
CSF	colony-stimulating factor
CSIRO	Commonwealth Scientific Industrial Research Organisation
DMSO	dimethyl sulfoxide
dsDNA	double stranded DNA
dsRNA	double stranded RNA
E.coli	Escherichia coli
EID_{50}	50% egg infectious dose
ELISA	enzyme-linked immunosorbent assay
EMSA	electrophoretic mobility shift assay
ERK	extracellular signal regulated kinase
HA	Hemagglutination Assay
HBV	Hepatitis B
HCV)	Hepatitis C
HPAI	highly Pathogenic Avian Influenza
IBDV	infectious bursal disease virus
IBV	infectious bronchitis virus
IFIT	interferon-induced protein with tetratricopeptide repeats
IFN	interferon
IFNα	interferon alpha
IFNαR1	interferon alpha receptor chain 1
IFNαR2	interferon alpha receptor chain 2
IFNγR1	interferon gamma receptor chain 1
IFNγR2	interferon gamma receptor chain 2
IFNλ	interferon lambda
IFNλR	interferon lambda receptor
IFNλR1	interferon lambda receptor chain 1
IL	interleukin
IL-10R2	interleukin 10 receptor chain 2
IRF-9	IFN regulatory factor-9
ISGF3	IFN stimulated gene factor 3
ISGs	IFN stimulated genes
ISREs	IFN stimulated response elements (ISREs)

JAK	Janus kinase
JNK	c-Jun N terminal kinases
LPS	lipopolysaccharides
LSD	least significant difference
MAPK	mitogen-activated protein kinase
MDA-5	melanoma differentiation-associated gene 5
MDV	Marek's disease virus
mRNA	messenger RNA
MX1	Myxovirus resistance gene 1 (Mx1
NA	neuroaminidase
NDV	Newcastle disease virus (NDV)
PAMPs	pathogen associated molecular patterns
PBMC	peripheral blood monocytes
PCR	polymerase chain reaction
PIC	poly I:C
PKR	IFN-induced double-stranded RNA dependent protein kinase
PRR	pattern recognition receptor
RIG-I	retinoic acid-inducible gene-I)
RT-PCR	real-time polymerase chain reaction
SEM	standard error of the mean
SFV	Semliki Forest virus (SFV)
SNP	single nucleotide polymorphism
ssRNA	single stranded RNA
STAT	signal transducer and activator of transcription
TLR	Toll-like receptor
TNF	tumor necrosis factor
TYK2	tyrosine kinase 2
Viperin	virus inhibitory protein, endoplasmic reticulum associated, interferon inducible
VSV	vesicular stomatitis virus

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4 Introduction

4.1 Avian influenza

Influenza A viruses are members of the *Orthomyxoviridae* family being lipid enveloped, negative-sense, single-stranded segmented RNA viruses, with eight segments encoding ten proteins [1,2]. Protruding from the enveloped surface are two distinct glycoproteins, the hemagglutinin (HA) and the neuraminidase (NA) on which subtypes and antigens are classified [3,4] (**Figure 4-1**). Influenza is capable of infecting a range of hosts including but not limited to humans, dogs, horses, pigs, ferrets, cats and a wide variety of domesticated and wild birds [5-12]. It is believed that wild aquatic birds act as a reservoir for influenza A viruses, since virtually all HA and NA subtypes have been isolated from that source [13]. The exceptions are the recently discovered subtypes H17 and H18 that seem to be limited to bats [14,15] (**Figure 4-2**). This suggests that spill-over hosts, like domesticated poultry and humans, will remain at risk due to the difficulties of eradicating a pathogen in a wild population.

There are two major mechanisms influencing viral diversification. Antigenic drift (**Figure 4-3**) describes the mechanism of generating seasonal influenza virus, where point mutations in the viral genome change the antigenic profile of the virus leading to immune evasion [16,17]. Antigenic shift (**Figure 4-3**) describes the rearrangement of the segmented genome when more than one strain infects one cell which forms a new virus with often unique properties [18,19]. This is thought to occur particularly in pigs, which can be infected by both avian and human virus strains, giving rise to new highly pathogenic influenza variants [20]. The selective pressure of the host immune system plays a key role in both antigenic drift and shift [21,22].

Infectivity of influenza virus in the host is influenced by the type of receptor the virus can bind [23]. The HA subtypes of avian influenza (AI) viruses, such as the H5 and H7 subtypes, are thought to preferentially attach to the α 2,3-linked sialic acid receptors present on the respiratory epithelium and intestinal tract tissue of many avian and terrestrial bird species [24] while HA subtypes adapted to humans preferentially bind the α 2,6-linked sialic acid receptors [25]. However, there are exceptions to this, with single amino acid substitutions at the receptor binding site of the HA molecule enough to allow HPAI H5N1 viruses to recognize the human α 2,6-linked sialic acid receptor on the surface of human respiratory epithelial cells and thereby assist in cross-species transmission [26]. It has similarly been shown in the recent H7N9 outbreak in China that the human virus isolates possessed two mutations

associated with increased α 2,6-linked sialic acid binding that could account for their human pathogenicity [27]. Therefore, both antigenic shift and drift can also influence host specificity.

AIs can cause significant mortality and morbidity in humans. Since 2003 851 human cases of H5N1 were reported, which led to 450 deaths [28]. More recently the H7N9 LPAI, which caused no death in chickens [29], led to severe symptoms and even death in humans [27]. Until now a total of 781 laboratory confirmed cases have been reported causing 313 deaths [30]. In 2014 a new H5 virus emerged, subtyped as an H5N6, which caused 14 confirmed cases and 6 deaths [28,31]. Evidently the AI viruses are still re-assorting and are likely to cause more mortality and morbidity since they have now become endemic in a number of countries and pose a serious risk of becoming a new pandemic strain [31-33].

Apart from the human cases, HPAI strains have killed millions of poultry worldwide directly and indirectly, since containment of the outbreaks has necessitated culling of additional birds. The impact of HPAI strains on the poultry industry has been substantial with the economic loss has been numbered in the billions (USD) [34,35]. Transmission of AI from poultry to humans usually occurs by close association [27,36]. Therefore, an AI virus with the ability to spread easily between host species poses a serious risk and could be a potential influenza pandemic virus candidate [37].

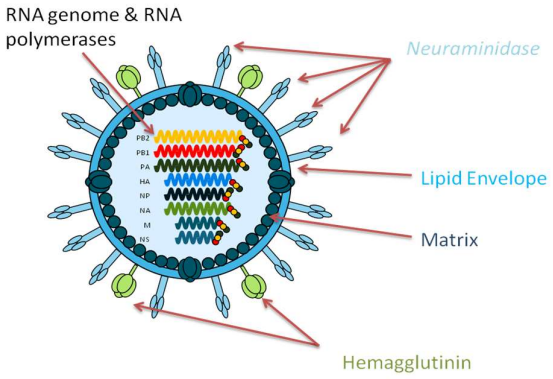

Figure 4-1 Schematic structure of influenza virus

Influenza viruses are negative-sense single-stranded RNA viruses. Their genome consists of eight RNA segments that encode 10 proteins, which are encapsulated by matrix proteins and surrounded by a host-derived lipid envelope from which the glycoproteins hemagglutinin and neuraminidase protrude.

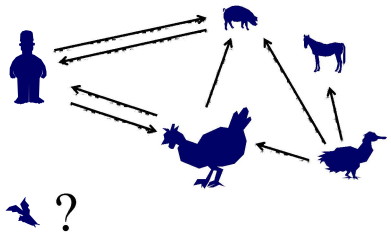

Figure 4-2 Host range and infection cycle of influenza virus

Water fowl, such as ducks, are thought to be the natural reservoir for influenza virus. Through the fecal/oral route they infect a range of domesticated animals like horses, pigs and chickens. The virus can then reach the human population by transmission via chickens or pigs. In contrast, the newly discovered H17 and H18 subtypes seem to be limited to bats.

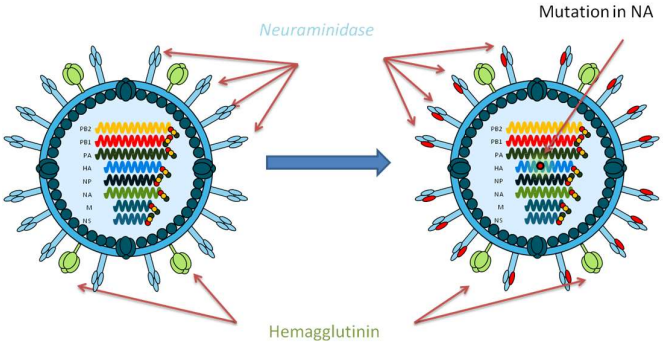

Figure 4-3 Antigenic drift of influenza virus

Seasonal influenza viruses typically acquire mutations through their error prone viral RNA polymerase. These mutations can alter antigenicity and enable the virus to escape the immune system.

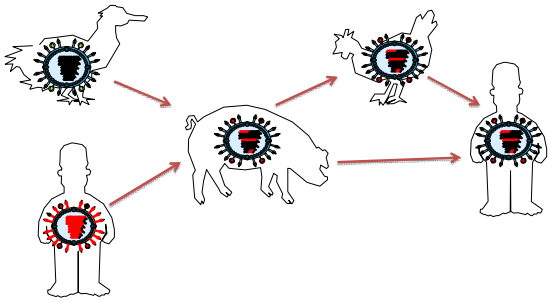

Figure 4-4 Antigenic shift of influenza virus

Different strains of influenza that infect the same cell can rearrange their segmented genomes, which can lead new gene combinations not previously encountered by the host immune system, potentially leading to new pandemic viruses. The pig is thought to be the ideal 'mixing vessel' since avian as well as human pathogenic influenza strains can infect it.

4.2 Host immune response to viral infections

The mammalian immune response to virus infections is well characterized. During viral invasion, innate immune recognition is mediated by a series of germ line encoded receptors, the pathogen recognition receptors (PRR), which detect conserved pathogen associated molecular patterns (PAMPs) [38-40]. One of the most prominent families of PRR are the Toll-like receptors (TLR) [41]. Up to thirteen conserved TLRs have been found in mammals that serve distinct functions in PAMP recognition and subsequent immune responses, although variation between species exist in the number of functional TLRs [42,43]. TLRs induce the expression of genes involved in both direct cellular defence and the mobilization of a wider immune response [38,44]. Infection with viral pathogens may be sensed through TLRs 2 and 4 that detect viral glycoproteins on the cell surface or through recognition of dsRNA through TLRs 3,7, 8 and 9 located in the endosome [45-51]. In addition to the TLRs, intracellular sensors such as retinoic acid-inducible gene-I (RIG-I) and melanoma differentiation-associated gene 5 (MDA-5) are involved in the cytosolic recognition of foreign nucleic acids [52]. PAMP recognition leads to activation of many cell types, such as phagocytes and macrophages and the expression of a number of genes including those encoding cytokines [53,54]. Cytokines are soluble, low molecular weight polypeptides and glycopeptides that interact with a multicomponent transmembrane receptor complex and activate intracellular signal transduction pathways that induce anti-viral genes as well as others that control the

complex interplay between various cell types involved in the immune response [55-57]. Cytokines represent a large family of signaling molecules, including the interferons (IFN), interleukins (IL), colony-stimulating factors (CSF), transforming growth factors (TGF) and tumor necrosis factors (TNF), each of which consists of multiple members [58]. However, the IFNs produced in the early stages of viral infection are of special interest since they play a crucial role in the broad spectrum cellular defence against the spread of virus [59] (**Figure 4-5**).

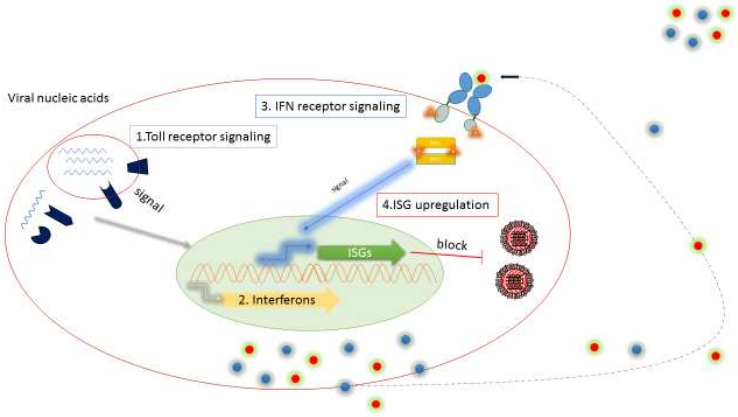

Figure 4-5 The IFN-mediated antiviral response

The antiviral actions of the type I/III interferon systems have been extensively studied in mammals. Upon sensing foreign nucleic acids host Toll-like receptors activate signals that induce the transcription of IFN genes, the products of which are secreted by the cell and serve to amplify the signal in an autocrine and/or paracrine manner. This is achieved by binding to cognate receptors that relay a signal via downstream signal pathways into the cell nucleus, which leads to the expression of ISGs. These genes encode proteins that block viral infection, growth and spread in a multitude of ways.

4.3 IFNs

IFNs were discovered by Isaacs and Lindenmann while studying influenza virus. They described the first IFN as a substance that was able to interfere with the ability of the virus to infect cells and thus coined the term “interferon” [60]. It is now recognized that there are in fact several IFNs that are collectively crucial for defence against pathogens, contributing to the induction and regulation of both innate and adaptive antiviral mechanisms [61-63]. Three distinct IFN families have been identified in vertebrate species. The families are defined by their use of specific receptor complexes, termed type I, II and III. These are distinguished by their distinctive signaling pathways and patterns of gene induction, although there is considerable overlap in these features between families [64-66]. All three IFN families are

important for an effective immune response but only type I and III are directly induced via the PRR pathway [59]. This suggests that they play a critical role in containing pathogens, providing the host with the means to survive otherwise lethal infections by delaying spread of the pathogen and enabling the adaptive immune system to mount an efficient response [67].

4.3.1 Type I IFN

Type I IFNs consist of at least 8 subclasses IFNα, IFNβ, IFNε, IFNκ, IFNω, IFNτ, IFNδ and IFNζ. The first 5 are found in humans with 13 subtypes of IFNα but only a single IFNβ, IFNε, IFNκ and IFNω [68,69]. The other subclasses of type I IFNs have so far only been identified in particular species: IFNτ in ruminants [70], IFNδ in pigs [71] and IFNζ in mice [72].

Following induction by viral infection, type I IFNs can act in both a paracrine and autocrine manner [73,74]. Of the type I IFNs, IFNα and IFNβ are well characterized, displaying potent antiviral activities. A broad range of cells can produce IFNα and IFNβ rapidly during the early stages of infection in response to the recognition of viral (and bacterial) products via PRRs [61,75,76] . In turn IFNs stimulate the expression of more than 300 genes that are called IFN stimulated genes (ISGs) many of which encode antiviral proteins that can collectively interfere with various stages of the viral life cycle [77-79]. This early antiviral response is critical to limit the spread of viruses and facilitate onset of the adaptive immune response [80]. Type I IFNs up-regulate the expression of the major immune histocompatibility complex I (MHC I) expression on many cells [81] as well as co-stimulatory molecules on antigen presenting cells (APC) [82] , enhance natural killer cell (NK) cytotoxicity, proliferation and memory functions [83-85] , induce differentiation of monocytes to dendritic cells (DC) [86], contribute to DC activation and priming abilities [87], augment the differentiation of T helper (Th) cells [88-90], modulate antibody production [91] and induce apoptosis of infected cells [92,93].

The anti-viral, immune stimulating and anti-proliferative properties of IFNs have made them attractive molecules as potential therapeutics for a variety of diseases [93,94]. Recombinant IFNα was the first approved bio-therapeutic and has been successfully used in the treatment of chronic hepatitis B (HBV) and hepatitis C (HCV) infections, where it leads to reduced viral loads and decreased incidence of liver cirrhosis and hepatocellular carcinomas [95,96]. Type I IFNs have also regularly been used in clinics as anti-cancer treatments, such as hairy cell leukaemia, Kaposi's sarcoma, chronic myelogenous leukaemia and metastatic malignant melanoma [97-100]. Likewise IFNβ forms a crucial part of long-term treatment for relapsing forms of multiple sclerosis [101]. Combination therapy of IFNα with anti-viral drugs like

ribavirin, can improve the treatment efficiency, although clinical drug resistance to IFNα has arisen in the setting of HCV infection [102,103]. The successful use of IFN alone and in combination to treat viral infections has been demonstrated experimentally both *in vitro* and *in vivo* [104-106]. However, type I IFNs represent a two-sided sword since they can also induce severe adverse effects ranging from diarrhoea, fatigue and depression to flu-like symptoms and hematologic toxicity [107,108].

4.3.2 Type II IFN

Only one type II IFN has been identified, IFNγ, which exists as a single gene in mammals and birds [109,110]. This is a pro-inflammatory cytokine that has an essential role in the activation of host defence against intracellular pathogens, representing a hallmark cytokine of Th1 cells [111,112]. In contrast to the type I and III IFNs, type II IFN is only produced by cells of the immune system such as NK cells, macrophages, dendritic cells and T cells [113,114]. IFNγ has similar actions to other IFNs in certain ways but it also has unique properties. For example, it is the only IFN that can enhance MHC class II expression [81,115] and is involved in the regulation of nitric oxide production and the promotion of Th1 differentiation [116,117].

4.3.3 Type III IFN

Type III IFNs were discovered in 2003 through computational analysis of the human genome, which identified three distinct proteins [118,119]. These were placed phylogenetically between type I IFNs and IL-10 related cytokines [120], leading to them being given alternate names. Sheppard and colleagues designated them IL-28A, IL-28B and IL-29 while others classified them as type III IFNs, IFNλ1 (IL-29), IFNλ2 (IL-28A) and IFNλ3 (IL-28B) with the latter names now taking precedence [118]. These IFNs share 15-19% amino acid (aa) homology with the type I IFNs but only 11-13% aa homology with IL-10, although their intron-exon structure resembles those of IL-10 and IL-10-related cytokines, while the crystal structure of IFNλ is structurally closest to the IL-10-related IL-22 [118,119,121,122]. The IFNλ genes are located in closely positioned clusters on human chromosome 19 and mouse chromosome 7, respectively [118,119,123]. In the mouse only two of the three IFNλ genes are considered functional [123,124] ,whereas a fourth IFNλ gene has been discovered in humans [120,125]. Due to their antiviral and anti-proliferative attributes, type III IFNs have also been used in viral [126] and cancer [127] treatment.

4.3.4 Expression and regulation

Co-induction of type I and III IFNs in response to a range of viral and bacterial components and TRL agonists has been reported in various *in vitro* and *in vivo* settings [64,119,128,129]. While

both IFN families can be expressed by almost all cell types, the plasmacytoid DC (pDC) lineage appears to be the greatest producers of both IFNs *in vitro* [120,130,131]. As mentioned earlier, expression of IFNs is triggered by PRRs, including the constitutively-expressed IFN regulatory factor (IRF) 3 and the IFN-induced IRF7 [132,133]. Many RNA viruses potently induce IFNλ expression including influenza A virus [130,134], Sindbis virus (SINV) and VSV [118]. Furthermore, stimulation with ligands for TLR3, TLR4 and TLR9 significantly increased IFNλ expression, whereas agonists for TLR7/8 were only weak inducers [130,134-136]. It has recently been demonstrated that murine macrophages express high levels of type I IFN mRNA but not type III IFN mRNA after herpes simplex virus (HSV) infection [135] and that influenza virus infection of human alveolar type II cells elicted high levels of IFNλ but not IFNβ [137]. Characterization of the human type III IFN promoters has revealed the presence of IRF and nuclear factor (NF)-κB binding sites [138]. IFNλ1 expression exhibited an IRF-3 dependence similar to that of IFNβ whereas IFNλ2/3 expression was controlled by IRF-7 similar to IFNα [139,140]. In addition, the NFκB sites in the promoter region of IFNλ1 were shown to be critical in DCs, suggesting that NFκB is a key regulator in these cells [141] and providing further evidence that the IFN induction pathways of type I and III IFNs differ. This indicates that more work is needed to fully understand the regulation of IFNλ.

4.3.5 Antiviral actions of IFNλ

IFNs can modulate the immune system, but can also directly interfere with the infection cycle of viruses [142], which has seen IFNs successfully used in a variety of antiviral treatments [102,143,144]. The characterization of IFNλ has largely focused on its antiviral role in the course of infection, where it displays activities reminiscent of type I IFN. Antiviral assays together with gene expression studies have demonstrated that IFNλ induces an antiviral state, with a large number of antiviral genes stimulated [137,145], which are mostly identical to those up-regulated upon type I IFN stimulation [146]. This could point towards redundancy of the immune system that could play a critical role in host survival, as many pathogens have developed mechanisms to evade or inhibit specific aspects of the host immune response [147]. However, differences in type I and type III activity can be observed. For example, studies have suggested IFNλ antiviral activity was weaker than type I IFNs, with higher concentrations required to achieve equivalent induction of antiviral genes [128,129]. In addition, the pre-treatment of cells with IFNλ before viral challenge can lead to reduced viral replication, as is also seen with IFNα pre-treatment. In contrast, IFNλ treatment post infection had no effect whereas IFNα was still able to inhibit virus replication to some extent [148]. A recent study also

found that rhinovirus infection of bronchial epithelial cells *in vitro* induced IFNλ mRNA and protein expression more strongly at all time points measured, compared to IFNα, which appeared early, and IFNβ, which was induced at later time points [149]. Other studies have shown that type III IFNs play a critical role in the control of rotavirus. Mice lacking functional IFNλ but competent for type I IFN could not control infection via the oral route, while administration of type III but not type I IFNs could induce an antiviral state in intestinal epithelial cells, providing evidence that this system is independent of type I IFNs [128]. Type III IFNs have also been shown to have a modulatory effect on the immune system. IFNλ has been shown to decrease IL-4, IL-5 and IL-13 production [150], thereby promoting a Th1 rather than Th2 response [150,151] *in vitro*.

Type III IFNs may provide an alternative therapeutic avenue for the treatment of virus infections such as hepatitis C in instances of type I IFN resistance [152,153]. Indeed IFNλ has been used in combination with ribavirin to control HCV infections with promising results [154]. There is thus potential for using different drug/IFN combinations, which could result in a further anti-viral synergy while decreasing the dose, diminishing side effects and reducing potential resistant virus isolates from developing [155,156]. Whilst IFNλ displays similar anti-viral and anti-proliferative activities to type I IFNs their respective outcomes differ in magnitude [146,152]. Thus, IFNλ may also elicit reduced adverse side effects that are associated with type I IFN therapy [157,158]. For example IFNλ was found to activate ISGs to a much lower extent in brain cells, suggesting that type III IFN therapy might lessen the neuropsychiatric effects associated with type I IFN therapies [136,159].

4.3.6 Apoptosis and anti-proliferative actions of IFNλ

Type III IFNs have been shown to exert anti-proliferative and pro-apoptotic effects in the human keratinocyte cell line (HaCaT) as well as suppress tumor growth and induce apoptosis in human glioblastoma, neuroendocrine, lung carcinoma and fibrosarcoma cells *in vitro* as well as in melanoma, fibrosarcoma and colon cancer *in vivo* and in some cases the ability to induce apoptosis was shown to surpass that of IFNα [152,160-162]. However, the anti-proliferative and pro-apoptotic actions appear independent, since IFNλ decreased cell proliferation of intestinal carcinoma cells but did not induce apoptosis [163]. Moreover, when responsive cells were treated with IFNλ and IFNα they showed a greater apoptotic effect than the respective single treatments [160]. This suggests a broad therapeutic potential for type III IFNs in combination with type I IFNs. However, further investigation is required to fully understand how type III IFNs might be used for cancer treatment.

4.4 Class II cytokine receptors

Members of the Class II cytokine receptor family (CRF2) are single pass transmembrane proteins defined by structural similarities in the extracellular domain, which includes the cytokine receptor homology domain (CHD) consisting of two tandem fibronectin type III repeats that is involved in ligand binding, and intracellular sequences[164]. They are distinguished from Class I cytokine receptors by differences in key amino acids in the CHD, notably alternative conserved cysteine residues and the absence of the hallmark WSxWS motif [164]. CRF2 chains combine to form heterodimeric receptor complexes. The ligands of CRF2 bind with high affinity to the R1/Rα chain, which characteristically has a large intracellular domain. However, binding of the ligand to the R1 chain is not enough to initiate signaling, which requires recruitment of an R2/Rβ chain that has a smaller intracellular region and lower ligand affinity. This trimeric complex then initiates specific intracellular signaling cascades [164]. This family is composed of 12 distinct receptor chains, which are used for signaling by members of the IFN, IL-10 and IL-10-related cytokines, as well as the unrelated factor VII (FVIIa) to mediate their biological activities [164,165].

4.5 IFN receptors

The three IFN families use distinct receptors to signal, which results in slightly different signaling pathways and ultimately variation in their respective biological activities[69]. The type I IFNs signal through the IFNαR1 and IFNαR2 complex [166], while IFNγ binds to a complex of IFNγR1 and IFNγR2 [167]. In contrast the type III IFNs bind to a receptor that utilizes a unique IFNλ receptor chain 1 (IFNλR1) in combination with the IL-10R2 chain, which is shared by IL-10, IL-22 and IL-26 receptor complexes [118,119,127]. Several of the receptors can be alternatively spliced which leads to both membrane-bound or secreted soluble forms [164]. The soluble receptor forms are usually identical in the extracellular domain but lack the transmembrane and intracellular domains [168], and are known to participate in signaling and its regulation [169]. For example, mice have an alternative soluble form of IFNαR2 that seems to be independently regulated from the membrane bound form [170,171]. These soluble forms have been thought to act as decoys, whereby they inhibit the binding of the ligand to the membrane bound form [168]. Thus the soluble form of IFNλR1 was found to antagonize IFNλ activity [172]. However, *in vitro* studies on IFNαR2 have suggested that the soluble receptor can interact with IFNα and the membrane bound IFNαR1 to promote signaling [171]. Therefore, while soluble receptors are clearly important in controlling cytokine signaling pathways and immune responses, their exact role is not yet well understood.

4.6 IFNλ receptor complex

Type III IFNs act through a unique transmembrane receptor complex which consists of the IFNλR1 and the IL-10R2 chains [118,119] (**Figure 4-6**).When either of these chains is absent or neutralized by an antibody, cells are unresponsive to IFNλ [119,159], showing that both IFNλR1 and IL-10R2 are needed to form a functional receptor [166]. The crystal structure of IFNλR1 indicates that it is most similar in structure to IL-10R1 and IL-22 binding proteins [121], which is perhaps not surprising since all interact with IL-10R2. Studies investigating the potential competitive inhibition of other IL-10R2 ligands, IL-10 and IL-22, on IFNλ signaling showed that while the presence of IL-10 was able to suppress the effects of IFNλ by unknown mechanisms, no direct competitive inhibition for binding to the IL-10R2 chain was found [173,174].

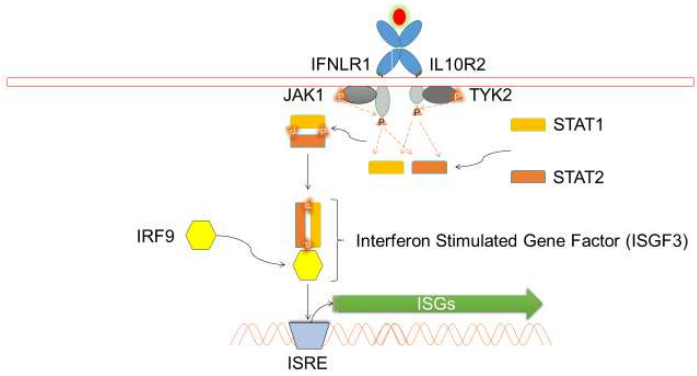

Figure 4-6 The IFN lambda receptor signaling cascade

IFNλ signals via a heterodimeric receptor complex consisting of IFNλR1 and IL-10R2. Ligand binding brings the associated Janus kinase 1 (JAK1) and Tyrosine kinase 2 (TYK2) proteins into close proximity, which leads to cross-phosphorylation and phosphorylation of the receptor complex. This facilitates the recruitment of signaling molecules, notably including STAT1 and STAT2. These also become phosphorylated allowing them to form a trimeric complex with the Interferon Response Factor 9 (IRF9) termed the Interferon Stimulated Gene Factor (ISGF3). ISGF3 can translocated to the nucleus interact with Interferon Stimulated Response Elements (ISREs) to mediate transcription of ISGs.

4.6.1 Expression and distribution

Ultimately the pattern of receptor expression determines which cells respond to a particular ligand. Although type III IFNs bind to a unique receptor complex, the downstream signaling is similar to that of type I IFNs including gene induction and biological activities [175]. However in mammals, unlike their type I counterparts, the IFNλR1 is predominantly expressed on epithelial cells, like skin, lung, intestine, colon, stomach liver and reproductive tract, as well as on specific subsets of immune cells [135,152,159,176-179]. It was believed that endothelial and fibroblast cell lines do not express IFN λR1 but evidence has emerged that show an effect on

endothelial cells of the blood brain barrier and in the neuro-invasion of West Nile virus, clearly demonstrating that endothelial cells have a response to IFNλ [180]. This suggests that the type III IFN system has evolved to protect the barriers like the mucosa and the blood brain barrier against pathogens [159], in contrast to the type I system, the receptors for which are expressed on all nucleated cells [181]. Liver, pancreatic and colorectal carcinoma cell lines also express IFNλR1 and are therefore susceptible to type III IFN signaling[142,159,179].

4.6.2 IFNλ receptor signal transduction

The binding of a cytokine to its cognate receptor initiates a signaling cascade which results in changes of the physiological state of the cell [56]. A variety of signaling cascades have been identified that are utilized by cytokine receptors to mediate the cell response [182]. One key pathway is named the Janus Kinase-Signal transducer and activator of transcription (JAK-STAT) pathway. The JAK family comprises four members, JAK1, JAK2, JAK3 and Tyrosine kinase 2 (TYK2), and the STAT family comprises seven members, STAT1-4 5a,5b and 6 [183,184]. IFNλ initially binds to the IFNλR1 chain, which causes a conformational change that enables recruitment of the IL-10R2 to form a trimer. This activates the intracellularly associated tyrosine kinases JAK1 and TYK2 to mediate phosphorylation of the receptor chains. This creates docking sites for various cytosolic signaling molecules including the latent transcription factors STAT1 and STAT2 [120]. Signaling through type III (and type I) IFN receptor complexes results in the formation of a transcription factor complex known as IFN stimulated gene factor 3 (ISGF3) [128,146]. This complex consists of 3 proteins, STAT1, STAT2, and IFN regulatory factor-9 (IRF-9) [120]. Once assembled, ISGF3 translocates to the nucleus where it binds to IFN stimulated response elements (ISREs) in the promoters of various interferon stimulated genes (ISGs) [185]. Despite different receptor complexes being employed there is a distinct signaling overlap between type I and III IFNs, largely due to convergent STAT activation, which helps explain why the antiviral outcomes are so similar [128,146,178,185]. However, other signaling molecules also contribute to the induction of antiviral genes. For example, activation of mitogen-activated protein kinases (MAPK) like ERK, p38 and JNK leads to recruitment of auxiliary transcription factors that co-operate with the ISGF3 complex to enhance its transcriptional activity [186,187]. The type III IFN activates a number of MAP kinases, relying to a greater extend on p38 and JNK for gene induction when compared to the type I IFNs [146,152].

4.7 The chicken IFN system

4.7.1 Chicken type I IFN

Chicken IFNs were those first discovered in 1957 by Isaacs and Lindenmann while growing influenza virus in chicken chorio-allantoic membrane [60]. Originally they were named IFN1 and IFN2 but Lowenthal and colleagues proposed the standard type I nomenclature be applied and therefore they were re-named IFNα and IFNβ, respectively [188].In chickens, IFNα consists of a multi-gene family, while only one member of IFNβ has been found. The encoded chicken IFNβ shares 58% homology to IFNα but neutralization studies with anti-IFNα antibodies confirmed that IFNβ was a distinct cytokine [189]. All type I IFNs are intron-less and located on the Z chromosome, which denotes the male sex chromosome in birds [189,190].

The chicken type I IFNs were found to be strongly induced in response to infection by a number of viruses, such as influenza A virus and Newcastle disease virus (NDV) [191]. Examination of the promoter regions of chicken type I IFNs has revealed putative binding sites for IRFs in all genes, and an NFκB site only in the IFNβ promoter, similar to the situation observed in mammals [192]. Recombinant chicken IFNα and IFNβ expressed in bacteria or COS cells displayed appreciable antiviral activity that was comparable to their mammalian counterparts [189]. Recombinant forms of chicken IFNα suppressed the growth *in vitro* of many viruses, such as Marek's disease virus (MDV) [193], infectious bursal disease virus (IBDV) [194] and infectious bronchitis virus (IBV) [195]. Chicken IFNα was also shown to also inhibit the replication of influenza A virus (H9N2) infection *in ovo* as well as *in vivo* [196].

4.7.2 Chicken type II IFN

Chicken IFNγ has been identified as a single-copy gene on chromosome 1 [197]. It demonstrates high homology to mammalian IFNγ and has been shown to have similar biological activities, such as induction of nitric oxide production in macrophages, antiviral activities against vesicular stomatitis virus (VSV) *in vitro,* and up-regulation of MHC class II expression [198-200].

4.7.3 Chicken type III IFN

Computational techniques enabled the identification of a single chicken IFNλ gene on chromosome 7 [65,201]. The genomic structures of both mammalian and chicken IFNλ genes are similarly organized into 5 exonic regions [118,119,201]. The encoded chicken IFNλ has a higher amino acid identity to human IFNλ2 compared to IFNλ1 and IFNλ3, and an even lower identity to the chicken type I or type II IFNs [201]. Chicken IFNλ has been recombinantly

expressed and its biological function investigated in a number of assays. Chicken IFNλ displayed inhibitory activity against Semliki Forest virus (SFV) and influenza A virus, in a similar manner to that observed for the type I chicken IFN. However, the overall activity of chicken IFNλ was lower than its type I chicken IFN counterparts, consistent with findings in human and mouse models [201].

4.7.4 Chicken IFN receptors

Only a few chicken CRF2 genes have been cloned or characterized to date. The IFNαR1, IFNαR2, IFNγR2 and IL-10R2 genes were found to cluster on chicken chromosome 1 in a similar fashion to that seen in the human [202], with IFNγR1 [203] also identified. Previous studies have also indicated the presence and activity of the JAK-STAT pathway components in birds [204,205], which would suggest conserved downstream signaling, although functional confirmation remains lacking. IFNλR1 mRNA expression has been described in chicken heart, liver, kidney, intestine, lung and trachea [206].

4.8 Interferon stimulated genes

The antiviral activity of IFNs is mediated by hundreds of genes upregulated upon IFN stimulation called ISGs [207]. These genes encode proteins that are able to interfere with the virus life cycle at different stages and so play a key role in IFN mediated antiviral defence. The antiviral effector ISGs can be grouped into three main categories [207]: (i) proteins that act as inhibitors of viral entry like the Myxovirus resistance gene 1 (Mx1) [208]; (ii) proteins that interfere with viral replication like the zinc finger antiviral protein (ZAP) [209], the IFN-induced double-stranded RNA dependent protein kinase (PKR) [210] or the interferon inducible protein with tetratricopeptide repeats (IFIT) gene family [211]; and, lastly (iii) proteins that inhibit viral budding, like the virus inhibitory protein, endoplasmic reticulum (ER) associated, interferon inducible (Viperin) [212].

4.8.1 Mx1

The mouse Mx1 gene belongs to the dynamin GTPase family [213] and was one of the first genes described to affect viral entry. Its encoded protein is specifically involved in blocking viral entry into the nucleus [214] via association with vesicular COP I, leading to sequestering of essential viral compartments within the cell [215]. The chicken Mx1 gene has been studied extensively for its role in influenza virus infections [216]. Some publications claim that chicken Mx1 is antiviral [217] while others show no antiviral benefit [216,218,219]. The chicken Mx1 gene is very polymorphic [218] with several different haplotypes identified across chicken breeds [220],

although potential differences between these haplotypes in antiviral protection has yet to be determined.

4.8.2 ZAP

ZAP is an accessory factor that recognizes viral RNA and promotes viral RNA degradation via the RNA exosome [221] thereby inhibiting translation of incoming viral RNA [222]. The chicken homologue of ZAP has previously been identified and characterized [223].

4.8.3 PKR

PKR is a RNA-dependent protein kinase that upon recognition of dsRNA phosphorylates the eukaryotic initiation factor-2α, which then blocks viral protein synthesis by disrupting delivery of tRNAs to the 40S ribosomal subunit [224]. PKR has also been shown to be involved in the activation of signal transduction pathways leading to IFNβ gene expression [225]. The chicken PKR gene has been identified and characterized [226].

4.8.4 IFIT

The various IFIT family members, IFIT1-5, have been shown to play critical roles in antiviral defence in humans and mice [211]. The mechanisms are not completely understood but IFIT1 and IFIT2 have been shown to suppress translation by binding to the eukaryotic initiation factor 3 [211]. Furthermore, IFIT family members can also directly bind to single-stranded RNA [211,227] and double-stranded DNA [228] and thereby reduce viral replication. The chicken IFIT has been mentioned in several publications[229] [230,231] but a comprehensive characterization of the gene has not been performed.

4.8.5 Viperin

Viperin has been shown to interfere with the viral life cycle of many viruses by as yet unknown mechanisms [212]. Some groups have suggested that viperin inhibited replication of certain viruses [232,233], while others have shown that it disturbs lipid rafts to restrict budding of influenza A viruses [234] and HIV [235]. Chicken viperin has been identified and characterized [236].

4.9 Rationale

Highly pathogenic avian influenza (HPAI) infections such as H5N1 and H7N9 cause significant morbidity and mortality in chickens worldwide [237]. This has widespread consequences, including devastating effects on the poultry industry [238] leading to severe economic losses [239] and bottlenecks in meat supply, since the method of choice to limit the spread of the virus is

the mass culling of infected chicken flocks [240]. There is also the potential for zoonotic transmission to humans [237,241-243], with most human infections of HPAI associated with direct transmission from avian hosts with little or no evidence of human-to-human transmission [244,245]. Therefore, the ability to stop HPAI infection in chicken would be a very attractive.

Knowledge in the field of mammalian antiviral defence is advanced, with studies in mice playing a crucial role in the advancement of knowledge regarding antiviral immunology and host pathogen interaction [246]. In contrast information on the avian immune response currently remains limited. Moreover, there are distinct differences in mouse physiology and immunology compared to natural pathogen reservoir species as well as spill over hosts [246,247]. This clearly indicates the need for more studies in avian species to better understand pathogenicity of viruses such as HPAI to underpin the development of means to prevent the spread of these pathogens [248].

4.9.1 Hypothesis

The studies in this thesis seek to investigate the hypothesis that the IFNλ signalling complex is involved in the antiviral immune response in chickens.

4.9.2 Aims

The research addresses following specific aims:

- To characterize the expression of chicken IFNλ and its effect on in vitro and in vivo ISG expression in comparison to IFNα.
- To identify and confirm the chicken IFNλR complex and downstream signaling effector molecules.
- To identify and characterize the chicken IFIT5 gene.

5 Material and Methods

5.1 Cell culture

5.1.1 Chicken splenocytes

Chicken splenocytes were purified as described previously [201]. Briefly, spleens from 4-6 week old (unless otherwise specified), specific-pathogen free (SPF) chickens were harvested and dispersed through a 70 µm mesh sieve (BD Falcon). The mononuclear cells were then purified using density gradient centrifugation (Lymphoprep – Nicomed Pharma). After washing, the cells were counted and $4x10^6$ cells per well transferred to 24 well plates (Nunc) and cultured in DMEM high glucose media (Life Technologies) containing 10% (v/v) fetal calf serum and 1000 U/mL penicillin and streptomycin (Sigma).

5.1.2 DF1 cells

DF1 cells were maintained in DMEM high glucose media (Life Technologies) containing 10% (v/v) fetal calf serum and 1000 U/mL penicillin and streptomycin (Sigma) at 37°C with 5% CO_2. After passaging the cells were counted and $6x10^4$ cells transferred into a 24 well plates (Nunc) and kept at 37°C with 5% CO_2 overnight.

5.1.3 Transfection

Cells were washed and cultured in 400 µl Optimem per well. Per well 2 µl Lipofectamine 2000 (Invitrogen) and 4 nM siRNA were incubated at RT for 30 min then 10% DMSO (v/v) was added and the mix transferred onto the cells. These were incubated at 37°C with 5% CO_2 for 12 h before the media was changed back to full growth media for 48 h.

5.1.4 Cell stimulation

After passaging the cells were counted and $6x10^4$ cells were transferred into a 24 well plates (Nunc) and kept at 37°C with 5% CO_2 overnight.

Cells were stimulated with 50 µg/mL of poly (I:C) (Invitrogen), 10 µg/mL lipopolysaccharide (LPS) purified from E. coli strain 0111:B4 (gamma irradiated, 500000 EU/mg) (Sigma Aldrich), 500 ng/mL recombinant chicken IFNα (Genway Biotech) or 50 µg/mL recombinant chicken IFNλ, kindly provided by Dr Tim Adams (CSIRO Manufacturing). The chIFNα was produced in an *E. coli* expression system while the chIFNλ was produced within an mammalian system utilizing CHO cells both were tested for the absence of endotoxins. In some experiments cells were pre-treated with 1 nM Ruxolitinib (Selleck Chemicals) per well for 2 h. The doses

for each compound were initially taken from literature or MSDS and confirmed by in vitro experiments on chicken primary or cell lines to be the most effective dose for (data not shown).

5.2 In vivo studies

5.2.1 Ethics

All animal work was conducted with the approval of the CSIRO Australian Animal Health Laboratory Animal Ethics Committee (permit number 1610). All procedures were conducted according to the guidelines of the National Health and Medical Research Council as described in the Australian code for the Care and Use of Animals for Scientific Purposes. All birds were obtained from Australian SPF Services P/L (Woodend, Australia).

5.2.2 Egg trial (H1N1)

D10 embryonated chicken eggs were inoculated with 100 µl of 1:10000 diluted virus stock A/Puerto Rico/8/1934 H1N1 of 6.4 x 10^8 pfu/mL (kindly provided by Dr. Siying Ye and Dr. John Stambas), incubated at 37°C for 24 or 48 h and then chilled at -20°C for 20 min and post mortems performed. The tissues were placed into 2 mL tubes (Sarstaed) with Graphite beads (Daintree Scientific) and RLT buffer (Qiagen) and homogenized twice for 20 s each. Tissue homogenate was then stored at -80°C for RNA extraction.

5.2.3 Virus propagation

A highly pathogenic avian influenza virus A/duck/Laos/XBY004/2014 (H5N6) (Lao/14), isolated from pooled duck tissues from Lao PDR [31], was used in this study. Virus was propagated according to accepted protocols [249], briefly: After wiping with 70% (v/v) ethanol a small incision was punched into the egg shell the virus was introduced by allantoic cavity inoculation of D9–11 embryonated SPF chicken eggs. The incision was then sealed with wood glue and the eggs incubated at 37°C for 48 h The virus stock was titrated in chicken eggs and the 50% egg infectious dose (EID_{50})/mL was calculated according to the method of [250].

5.2.4 Animal trial (H5N6)

Six 5-week-old SPF chickens were used for experimental infections. Samples from six uninfected chickens from the same cohort were used as controls. Prior to challenge, serum was collected from each chicken to confirm that birds were serologically negative for avian

influenza A virus, as determined by blocking ELISA [251]. Each chicken was inoculated with a dose of 10^6 EID_{50} of Lao/14 in 0.2 mL by the oral-nasal-ocular route. Chickens were observed closely from 22 hpi and were euthanized at a humane endpoint defined as progression to moderate signs of disease, including facial swelling, diarrhoea, hunched posture with ruffled feathers, drooping wings, huddling, recumbency, depression and slow response to stimulation. In accordance with Institutional Animal Welfare Policies, chickens were euthanized by cervical dislocation following heart bleed under anesthesia (44 mg/kg ketamine, and 8 mg/kg xylazine injected intramuscularly). Immediately after euthanasia, swabs (oral and cloacal) were taken and approximately 100 mg of tissue from the spleen and lung were collected into sterile 2 mL tubes containing PBS with antibiotics and a small quantity of 1 mm silicon carbide beads (BioSpec Products). Tissue samples were homogenized twice for 20 s in a FastPrep24 tissue homogenizer (MP Biomedicals) for bioassays. The presence of influenza viral genome within swabs, tissues samples was assessed by extracting total RNA from each sample (MagMax-96 Total RNA Isolation Kit, Life Technologies) for testing using a pan-influenza A matrix gene real-time RT-PCR assay [252]. Cycle threshold (Ct) values for each sample were compared to those obtained for a set of RNA transcripts encoding the Lao/14 matrix genome segment to convert each sample Ct value into a value representing the number of copies of the matrix genome segment per µl of sample. These RNA transcripts were generated using T7 RNA polymerase (Promega) and a plasmid encoding the Lao/14 matrix genome segment cloned into the pGEM-T-Easy cloning vector (Promega). The complete data set can be found in Butler et al 2016 [253]

5.3 Bioinformatics

5.3.1 Primer design and domain prediction

Protein sequences were retrieved from the Ensembl genome database (www.ensembl.org) except for KF956064, which was retrieved from Genbank (www.ncbi.nlm.nih.gov/genbank). Primer design, sequence assembly and initial alignment was performed using CLC-Main Workbench 7.6.3.

5.3.2 Phylogenetic analysis

All Sequences were retrieved from Genbank (www.ncbi.nlm.nih.gov/genbank). Protein alignments used the MUSCLE algorithm and phylogenetic trees were calculated using MEGA 6.0. Accession Numbers of all sequences used in this analysis are given in the Appendix.

Table 2 (IFNs)

Table 3 (IFNλR)

Table 4 (IFNAR1)

Table 5 (IL10R2)

Table 6 (IFITs)

5.4 Molecular methods

5.4.1 RNA isolation and reverse transcription

Total RNA was harvested from cultured cells using the Qiagen RNAeasy extraction kit according to the manufacturer's instruction, and from tissues using TRIzol (Life Technologies). Prior to qRT-PCR for gene expression analysis, RNA samples were subsequently treated with RNAse-free DNase (Promega) and reverse transcribed with a Superscript III cDNA synthesis kit (Life Technologies).

5.4.2 Cloning and sequencing

The chIFIT5 gene was amplified using Platinum *Taq* Master mix (Life Technologies) with gene specific primers (forward primer: 5'-ATGAGTACCATTTCCAAGAAT, reverse primer: 5'-TAGCTTGAGAGGGAAAG) and cloned into the pGEM-T-Easy vector (Promega) using T7 Ligase (Promega) and transformed into *Escherichia coli* DH5α (Life Technologies), according to manufacturer's instructions. Plasmids were purified using a Qiagen Plasmid Miniprep kit and sequencing was performed by the Micromon Sequencing Facility (Monash University).

5.4.3 Quantitative Real Time PCR (RT-PCR)

Gene expression was quantified in DF1s, splenocytes and lung and spleen tissues by RT-PCR using TaqMan Universal PCR master mix (Life Technologies) with FAM reporter dye and NFQ quencher and primer probe pairs obtained from Applied Biosystems (**Table 7**). Temperature profile for the RT PCRs was: 50°C 2 min, 95°C 10 min, 40 cycles of 95°C 15 sec and 60°C 1 min.

5.4.4 Electrophoretic mobility shift assay (EMSA).

Nuclear extracts were prepared as described previously [254]. Briefly, cells were stimulated , pelleted and resuspended in ice-cold hypotonic buffer (2 mM HEPES pH 7.8 (Sigma), 20 mM NaF (Sigma), 1 mM Na_3VO_4 (Sigma), 1 mM DTT (Sigma), 1 mM EDTA (Sigma), 50 µg/mL

Protease inhibitors (Sigma), 1 mM Tween-20). Cells were then briefy and the nuclei pelleted by centrifugation at 15,000g for 30 s. Nuclear extracts were prepared by resuspension of the nuclei in high-salt buffer (hypotonic buffer with 420 mM NaCl (Sigma) and 20 % (v/v) glycerol) and extraction of proteins by rocking for 30 min at 4°C. Insoluble materials were removed by centrifugation at 4°C for 15 min at 15,000g and nuclear extracts were stored at -80°C for analysis.

Nuclear extracts were incubated for 20 minutes at room temperature with 32P-labeled double-stranded m67 oligonucleotide (5'-CATTTCCCGTAAATC), a high-affinity mutant of the sis-inducible element (SIE) and poly(dI-dC) in binding buffer (13 mmol/L HEPES, pH 7.8, 80 mmol/L NaCl, 3 mmol/L NaF, 3 mmol/L NaMoO4, 1 mmol/L DTT, 0.15 mmol/L EDTA, 0.15 mmol/L EGTA, and 8% glycerol). The DNA-protein complexes were separated by electrophoresis on 5% polyacrylamide gels containing 5% glycerol in 0.25 x Tris-buffered EDTA (TBE). The gels were dried and subsequently exposed to phosphoimager screens and analyzed with ImageQuant software (Molecular Dynamics).

6 Characterization of chicken IFNλ signaling

6.1 Introduction

The interferons (IFNs) were discovered by Isaacs and Lindenmann in the chorio-allantoic membrane of chicken eggs with the isolated protein first described as "a substance that was able to interfere with the virus' ability to infect cells"[60]. Since then the knowledge about IFNs has increased dramatically, although most these studies have been performed in mammals.

IFNs are expressed by virtually all cells of the body upon sensing of viral PAMPs via PRRs such as TLRs [38,44]. Of the thirteen TLRs identified six are considered to function as antiviral sensors either through the detection of viral glycoproteins on the cell surface (via TLR2 and 4) or through recognition of dsRNA in the endosome (via TLRs 3,7, 8 and 9) [45-51]. In addition to the TLRs, intracellular sensors such as RIG-I and MDA-5 are involved in the cytosolic recognition of foreign nucleic acids [52] although in chickens MDA-5 appears to have taken over some of the roles of RIG-I that it is absent in this species [255].

Three IFN families have been identified which are called type I, type II and type III. IFNs are assigned to these families according to their receptor specificity and signaling pathways, which defines downstream gene induction although overlaps occur in these features between families [64-66]. Type I IFNs consist of at least 8 subclasses with the IFNαs and IFNβ as their most prominent members [1,22], type II consists of IFNγ and type III comprises IFNλs of which there are four in humans but only one in the chicken [201]. All types of IFN contribute to the antiviral immune response but only type I and III have a direct antiviral effect [59], which is mediated by a group of hundreds of antiviral genes called ISGs [207].

Similarities between type I and type III IFNs have been described concerning gene induction and antiviral properties [146,175]. However, each of these families signal through a unique receptor complex consisting of two chains; for type I IFNs these are IFNAR1 and IFNAR2[256] and for type III IFNs these are IFNλR1 and IL-10R2 [118,119]. Recent studies have identified unique roles for IFNλs in antiviral defense, and there is emerging evidence that IFNλs may have functional importance beyond innate antiviral protection [128].

This Chapter focuses on advancing our understanding of the biological activities of chicken type III IFN particularly the similarities and differences with respect to type I IFN.

6.2 Results

6.2.1 Phylogenetic analysis of the chicken type I/III IFNs

To obtain an initial insight into the relative homology between the chicken and mammalian IFN systems phylogenetic analysis of type I and type III IFNs from chicken, human and mouse was performed (**Figure 6-1**). Unsurprisingly, the IFNαs of both human and mouse clustered together in separate groups, which collectively were distinct from other human and mouse type I IFNs like IFNo, IFNζ and IFNκ. Chicken type I IFNs, IFNα and IFNβ, clustered together in a subgroup linked with human and mouse IFNβ and IFNε. The chicken IFNλ grouped with the mammalian IFNλ proteins that clustered by species. This suggested overall conservation, including distinct type I and type III members between chickens and mammals.

6.2.2 Dose response of chicken IFNs to poly (I:C)

Poly (I:C) (PIC) is a powerful inducer of IFNs [257]. It serves as a mimic of viral RNA and so is able to trigger PRRs like the TLR family and MDA-5. To examine induction of IFNα and IFNλ by PIC purified chicken splenocytes were cultured in vitro with different concentrations of PIC for 3 h and IFN mRNA levels analyzed by qRT-PCR (**Figure 6-2**). Both IFNs were induced even by low concentrations of PIC. Indeed, IFNα reached its peak expression with a 5-fold upregulation at 1 μg/mL that was similar up to 100 μg/mL. In contrast IFNλ expression increased up to 10 μg/mL where it maintained a peak level of expression at around 600-fold upregulation compared to the untreated control.

6.2.3 Time course of TLR-dependent stimulation of chicken IFNs

To further investigate the type I versus type III IFN response, the timing of TLR-mediated IFN induction was analyzed following stimulation with PIC as well as lipopolysaccharide (LPS), another TLR agonist [258]. Chicken splenocytes were treated with either PIC (**Figure 6-3A**) or LPS (**Figure 6-3B**) and IFN expression measured at different time points. PIC treatment led to a rapid and robust upregulation of both IFNs, being increased after 0.5 h and peaking at 30-fold for IFNα and 100-fold for IFNλ at 1.5-3 h, followed by a decline to close to base-line expression by 24 h post stimulation. LPS treatment, in contrast, failed to induce any significant upregulation of either IFNα or IFNλ at any time points measured.

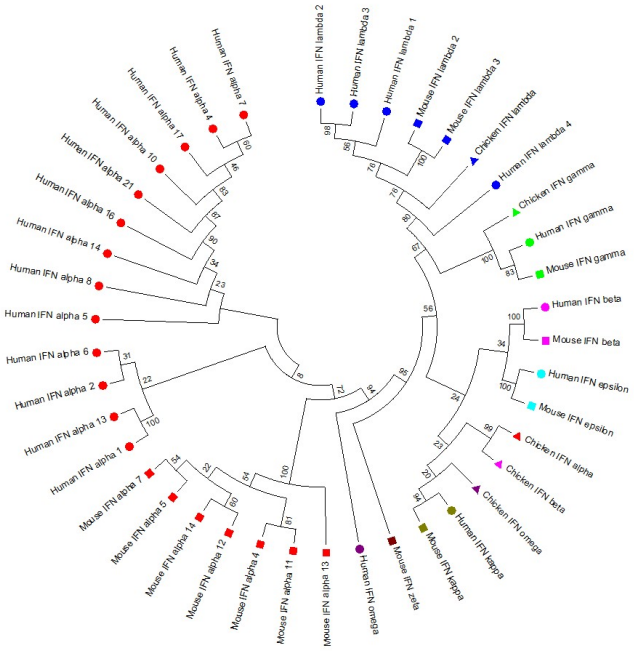

Figure 6-1 Phylogenetic analysis of the type I/III IFNs

The evolutionary history of the type I and type III IFNs from human, mouse and chicken was inferred by using the Maximum Likelihood method based on the JTT matrix-based model [259]. The tree with the highest log likelihood (-10509.0810) is shown. The accession numbers of all sequences used can be found in the Appendix (Table 1).

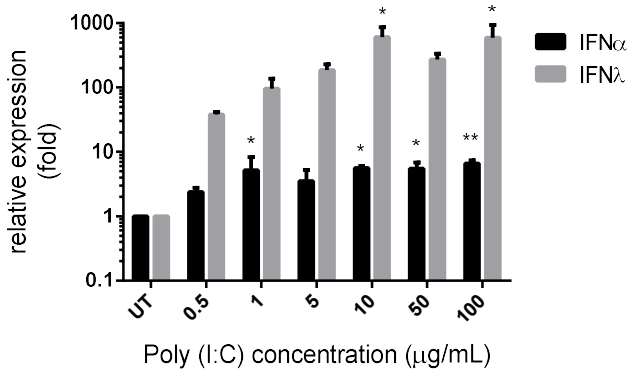

Figure 6-2 Dose response of chicken IFNs to poly (I:C)

Expression of IFNα and IFNλ mRNA in purified chicken splenocytes from SPF chickens stimulated with poly (I:C) at the concentrations indicated. The bars represent the mean fold change of 3 chicken spleens with the standard error of the mean (SEM) compared to the untreated sample, normalized against the housekeeping gene GAPDH (* p value < 0.05 using a one-way ANOVA test with Fischer's uncorrected LSD test).

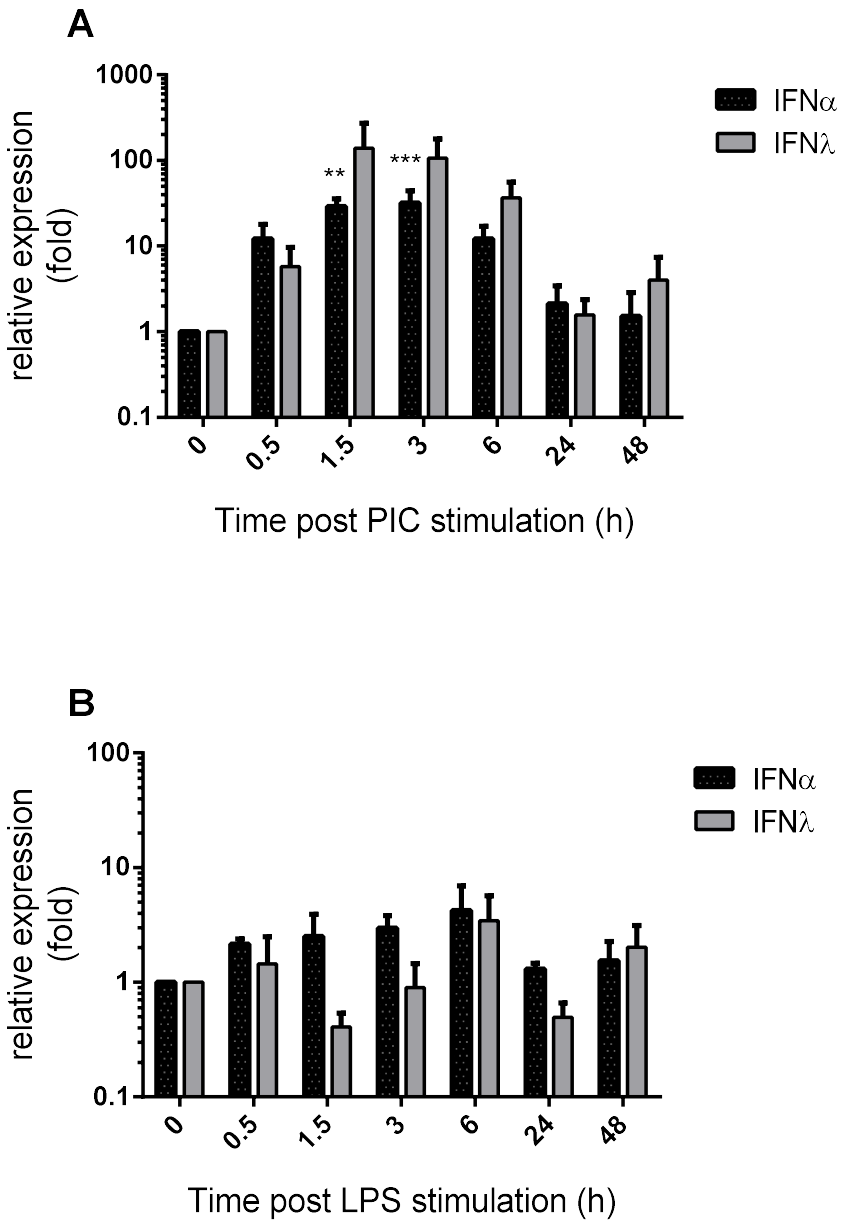

Figure 6-3 Time course of TLR-dependent stimulation of chicken IFNs

Expression of IFNα and IFNλ mRNA in purified splenocytes from SPF chickens stimulated with 50 µg/mL PIC (A) and 10 µg/mL LPS (B) over the indicated times. The bars represent the mean fold change of 3 chicken spleens with the standard error of the mean (SEM) compared to the untreated sample, normalized against the housekeeping gene GAPDH (* p value < 0.05, *** p value < 0.001 using a one-way ANOVA test with Fischer's uncorrected LSD test).

6.2.4 Time course of IFN mediated induction of chicken IFN expression

Self-induction and/or positive feedback loops for IFNs exist in mammals, where they play an important role in signal amplification [260]. Since this has not previously been investigated in birds, splenocytes from SPF chickens were exposed to recombinant chicken IFNα **Figure 6-4A**) and recombinant chicken IFNλ (**Figure 6-4B**) and levels of IFN mRNAs quantified. IFNα stimulation did not significantly induce IFNα or IFNλ mRNA expression across all chickens at any time points measured although induction was observed in a subset (two) of chickens. IFNλ treatment in contrast upregulated IFNλ slightly up until 6 h but by 48 h a 3000-fold increase was observed. IFNλ stimulation caused IFNα mRNA levels to decrease until 24 h, but with a later upregulation to 350-fold at 48 h.

6.2.5 Characterization of TLR-dependent ISG responses

TLR responses in chicken cells have been previously described [261], but the timing of ISG induction has not been well characterized. Splenocytes from SPF chickens were stimulated with PIC (**Figure 6-5A**) and LPS (**Figure 6-5B**) and ISG expression quantified. The PIC treatment led to a sharp increase in ISG expression at 3 h, with a peak expression level for all ISGs at 6 h post stimulation, followed by a decline to base-line levels after 48 h. Mx1 showed the highest induction of approximately 170-fold followed by Viperin at around 70-fold, with induction of Zap and PKR peaking at around 10-fold at the 6 h time point. LPS stimulation gave a similar kinetics of ISG induction, with peak expression at 6 h and then a decline to base-line expression around 48 h. The magnitude of induction was similar for Viperin (approximately 50-fold) as well as PKR and ZAP (around 12-15-fold), but Mx1 induction was substantially reduced (around 20-fold).

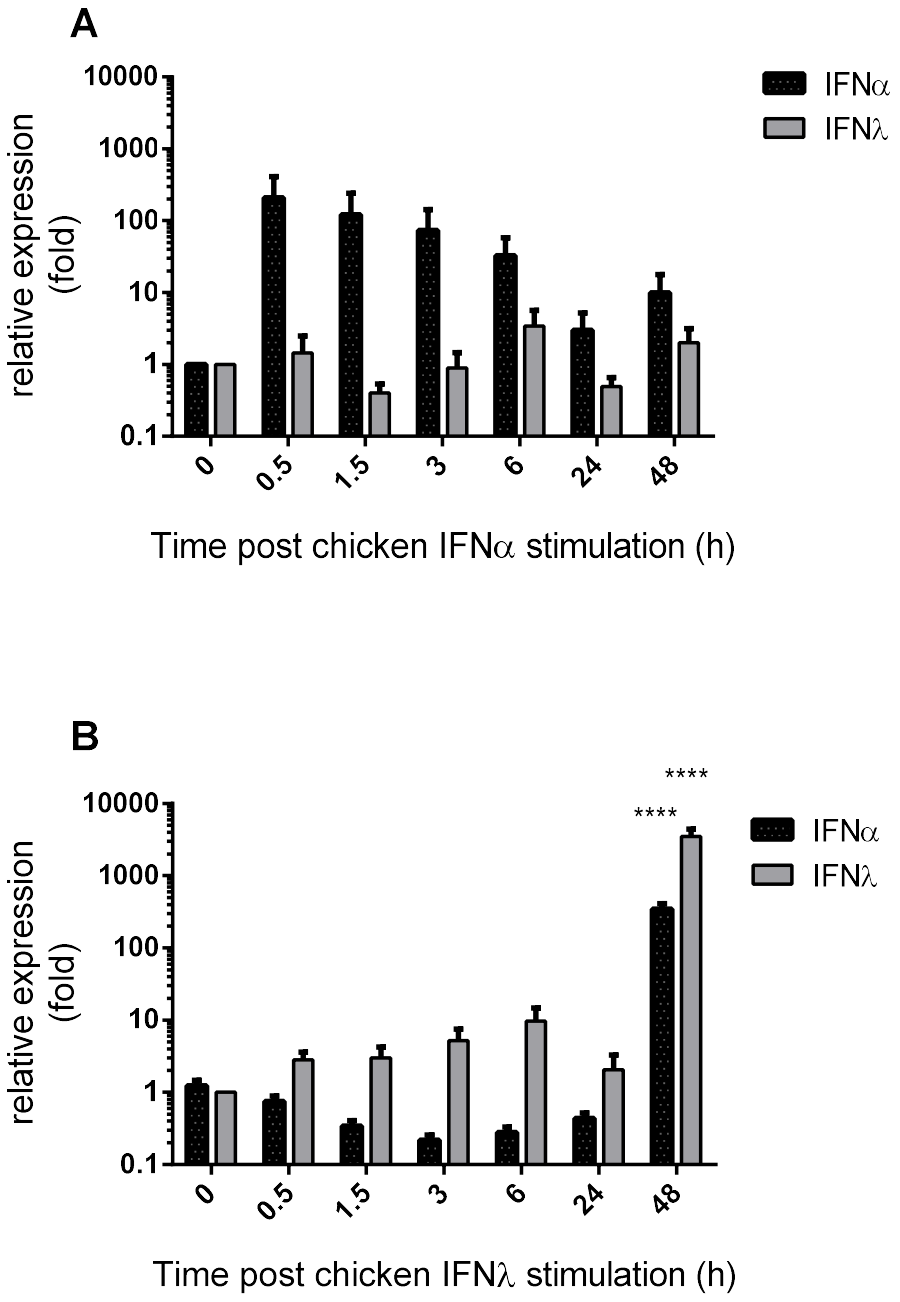

Figure 6-4 Time course of IFN-mediated induction of chicken IFN expression

Expression of IFNα and IFNλ mRNA in purified splenocytes from SPF chickens stimulated with 500 ng/mL IFNα (A) and 50 µg/mL IFNλ (B) over the indicated time course. The bars represent the mean fold change of 3 chicken spleens with the standard error of the mean (SEM) compared to the untreated sample, normalized against the housekeeping gene GAPDH (*** p value < 0.001 using a one-way ANOVA test with Fischer's uncorrected LSD test).

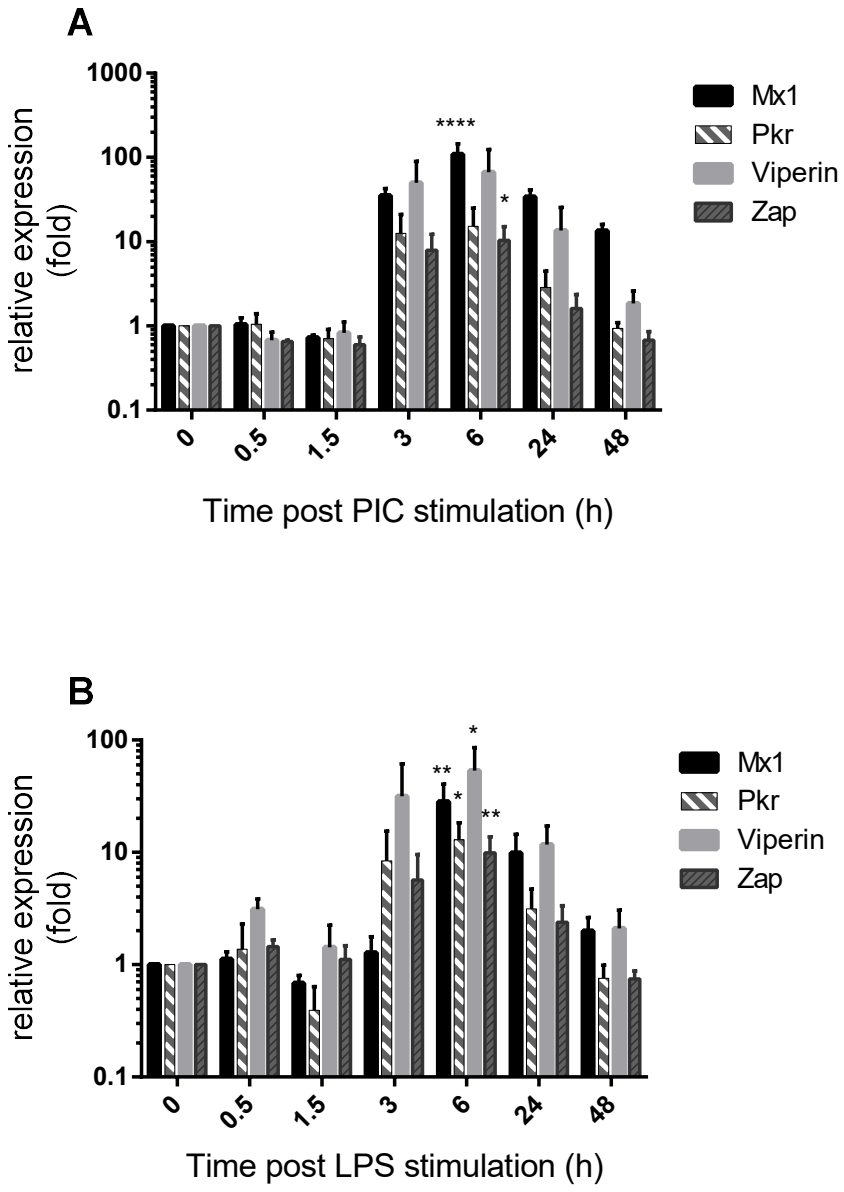

Figure 6-5 Characterization of TLR-dependent ISG response

Expression of Mx1, PKR, Viperin and Zap mRNA in purified splenocytes from SPF chickens stimulated with 50 µg/mL PIC (A) and 10 µg/mL LPS (B) over the indicated time course. The bars represent the mean fold change of 3 chicken spleens with the standard error of the mean (SEM) compared to the untreated sample, normalized against the housekeeping gene GAPDH (* p value < 0.05, *** p value < 0.001 using a one-way ANOVA test with Fischer's uncorrected LSD test).

6.2.6 Characterization of the type I/III IFN-mediated ISG response

In mammalian systems profound differences between type I and type III interferons have been described, both in the magnitude of the ISG response as well as its timing [157]. To better understand the IFN response in chickens, the time-dependent expression of several well characterized ISGs was evaluated. Splenocytes from SPF chickens were treated with chIFNα (**Figure 6-6A**) or chIFNλ (**Figure 6-6B**). ChIFNα treatment led to a rapid increase of all ISGs investigated with similar kinetics, a sharp rise in expression levels after 3 h with a peak at around 6 h and then a decline to 48 h. Although having similar induction kinetics, the magnitude of the responses differed between the genes, with Mx1 and Viperin showing highest upregulation at around 40 and 30-fold increase, respectively, while both PKR and ZAP expression levels showed an approximately 8-fold increase during the peak at 3 h post stimulation. In contrast, chIFNλ stimulation of these cells led to a considerably delayed induction of ISGs. A small increase was apparent by 1.5 to 3 h, with peak induction at 48 h post stimulation. By this time, both Viperin and Mx1 were highly induced, with a 20-fold and 10-fold increase at the 48 h time point, respectively. No significant increase in ZAP or PKR expression was observed following chIFNλ stimulation.

6.2.7 Characterization of the IFN response following acute HPAI H5N6 infection

To evaluate the involvement of type I and III IFNs in vivo, RNA was extracted from spleens and lungs of H5N6 infected and uninfected chickens at 24 h post infection and the levels of IFNα and IFNλ measured by qRT-PCR. In the spleen of infected chickens (**Figure 6-7A**) IFNα was induced 9-fold and IFNλ almost 70-fold compared to uninfected animals. In the lungs (**Figure 6-7B**), the primary site of infection, both IFNs were highly induced, with a 25-fold increase of IFNα and a 60-fold increase in IFNλ compared to uninfected birds.

6.2.8 Characterization of ISGs expression following acute HPAI H5N6 infection

ISGs are key mediators of the antiviral immune response mediated by IFNs. Therefore, the expression of some of the most important antiviral genes was examined during acute HPAI infection. In the spleen of infected chickens Mx1 gene expression was induced 230-fold compared to uninfected controls with PKR being slightly lower at around 40-fold, but no significant upregulation of ZAP expression was observed (**Figure 6-8A**). In the lungs, expression of Mx1 was induced 65-fold and PKR around 60-fold with ZAP again not upregulated (**Figure 6-8B**).

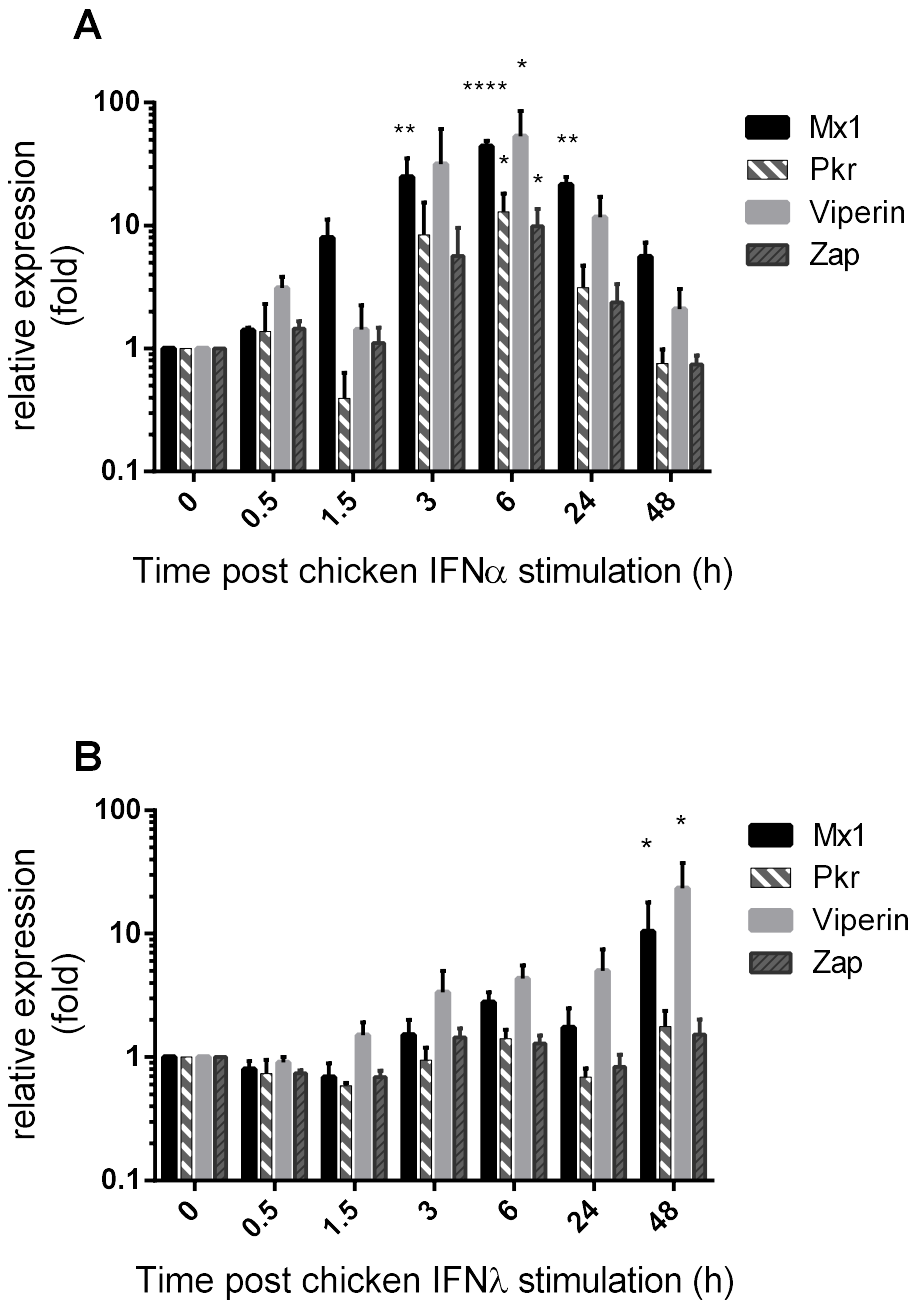

Figure 6-6 Characterization of the type I/III IFN-mediated ISG response

Expression of Mx1, PKR, Viperin and Zap mRNA in purified splenocytes from SPF chickens stimulated with 500 ng/mL IFNα (A) and 50 µg/mL IFNλ (B) over the indicated time course. The bars represent the mean fold change of 3 chicken spleens with the standard error of the mean (SEM) compared to the untreated sample, normalized against the housekeeping gene GAPDH (* p value < 0.05, **** p value of <0.0001 using a one-way ANOVA test with Fischer's uncorrected LSD test).

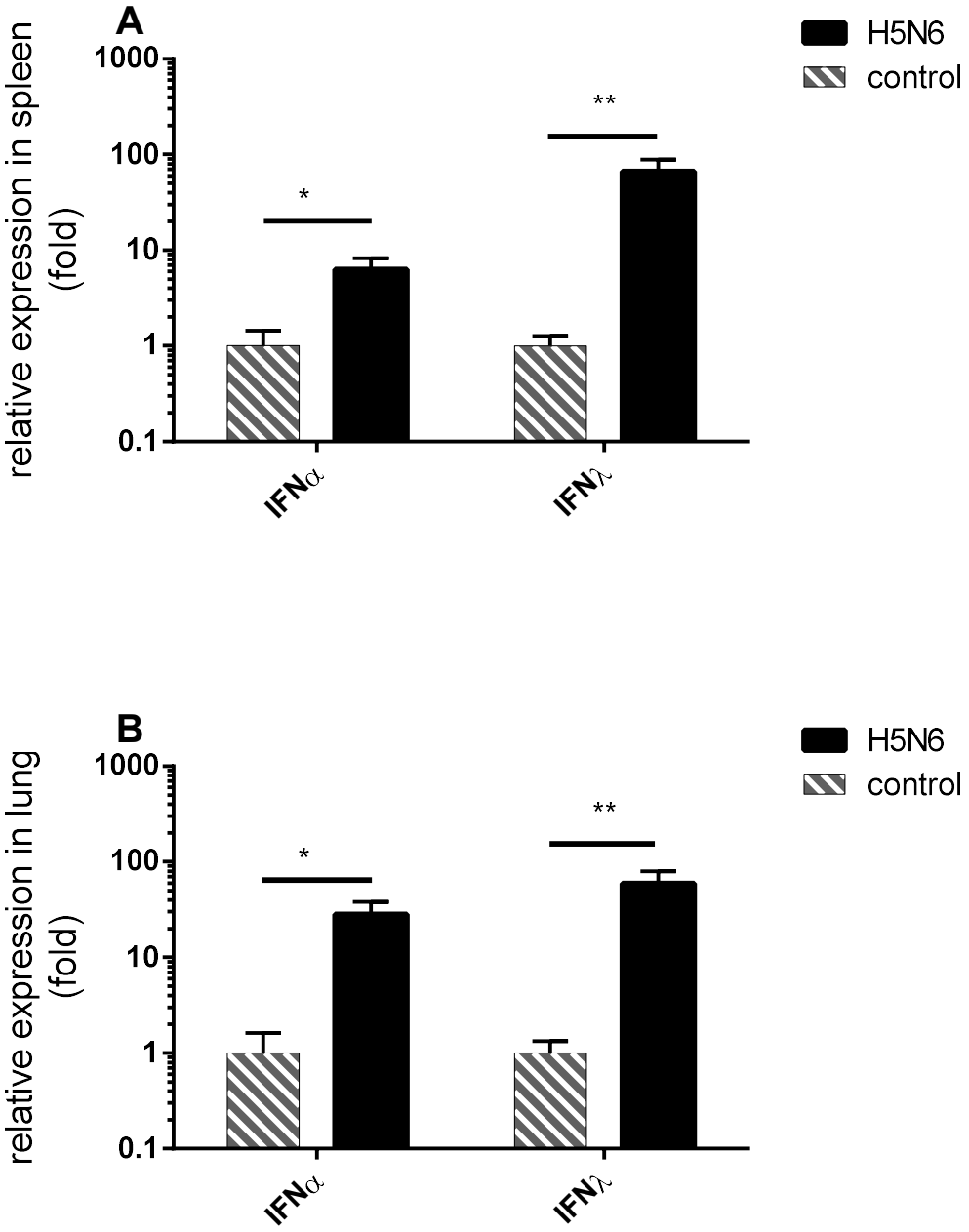

Figure 6-7 Characterization of IFN response following acute HPAI H5N6 infection

Expression of IFNα and IFNλ mRNA in the spleen (A) and lung (B) of six 5-week old SPF chickens 24 hours post infection with Influenza virus A/duck/Laos/XBY004/2014 (H5N6). Data is shown as the mean fold change of mRNA expression with the SEM compared to the same tissue of the uninfected birds, normalized against the housekeeping gene GAPDH (* p value < 0.05 using the Students *t* test with Mann Whitney U test).

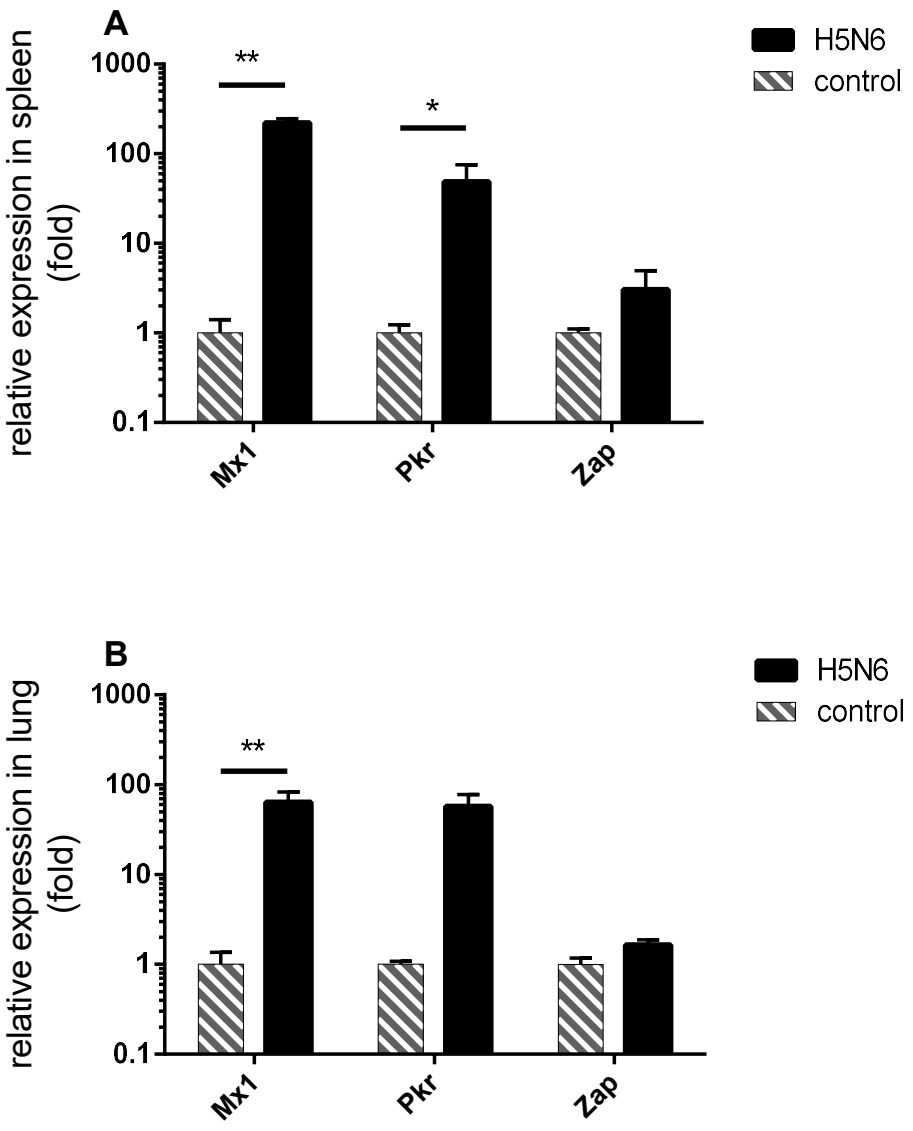

Figure 6-8 Characterization of ISG response following acute HPAI H5N6 infection

Expression of Mx1, Pkr and Zap mRNA in the spleen (A) and lung (B) of six 5-week old SPF chickens 24 hours post infection with Influenza virus A/duck/Laos/XBY004/2014 (H5N6). Data is shown as the mean fold change of mRNA expression with the SEM compared to the same tissue of the uninfected birds, normalized against the housekeeping gene GAPDH (* p value < 0.05 using the Students *t* test with Mann Whitney U test).

6.2.9 Comparison of type I and III IFN expression between male and female chickens.

Recent evidence from mice and humans suggests that females and males differ in the magnitude of immune responses particularly with regards to IFNs, with a link to female hormone levels also suggested [262,263]. To examine this possibility in chickens, splenocytes from immature female and male chickens were treated with PIC (**Figure 6-9A/B/C**) with blood estradiol levels of each bird measured in parallel (**Figure 6-9D**). IFNα mRNA levels (**Figure 6-9A**) were relatively tightly grouped: males tended to have higher levels earlier but females tended to show a significantly higher peak at 2 h. The IFNβ mRNA levels (**Figure 6-9B**) followed the same trend as IFNα, with significantly higher female expression at 2 h. Interestingly the opposite trend was observed for IFNλ (**Figure 6-9C**) with males having a generally higher expression albeit only reaching significance at the 2 h time point. The inability to reach significance at other time points was in part due to highly variable IFNλ expression in females compared to the males with several individuals showing negligible IFNλ induction. The concentration of serum estradiol was measured in both groups and indicated that the immature female chickens had higher levels, ranging from 70-90 pg/mL while all the male samples clustered around 60 pg/mL. The experiment was repeated with chicken splenocytes from sexually mature male and female chickens. PIC stimulation (**Figure 6-10A**) lead to higher mean IFN expression in females which reached statistical significance for IFNλ. The estradiol levels (**Figure 6-10B**) were significantly higher in females ranging from 300 -1400 pg/mL, with in males again around 60 pg/mL. Single bird correlation of estradiol levels and IFN mRNA expression showed no conclusive pattern in female or male birds (**Figure 6-10C/D**).

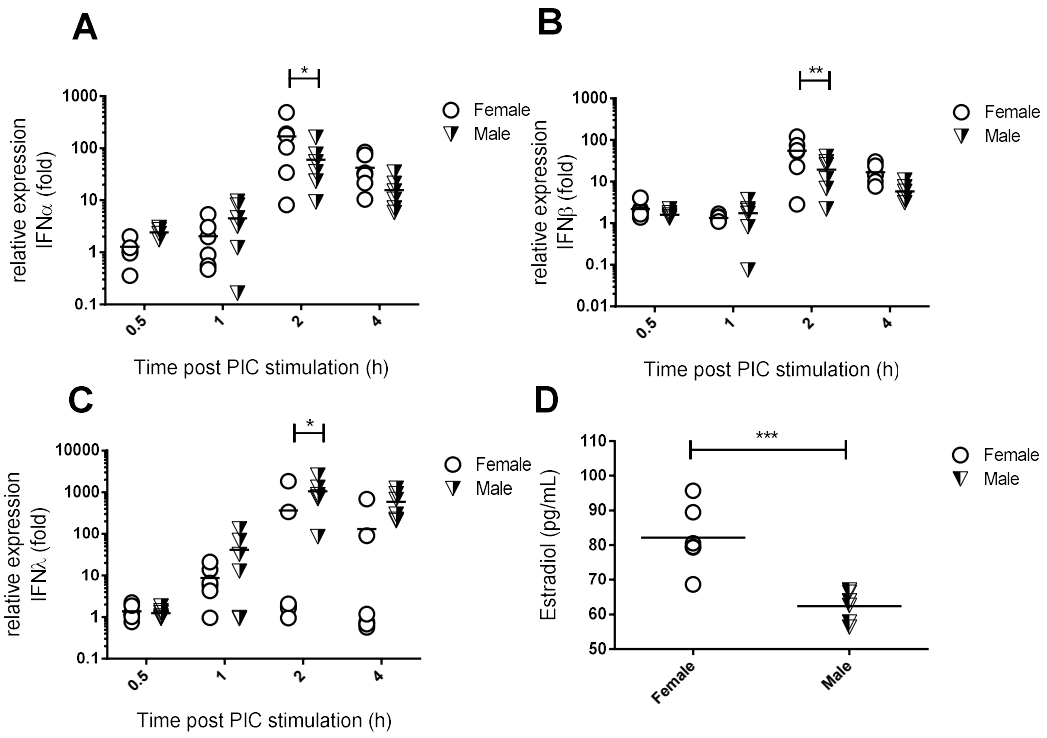

Figure 6-9 Comparison of type I and III expression in immature male and female chickens.

Expression of IFNα (A), IFNβ (B), IFNλ (C) mRNA in purified chicken splenocytes from 6 female (circles) and 6 male (triangles) 5-week old SPF chickens stimulated with 50 µg/mL PIC over the time course indicated. Symbols represent fold upregulation of each stimulated splenocytes sample compared to the untreated sample, normalized against the housekeeping gene GAPDH. (For A-C: * p value < 0.05, ** p value < 0.005, *** p value < 0.001 using a two-way ANOVA with Bonferroni's multiple comparisons test) (D) Blood estradiol levels chickens at the time of sacrifice (*** p value < 0.001 using the Students *t* test with Mann Whitney U test).

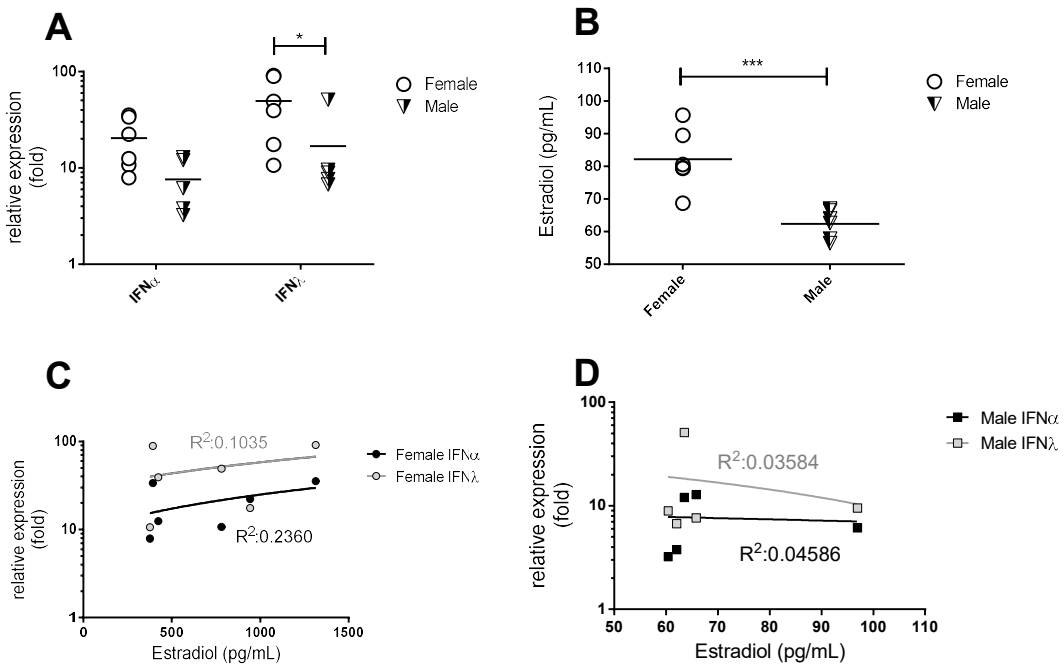

Figure 6-10 Comparison of type I and III IFN expression in mature male and female chickens.

Expression of IFNα and IFNλ mRNA in purified chicken splenocytes from 6 female (circles) and 5 male (triangles) 12-week old SPF chickens stimulated with 50 µg/mL PIC for 2 h. Symbols represent fold upregulation of each stimulated splenocytes sample compared to the untreated sample, normalized against the housekeeping gene GAPDH (A). (B) Blood estradiol levels of the chickens at the time of sacrifice. (For A: * p value < 0.05 using a two-way ANOVA with Bonferroni's multiple comparisons test, for B ** p value < 0.005 using the Students *t* test with Mann Whitney U test). Depiction of IFNα and IFNλ mRNA levels for females (C) and males (D) (R^2 values indicate the curve fit for a linear regression).

6.3 Discussion

Like other bird species, chickens have a single IFNλ, compared to four members in humans [125] and two in mice [124]. This Chapter sought to further the knowledge base about chicken IFNλ, particularly in comparison to type I IFNs.

Phylogenetic comparison of chicken type I and III IFNs with those of mice and humans provided insights into their evolution. IFNαs have separately expanded in both humans and mice, forming distinct sub-clades that group together. These formed a larger clade with other type I IFNs, including the single IFNβ and numerous alternate IFNs. It has been argued that this reflects the strong evolutionary pressure on IFN evolution that is thought to be unequal between IFNs [264]. Notably, chicken IFNα and IFNβ clustered together, confirming their presumed divergence from a common precursor, but also suggesting that species-dependent evolutionary pressure has driven them to remain relatively highly conserved compared to those of other species [68]. The IFNγ proteins formed a distinct cluster, indicative of a conserved role across species [265], with the human and mouse IFNλs again forming separate sub-clades.

Since their discovery, type III IFNs have been compared to their type I counterparts [118,119]. Under certain experimental situations, type III IFNs showed similar gene induction to type I IFNs but elicited a reduced magnitude of antiviral effect [266,267]. This has led to the assumption that IFNλs are just a 'weaker' form of type I IFNs. However, other studies have found significant differences between type I and type III IFNs, including the identification of novel roles for the latter [268-270]. Whether such differences were present in chickens remained an unanswered question. Therefore, the induction and effects of IFNλ were directly compared to IFNα in primary chicken splenocytes. Treatment with well-characterized TLR agonists revealed differences in dose-response, magnitude and timing of IFN induction. It is worth to mention here that all stimulations were executed at 37°C and not the usual temperature for avian cells (42°C) due to limitations of incubation space, which may influence the magnitude of the response to stimuli. PIC is a viral RNA mimic that triggers TLR3 and initiates a strong antiviral response in most cells [47]. This was shown to induce IFNα by 3 to 8-fold, whereas IFNλ induction was 30-500-fold. Moreover, peak IFNα induction occurred at a lower dose of PIC (0.5 µg/mL) compared to IFNλ (10 µg/mL). In contrast the timing of PIC-mediated induction of IFNα and IFNλ was similar, with a peak at 1.5-3 h, followed by a decline. These results suggest that the two IFNs may have different biological roles, with moderate IFNα induction triggered by even low levels of pathogen, whereas a stronger IFNλ induction

requires higher pathogen levels. This data is consistent with human and mice experiments [257,271], suggesting that the pattern of IFN induction is conserved. LPS treatment did not upregulate IFNα or IFNλ at any time point, which is in line with observation that infection with gram positive bacteria strongly induce IFNλ in lung epithelia and placenta cells but not gram negative bacteria [272]. Further, studies in human immune cells have shown that only IFNλ1 can be upregulated by LPS stimulation while IFNλ2 and λ3 remain unresponsive in [273]. This suggests that IFNλ1 may have acquired this feature, which is not a general property of type III IFNs. It is worth noting that large variations in IFNλ induction were observed between birds that limited finer analysis, this may be due to genetic factors as is the case for IFNγ in humans [274]. In addition, the reduced fold induction of IFNα is likely due, at least in part, to the higher basal levels of this IFN.

Amplification of antiviral signals plays an important role in immune defence, not only ensuring that the signal reaches sufficient strength to exert an effective response, but also that it is spread to neighbouring cells. These positive feedback loops have been described extensively for mammalian IFNs [128,260,275].To investigate whether this mechanism was conserved in chickens, purified splenocytes were treated with recombinant IFNα or IFNλ. IFNα treatment showed a trend for rapid IFNα upregulation, which then slowly declined. Although the results lacked statistical significance due to considerable bird-to-bird variation the suggested time frame of self-induction was in agreement with mammalian studies [275,276]. IFNλ, in contrast, showed no induction by IFNα at any time point, which is different to studies in mice where IFNα induced a strong IFNλ induction [135]. However, this could be due to cell-type specific effects, since IFNλ expression is known to vary between cell types [137,273]. IFNλ stimulation resulted in a trend for differential regulation of IFNα and IFNλ early, but by 48 h led to a significant upregulation of both IFNα and IFNλ, which was higher for the latter. Based on the time lag involved, this is likely to be an indirect effect, the mechanism of which would require additional study.

ISGs represent the key effectors of the IFN system and as such their expression in response to pathogen related stimuli provides important insight into the chicken innate immune response. Therefore, the expression levels of four well known ISGs were measured in chicken splenocytes stimulated with PIC and LPS. In both cases, Mx1 and Zap showed strong induction that peaked at 3 h. Pkr and Viperin followed a similar trend albeit at a lower fold induction that failed to reach significance in the PIC stimulated cells. The pattern of ISG induction is in broad agreement with published data from mammal and avian in vitro systems, although it is clear that differences exist between cell types [223,236,267,277-279]. Although

LPS did not induce IFNα or IFNλ it has previously been shown that IFNβ is in fact stimulated by LPS and responsible for the ISG upregulation [280].Treatment of splenocytes with recombinant IFNs revealed a difference between IFNα and IFNλ. IFNα was able to stimulate ISGs in a similar manner to PIC with respect to timing and breadth of genes. In contrast, IFNλ was only able to induce Mx1 and Viperin, reaching statistical significance at around 48 h. The rapid but transient induction of ISGs with IFNα but slower and more sustained induction with IFNλ is consistent with observations in mammals [267,271,281]. It seems likely that the peak in ISG expression 48 h post IFNλ treatment is a consequence of the large upregulation of IFNs seen concomitantly at this time point, although the specificity of ISG induction would indicate that the effects of IFNα have been modulated in some manner.

To provide more biologically-relevant insights, the effects of influenza virus infection were investigated. There studies utilized a H5N6 influenza A strain isolated from duck that was previously shown to elicit moderate signs of disease after 48 h. HPAI infection induced a significant but moderate upregulation of IFNα (8-fold) with a stronger induction of IFNλ (50-fold) in the spleen. In the lungs, induction of IFNα was comparably higher (25-fold), with IFNλ upregulation comparable to the spleen (50-fold). The higher IFNα induction in the lung probably reflects this tissue being the primary site of infection, whereas the higher relative IFNλ induction in both organs is likely due the high base-line IFNα expression that reduced the fold change. The upregulation of both IFNs is, however, consistent with other studies investigating HPAI infection in birds [205,282]. Moreover, the fact that both IFNs are induced indicates that this HPAI virus might not interfere significantly with the IFN induction, at least at the mRNA level. HPAI infection resulted in strong induction of Mx1 in both the spleen and lung, whereas Pkr was only significantly upregulated in the spleen. These expression patterns are consistent with the IFN induction observed as well as results in the literature [283,284]. In contrast, Zap was not significantly upregulated at the mRNA level in either the spleen or the lung despite previous studies showing high Zap expression that correlated with a shorter survival time during HPAI infections in chickens [285]. The reason for this lack of Zap induction remains unknown.

Substantial variability was observed in immune gene expression between birds, which represented a significant issue in this study. Not only did it interfere with the ability to obtain statistically significant results and increase bird numbers required in experiments, but also pointed to a biological cause. Differences in the female and male immune systems have previously been noted in both humans and mice [263,286-290]. Exploring this as a potential cause of the variability of IFN expressions in chickens was interesting, since this organism has some

key genetic differences in this regard. In particular, the type I IFNs are located in one cluster on the sex (Z) chromosome of chicken, in which ZZ determines males and ZW determines females. Therefore, splenocytes from male or female chickens, both immature and mature, were tested for IFN responsiveness to PIC. Immature chicken splenocytes responded to PIC treatment with a time dependent activation of IFNs, and greater variability amongst females. For IFNα, males responded earlier, but induction in females was significantly higher and more sustained – a trend that also held true for IFNβ. One hypothesis for that observation would be that the presence of additional copies of type I IFNs in males enables a more rapid response. Induction of IFNλ was also earlier in males, but in this case the peak was significantly higher compared to females. As expected, estrogen levels were significantly higher in females than in males, being much more variable in the former. In mature chickens, induction was higher for both IFNα and IFNλ in females but only reached significance for IFNλ. Consistent low estrogen levels were again observed in mature males (around 50 pg/mL) with much higher and more variable levels in females (450-1400 pg/mL). Investigation of a possible correlation of estradiol levels and induction levels in mature females as in males showed no significant trend. Further research is required to understand the interactions between sex hormones and IFN responses and to identify causes of the observed variability of IFN induction in birds.

7 Characterization of chicken IFNλR

7.1 Introduction

Members of the Class II cytokine receptor family (CRF2) are heterodimeric receptors composed of two receptor subunits, denoted as R1/Rα and R2/Rβ. Each subunit is a single pass transmembrane protein defined by structural similarities in the extracellular domain, which includes the cytokine receptor homology domain (CHD) that is involved in ligand binding and certain sequences in the intracellular domain [164]. CRF2 ligands bind with high affinity to the R1 chain, which characteristically has a larger intracellular domain, whereas the R2 chains have smaller intracellular domain and lower ligand affinities. The binding of the ligand to the R1 chain leads to recruitment of the R2 chain and the trimeric complex then initiates specific intracellular signaling cascades [164]. The family is composed of 12 distinct receptors, which are used for signaling by members of the IFN, IL-10 and IL-10 related cytokines [164,165]. Although type I and type III IFNs share similarities in biological activity they utilize different receptors. Unlike type I and type II IFN receptors, which signal through exclusive R1 and R2 subunits, IFNλR consists of the ligand specific IFNλR1 along with a IL-10R2, which is shared among IL-10R, IL-22R and IL-26R [119,120,166]. When one of these chains is absent from the cell surface, or neutralized by an antibody, cells are unresponsive to IFNλ [119,159], showing that both IFNλR1 and IL-10R2 are needed to form a functional receptor complex.

IFNλ initially binds to the IFNλR1 chain, which causes a conformational change that enables recruitment of the IL-10R2 to form a trimer. This activates the pre-associated intracellular tyrosine kinases JAK1 and TYK2 to mediate phosphorylation of the receptor chains, which creates docking sites for cytosolic STAT1 and STAT2 [120]. Signaling through type I and type III IFN receptor complexes results in the formation of a transcription factor complex known as IFN stimulated gene factor 3 (ISGF3) [128,146]. This complex consists of three proteins, STAT1, STAT2, and IFN regulatory factor-9 (IRF-9) [120]. Once assembled, ISGF3 then translocates to the nucleus where it binds to IFN stimulated response elements (ISREs) in the promoters of various interferon-stimulated genes (ISGs) [185]. Despite different receptor complexes being employed there is a clear signaling overlap between type I and III IFNs, which is why the antiviral outcomes are so similar [128,146,178,185].

Ultimately receptor expression determines which cells respond to particular IFNs. Different tissues and cell types have a highly specialized need to recognize, and be able to act on

specific signaling molecules. The IFNλR subunit, IFNλR1, is predominantly expressed on epithelial cells like these found in skin, lung, intestine, colon and stomach [152,159,179] but is undetectable in other cell subtypes, particular fibroblastic and endothelial cells. This contrasts with the type I IFNRs that are expressed on all nucleated cells [181]. Skin and mucosal surfaces provide a barrier between the host and the environment which suggests that the type III IFN system has evolved to protect the epithelia against pathogens [159].

The antiviral activity of type III IFNs compared to type I IFNs is reported to be 'weaker', which appears to be closely related to IFNλR1 expression [64,291]. Initial studies showed that IFNλR1 knock-out mice were indistinguishable from the wild type mice when challenged by a panel of viruses, unlike IFNαR1 knock-out mice, which were highly susceptible [135,176]. However, IFNλR1 knock-out mice were later found to be susceptible to rotavirus infection whereas type I IFN knock-out mice were similar to the wild type [270].

The results from mammalian studies provides clues as to the roles of IFNs in the chicken. However, the identity of the IFNλR complex in chickens has not been functionally confirmed and its expression remains poorly characterized. In addition, downstream signaling of IFNλR is not documented in the avian host with few studies looking at ISG regulation. This Chapter aims to provide a clear picture of the IFNλR and its downstream signaling cascade to better understand the chicken IFNλ mediated responses.

7.2 Results

7.2.1 Phylogenetic analysis of the type III IFN receptor chains

To evaluate the extent of conservation of the type III IFNR system, a phylogenetic tree was constructed using the Neighbour Joining method with all published and predicted IFNλR1 sequences (**Figure 7-1**). This revealed that chicken IFNλR1 clustered with those of other birds which were most closely related to reptile sequences that clustered together, the exception being the anole lizard sequence. Mammals clustered together with a significant distance from the bird-reptile sequences, with fish and amphibian sequences found to be more distantly related. Closer analysis of the bird sequence cluster showed the chicken sequence was most closely related to that of turkey, duck and goose.

Phylogenetic analysis of IL-10R2 sequences gave a tree with similar topology (**Figure 7-2**). Bird sequences again clustered and were most closely related to reptile sequences. Mammal sequences clustered together in a tight group, with amphibians and *Carolina anole* between birds and mammals and fish sequences the most distant. Closest related to the chicken sequence were those of turkey, duck and goose.

7.2.2 Synteny of genes encoding the type III IFN receptor chains

To provide evidence of orthology the conservation of flanking genes was investigated. The human IFNλR1 gene is located on chromosome 10 and is flanked by the MYOM3 and IL-22RA genes upstream and the GRHL3, STPG1, NIPAL3 and RCAN3 downstream (**Figure 7-3A**). The chicken gene was found to be located on chromosome 23 with the predicted upstream flanking genes also MYOM3 and IL-22RA and the downstream genes GRHL3, NIPAL3 and RCAN3. Both the chicken and human IFNλR1 genes consist of 7 exons.

The human IL-10R2 is located in the IFN receptor cluster on chromosome 21 and is flanked by IFNαR2 upstream and IFNαR1 and IFNγR2 downstream (**Figure 7-3B**). The chicken orthologue was also found to be located in the IFN receptor cluster on chicken chromosome 1 and was flanked by IFNαR2 upstream and IFNαR1 and IFNγR2 downstream. The chicken and human IL-10R2 also consisted of 7 exons. This suggests both genes are orthologous with their human counterpart.

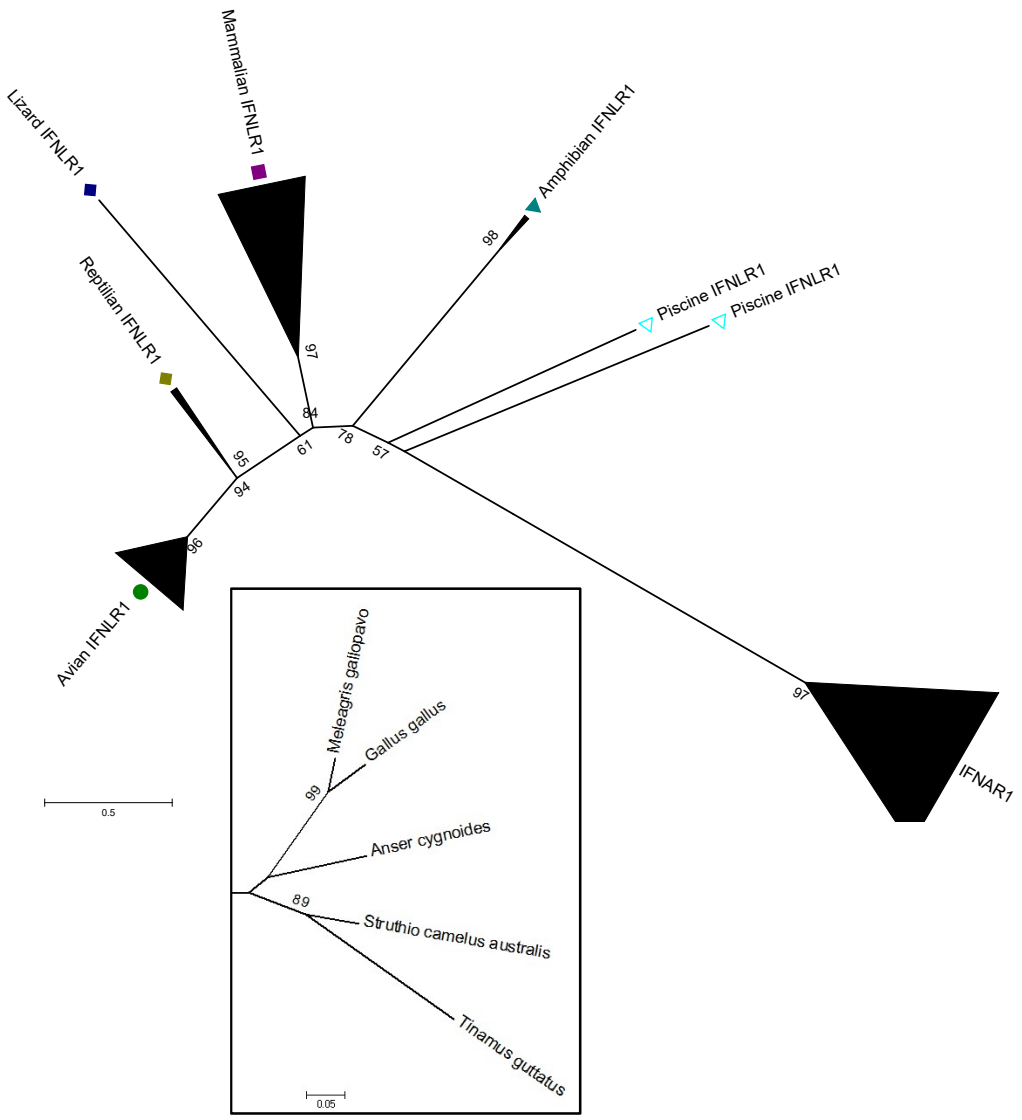

Figure 7-1 Phylogenetic analysis of IFNλR1 proteins

Phylogenetic tree of all IFNλR1 protein sequences constructed using the Neighbor-Joining method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The insert shows the closest relatives to the chicken sequence in the avian clade.

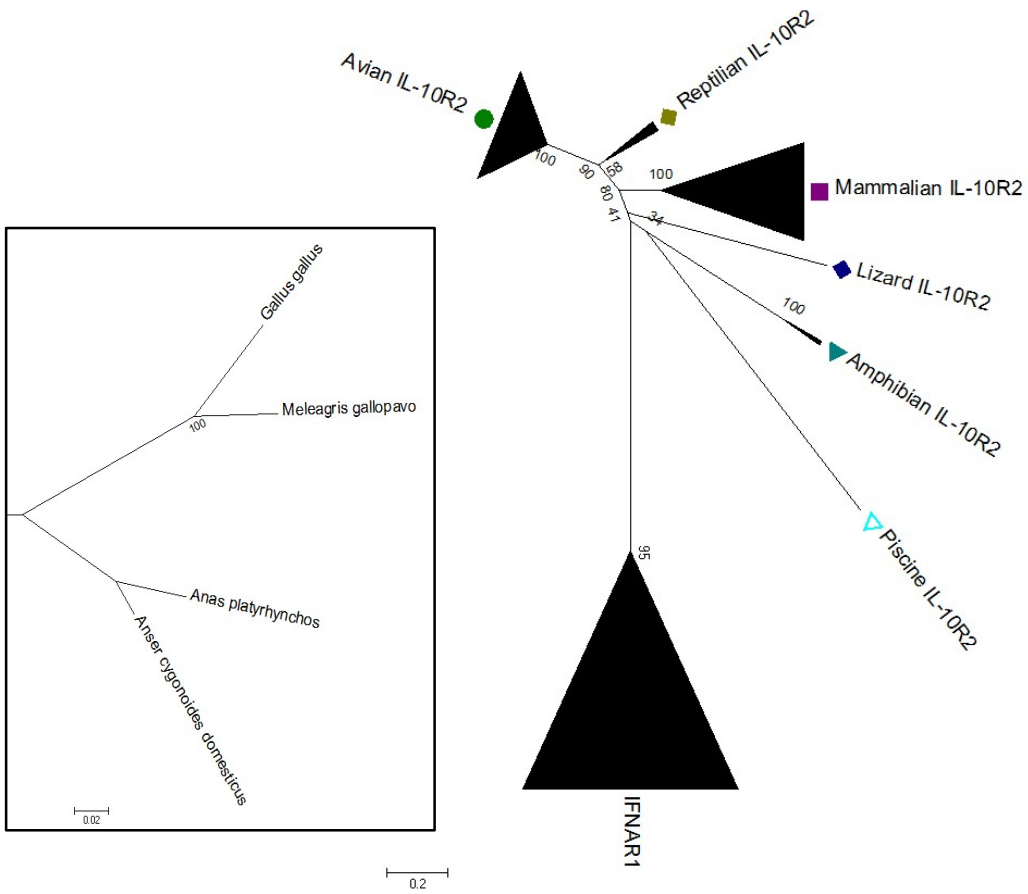

Figure 7-2 Phylogenetic analysis of IL-10R2 proteins

Phylogenetic tree of all IL-10R2 protein sequences constructed using Neighbor-Joining method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The insert shows the closest relatives of the IL-10R2 gene in the avian clade.

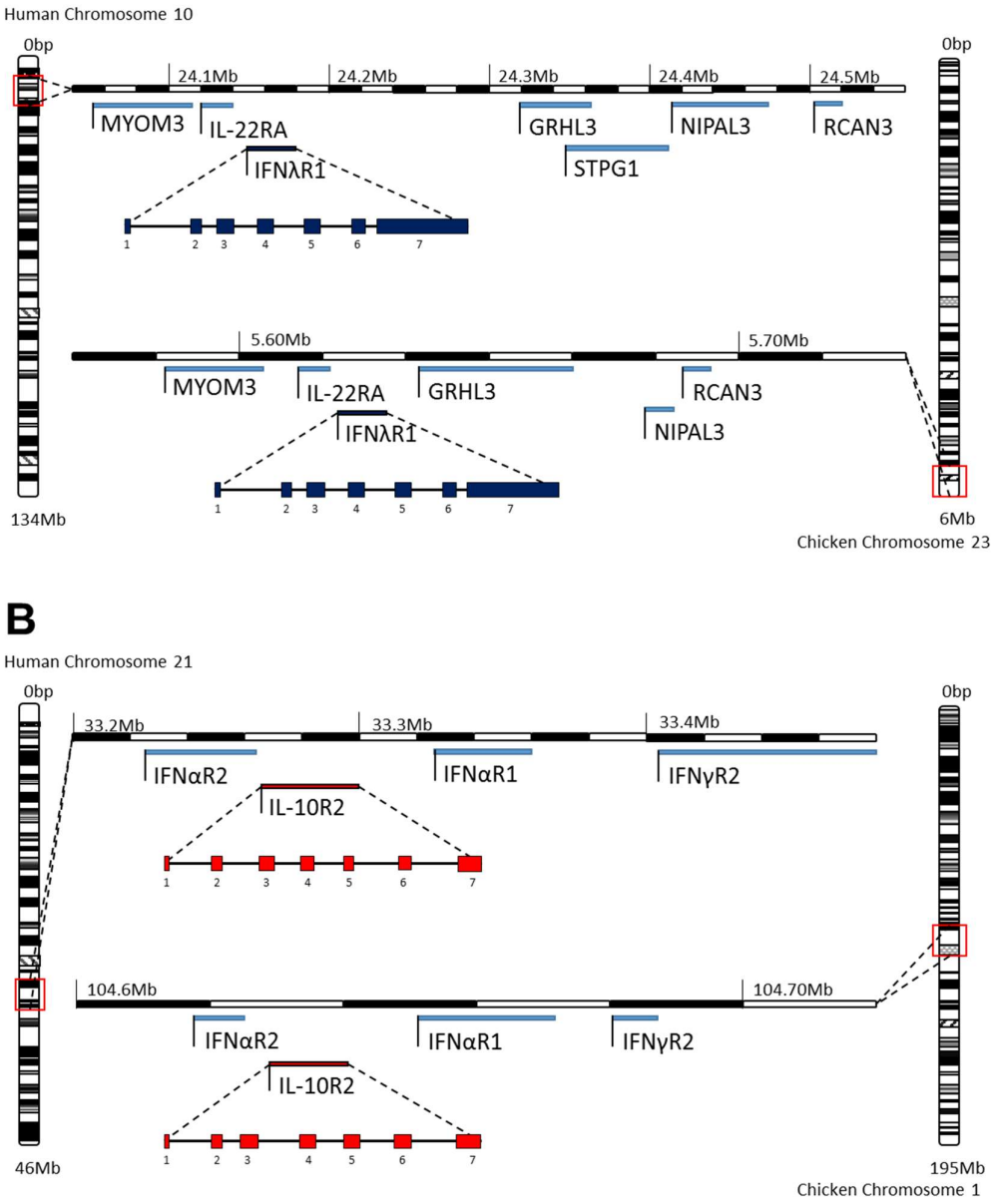

Figure 7-3 Synteny of IFNLR1 and IL10R2

(**A**) Location of the human IFNλR1 gene on chromosome 10 and the chicken IFNλR1 gene on chromosome 23 . The flanking regions show conserved genes upstream (MYOM3, IL-22R1) and downstream (GRHL3, NIPAL3 and RCAN 3. The predicted gene structure of chicken IFNλR1 shows seven exons and six introns like human IFNλR1. (**B**) Location of the human IL-10R2 on chromosome 21and the chicken IL-10R2 on chromosome 1. Conserved flanking genes upstream (IFNαR2) and downstream (IFNαR1, IFNγR2) genes are shown. The IL-10R2 gene has seven exons and six introns in both species.

7.2.3 Tissue distribution of the type III IFN receptor chains

To gain insight into the role of the IFNλR complex, its tissue distribution was evaluated. Total RNA was isolated form chicken tissues and purified mononuclear cells from the bone marrow and blood and subjected to qRT-PCR to assess the expression of the genes encoding the two IFNλR chains (**Figure 7-4**) with the relative expression levels calculated using GAPDH as a housekeeping gene. Assuming comparable amplification efficiency, the expression of IFNλR1 was universally lower than that of IL-10R2 in all the tissue types. The highest IFNλR1 expression was seen in the spleen, bursa and particularly PBMCs. The lowest expression of both receptors was found in the brain. To provide additional perspective the relative receptor chain expression was analyzed. The ratio of IL10R2 to IFNλR1 was found to be 5-fold in spleen, bone marrow and bursa, around 3-fold in the thymus and 7- and 15-fold in PBMCs and brain respectively.

7.2.4 Functional analysis of the chicken type III IFN receptor complex

While there was good circumstantial evidence that a functional chicken IFNλR complex existed, to provide definitive support a siRNA knockdown strategy was developed in the chicken fibroblast cell line (DF1). Chicken IFNλR specific siRNA (**Figure 7-5A**) was able to knockdown IFNλR1 mRNA expression by 60% at 48 h post transfection compared to the non-target control siRNA (eGFP). Similarly, a IL-10R2 siRNA (**Figure 7-5B**) resulted in an 80% knockdown of IL-10R2 gene expression compared to an eGFP siRNA treated control. These cells were subsequently stimulated with chicken IFNλ (chIFNλ) and analyzed for STAT activation by EMSA (**Figure 7-6**) and Mx1 gene expression by qRT-PCR (**Figure 7-7**) as a measure of receptor activation. Strong STAT activation was observed in the control group, which was greatly reduced in the IFNλR1 siRNA group and almost undetectable in the IL-10R2 siRNA treated group. Similarly, significantly lower expression level of Mx1 was observed post IFNλ stimulation in both the IFNλR1 siRNA and the IL-10R2 siRNA treatment groups, respectively, when compared to the eGFP (non-target) siRNA treated group.

To verify that the signal downstream of the chicken IFNλR complex utilized JAK1, the selective JAK1 inhibitor Ruxolitinib, was employed. Stimulation of DF1 cells with chIFNλ led to a significant upregulation of Mx1 from the unstimulated state while the cells treated with Ruxolitinib and chIFNλ showed no upregulation (**Figure 7-8**).

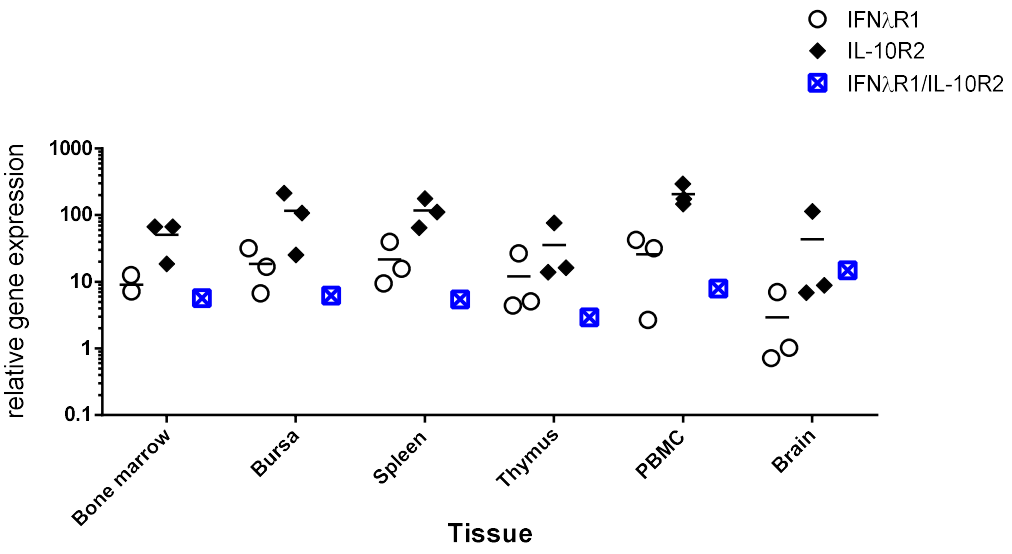

Figure 7-4 Tissue distribution of IFNλR chains

Expression of IFNλR1 and IL-10R2 mRNA in tissues and cell populations of 6-week old SPF chickens. Relative expression levels of IFNλR1 (white dots) and IL-10R2 (black diamonds) normalized against the housekeeping gene GAPDH. IFNλR1/IL-10R2 ratio in each tissue are indicated by blue squares.

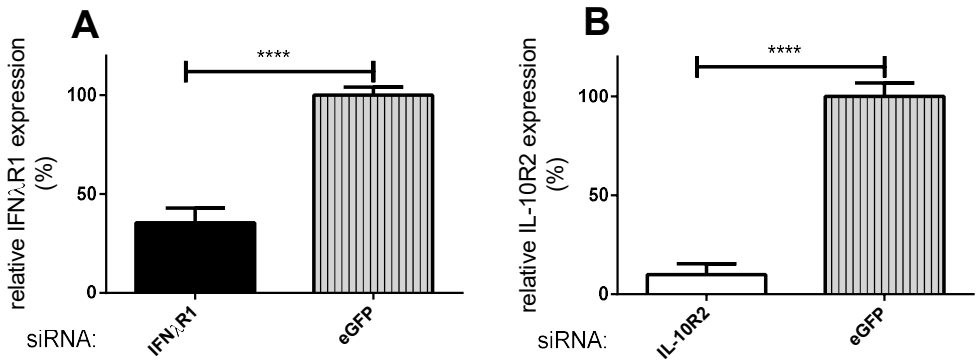

Figure 7-5 Confirmation of successful siRNA mediated gene knockdown

Expression of IFNλR1 (A) and IL-10R2 (B) mRNA in DF1 cells treated with the respective siRNAs and a non-target control siRNA (eGFP). The bars represent relative expression levels of mRNA, normalized against the housekeeping gene GAPDH as a percentage of expression compared to the control. (**** p value < 0.0001 compared using Students *t* test).

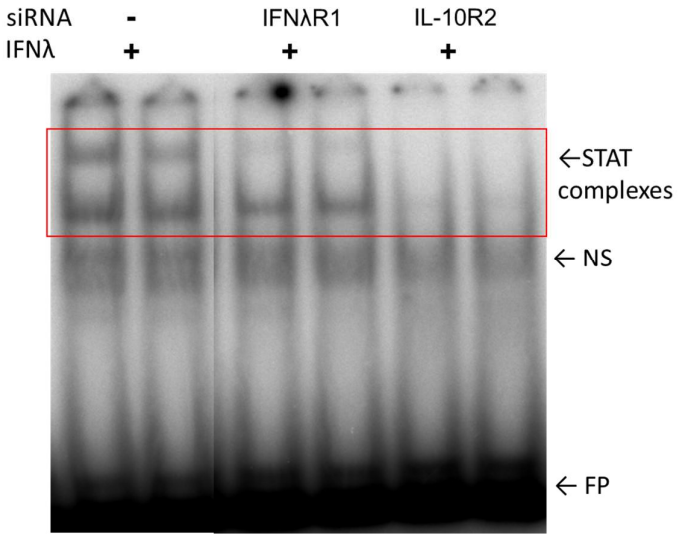

Figure 7-6 Effect of IFNλR chain knockdown on STAT activation

EMSA analysis of STAT activation following IFNλ stimulation of DF1 cells with no siRNA (-) or transfected with IFNλR1 siRNA or IL-10R2 siRNA as indicated in duplicates. Red box indicates the stat complexes, NS indicates nonspecific bands, FP indicates free probe.

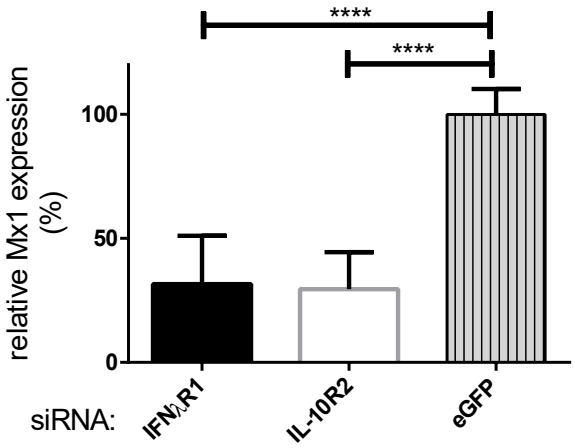

Figure 7-7 Effect of IFNλR chain knockdown on Mx1 expression

Expression of Mx1 mRNA in DF1 cells treated with siRNAs targeting IFNλR1 (black), IL-10R2 (white) or control siRNA (eGFP). The bars represent relative expression levels of mRNA, normalized against the housekeeping gene GAPDH as a percentage of expression compared to the control. (**** p value < 0.0001 compared using a one-way ANOVA test with uncorrected Fischer LSD post-test).

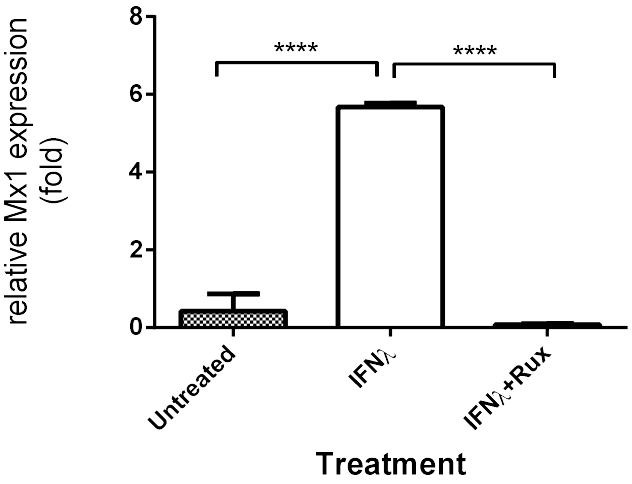

Figure 7-8 Effect of JAK inhibition on IFNλ-mediated Mx1 induction

Expression of Mx1 mRNA in untreated and unstimulated (grey), untreated and IFNλ stimulated (white) and Ruxolitinib-treated and IFNλ stimulated (black) DF1 cells. The bars represent fold change in levels of mRNA expression, normalized against the housekeeping gene GAPDH compared to the untreated/unstimulated group. (**** p value < 0.0001 using a one-way ANOVA test with Bonferroni posttest).

7.3 Discussion

Translated IFNs are transported out of the cell and stimulate cell surface IFN receptors [164]. For IFNλ, the receptor complex comprises IFNλR1 and IL-10R2 , which relays the signal through the intracellular JAK-STAT pathway [118,119]. Type I and type III IFNs stimulate highly similar biological effects largely through their shared downstream signaling components JAK1 and TYK2, which phosphorylate and activate STAT1/STAT2 heterodimers that associate with IRF9 to form the transcriptional complex ISGF3 [292]. ISGF3 upregulates the expression of hundreds of antiviral genes that block the virus lifecycle at multiple stages [207]. The IFNλ mediated signaling cascade has been very well characterized in mouse and human but little is known about this pathway in chicken. The IL-10R2 was previously characterized and a candidate IFNλR1 gene had been identified using BLASTN. However, confirmation of the identity of this candidate gene and the dependency of both chains for chicken IFNλ signaling via the JAK/STAT pathway was missing.

The human IFNλR1 gene has 7 exons and is flanked upstream by MYOM3 and IL-22RA genes, and downstream by GRHL3, STPG1, NIPAL3 and RCAN3 genes. The candidate chicken IFNλR gene showed identical synteny except for STPE1 for which no chicken homologue could be identified. As shown previously by Reboul et al [202] the structure and location of the second receptor chain gene, IL-10R2, is conserved between chicken and human consisting of seven exons and is located in an IFN receptor cluster, flanked upstream by IFNαR2 and downstream by IFNαR1 and IFNγR2.

Phylogenetic analysis of the IL-10R2 and IFNλR1 chains revealed that both chicken sequences clustered with those from other birds. These were collectively most similar to reptiles, which is expected given their shared evolutionary history [293]. For both receptor chains, mammalian sequences clustered tightly together at a significant distance from the avian-reptilian sequences. Fish sequences were found to be the most distant in this analysis, consistent with other published work [56].

Expression of both IFNλR chains was investigated in immune relevant tissues to identify those likely to be responsive to IFNλ in the chicken. In all tissues examined, IL-10R2 was more highly expressed than IFNλR1. The highest IFNλR1 expression levels were observed in splenic, bursal tissues and PBMC; similar to results observed in humans [172]. Interestingly purified splenocytes showed even higher IFNλR1 expression than the whole spleen which may indicate increased expression of IFNλR1 by white blood cells (data not shown). The highest IL-10R2 expression was in the brain followed by PBMCs, spleen, bone marrow and

bursal tissue, and the lowest thymic tissue. As both chains must be present for an IFNλ-mediated immune response these findings indicate that the IFNλR1 is likely the restrictive receptor as it is universally expressed at a lower level. Although the levels of expression were only investigated at the level of mRNA, previously published work has shown a direct correlation between IFNλR mRNA expression and responsiveness to chIFNλ [206].

Two chicken cell lines were tested for IFN λR1 expression and both the Fibroblastic cell line DF1 and the Macrophage cell line HD11 were found to express low levels of the receptor, however as the transfection efficiency was higher in DF1s the work was continued with these cells. An siRNA mediated knockdown strategy was used to target both IFNλR chains separately. This blunted chIFNλ-induced STAT activation and Mx1 expression by up to 60% compared to controls which corresponded with the knockdown efficiency of the respective siRNAs. The involvement of JAK1 was examined with Ruxolitinib, a selective JAK1 inhibitor used as a treatment for myelofibrosis [294]. DF1 cells treated with chIFNλ showed a 6-fold increase in Mx1 mRNA levels, which was completely blocked with Ruxolitinib. These results collectively confirm that chIFNλ requires IFNλR1, IL-10R2 and JAK1 for STAT complex activation and the subsequent upregulation of ISGs like Mx1.

8 Characterization of chicken IFIT5

8.1 Introduction

Avian influenza (AI) viruses, especially the highly pathogenic subtypes, cause significant morbidity and mortality in chickens worldwide [237]. This has widespread consequences, notably including severe economic losses [239] and the potential of zoonotic transmission to humans [237,241-243]. To fully understand how the disease might be combatted in poultry it is essential to characterize the parts of the host immune system responding to influenza virus infection [248].

The innate immune system forms a critical part of the host defence against pathogens and several pathways have been characterized to date. In mammals, innate immune recognition is mediated by a series of germ-line encoded receptors called pathogen recognition receptors (PRR) that detect conserved pathogen-associated molecular patterns (PAMPs) [40]. One of the most prominent families of PRR are the Toll-like receptors (TLR) [41]. To date, nine conserved TLRs have been found in humans and mice and each one serves a distinct function in PAMP recognition and immune response [53]. TLRs essentially function as sentinels, which when activated modulate the expression of multiple immune genes that are involved in the mobilization of a wider immune response [38,44]. Infections with viral pathogens may be sensed through TLR3, which recognizes double stranded RNA (dsRNA) and TLRs 7, 8 and 9, which recognize non-self nucleic acids [45-50]. Upon viral PAMP engagement, TLRs trigger intracellular signaling cascades that lead to the expression of a variety of pro-inflammatory cytokines and chemokines as well as antiviral genes, which together orchestrate the early host response to infection [53,295]. However, important differences between the mammalian and the avian hosts have been found like the absence of TLR9 and RIG-1, while chicken TLR8 seems to be non-functional. Notwithstanding this, TLR3, the most important receptor in dsRNA sensing has been found and characterized [261,296,297]. Furthermore, there is evidence to suggest that chicken MDA5 is also a functional analogue of mammalian RIG-1 [255].

Cytokines comprise a large family of molecules, including interferons (IFNs), interleukins (ILs), colony-stimulating factors (CSFs), transforming growth factors (TGFs) and tumour necrosis factors (TNFs) [58]. It is now recognized that several IFNs are collectively crucial for defence against viral pathogens, contributing to the induction and regulation of both innate and adaptive antiviral mechanisms [61-63]. Three distinct interferon families, type I , II and III, have been identified in mammalian and avian species, which differ in their amino acid

sequence, receptor specificity, signaling pathways and gene induction, but there is overlap between families [64-66]. Although only type I and III IFNs are directly produced in response to viral infections, all types significantly contribute to the generation of an effective antiviral immune response against a wide range of pathogens [59]. This is achieved by upregulation of hundreds of antiviral genes that act in concert to limit viral spread and replication [207], which includes members of the IFIT family. Human and mouse IFIT1-5 have been shown to play critical roles in antiviral defence [211]. The mechanisms are not completely understood but IFIT1 and IFIT2 have been shown to suppress translation by binding to the eukaryotic initiation factor 3 [211]. IFIT family members can also bind to single stranded RNA [211,227] and double stranded DNA [228] directly and thereby reduce viral replication.

A better understanding of the innate immune system particularly in acute AI infection is needed for the development of targeted therapeutic interventions. In this Chapter the IFIT5 gene of chickens was investigated to gain further insights into the innate defence of this important production animal against viral pathogens. In particular, the response of the chicken IFIT5 gene to different immune stimuli and acute influenza virus infection was assessed.

8.2 Results

8.2.1 Identification and characterization of a putative chicken IFIT5 gene

Bioinformatic analysis of the chicken genome with the human IFIT5 gene sequence using BLASTN identified a potential chicken IFIT5 gene located on chromosome 6 (**Figure 8-1**). It was flanked by CH25H and LIPA upstream and SLC16A and PANK 1 downstream, similar to the human IFIT gene cluster located on chromosome 10. Like human IFIT5, the chicken gene also consisted of 2 exons and 1 intron.

Gene specific primers were used to amplify cDNA from 4-week old White Leghorn chicken splenocytes stimulated with poly (I:C) for 3 h. This generated an approximately 1440 base pair (bp) amplicon (**Figure 8-2**) that was not observed in the no template control. This amplicon was cloned and sequenced (Accession No.: KT180229) revealing nine single nucleotide polymorphisms (SNPs) when compared to the predicted sequence, five of which would lead to amino acid changes in the 470 residue protein.

8.2.2 Conservation of chicken IFIT5

Phylogenetic analysis of IFIT protein sequences (**Figure 8-3A**) showed that the putative chicken IFIT5 clustered with similar genes from other bird species as well as from turtle. In contrast, mammalian sequences clustered together in distinct families of IFIT1-5 with the exception of chimpanzee IFIT3 and marsupial IFITs, which formed their own clade. Fish sequences were the most distantly related and formed two distinct clades, with reptile (lizard) and amphibian (frog) sequences in between. To illustrate the sequence divergence of bird IFITs compared to human IFIT1-5 a smaller tree was constructed (**Figure 8-3B**). The chicken IFIT5 protein sequence clustered with the other bird IFITs, whereas the human sequences formed a separate clade. Chicken IFIT5 was most closely related to the turkey IFIT5 sequence with 0.10 amino acids substitutions per site (aas/s) (**Table 1**) followed by the recently cloned duck IFIT5 [298] with 0.47 aas/s. Of the human IFIT proteins, the chicken IFIT5 was most closely-related to human IFIT5 with 0.89 aas/s and IFIT1B with 0.92 aas/s. Domain prediction of the chicken IFIT5 protein sequence identified multiple Tetratricopeptide repeats (TRPs), a hallmark of IFIT genes, with an arrangement of TRPs similar to other bird and human IFIT genes (**Figure 8-4A**). Protein sequence alignment with human IFIT5 (**Figure 8-4B**) revealed that 13 out of 16 amino acids previously shown to be critical for function were conserved in chicken IFIT5, including two residues that were important for ssRNA/dsDNA binding selectivity [228].

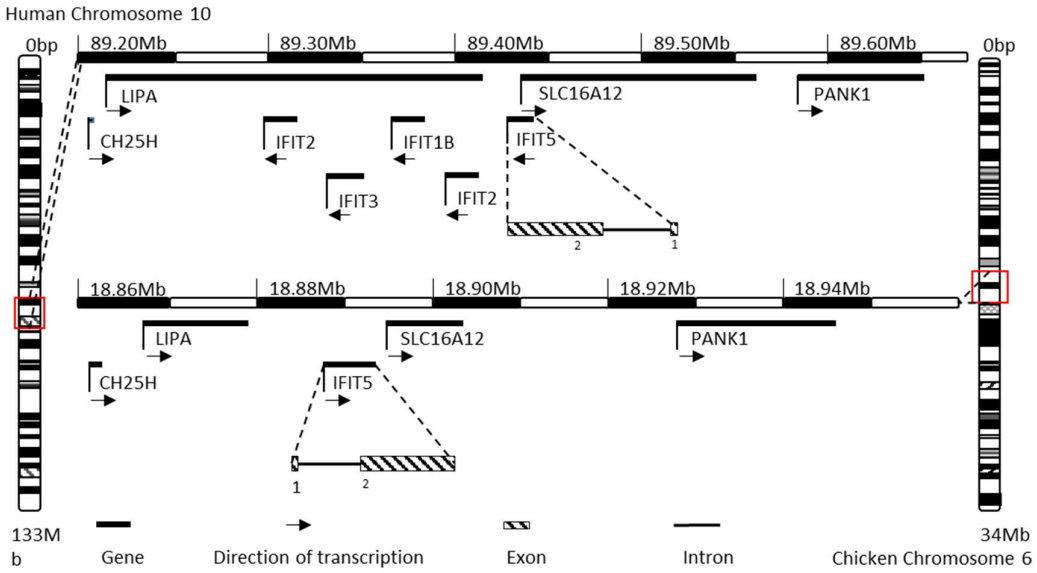

Figure 8-1 Chromosomal location and predicted gene structure of chicken IFIT5

Location of the chicken IFIT5 gene on chromosome 6 and the human IFIT5 gene cluster on chromosome 10. Conserved flanking genes upstream (CH25H, LIPA) and downstream (SLC16A, PANK1) and additional human IFIT genes are shown. The predicted gene structure of chicken IFIT5 with 2 exons and one intron is presented.

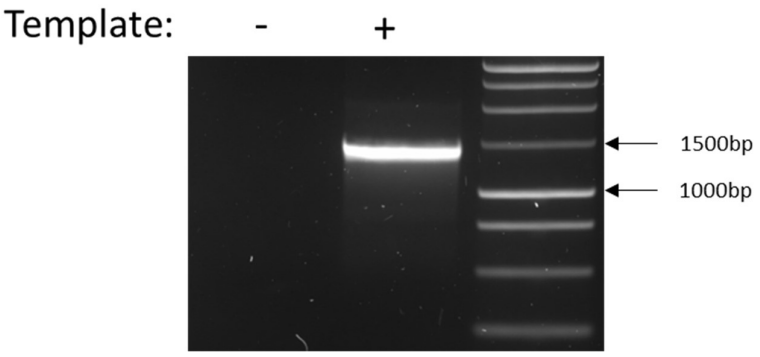

Figure 8-2 Amplification of the chicken IFIT5 gene

RT-PCR amplification of the ~1440 bp chicken IFIT5 gene using specific primers from poly (I:C) stimulated splenocytes (+). No amplification was observed when the template was omitted (-).

Table 1 Evolutionary distance of homologs from chicken IFIT5 gene

The number of amino acid substitutions per site from between sequences are shown. Analyses were conducted using the JTT matrix-based model. The analysis involved 10 amino acid sequences. All positions with less than 85% site coverage were eliminated. The analyses were conducted in MEGA 7.

Chicken IFIT5									
Turkey IFIT5*	0.10								
Duck IFIT5	0.47	0.47							
Flycatcher IFIT5*	0.67	0.66	0.66						
Zebrafinch IFIT5*	0.65	0.62	0.60	0.33					
Human IFIT1	0.98	0.99	0.99	1.04	1.01				
Human IFIT1B	0.92	0.95	0.97	1.02	0.97	0.40			
Human IFIT2	1.23	1.22	1.18	1.19	1.18	0.93	0.98		
Human IFIT3	1.26	1.22	1.22	1.30	1.25	0.98	0.93	0.58	
Human IFIT5	0.89	0.92	0.90	1.00	0.94	0.58	0.64	0.95	0.98

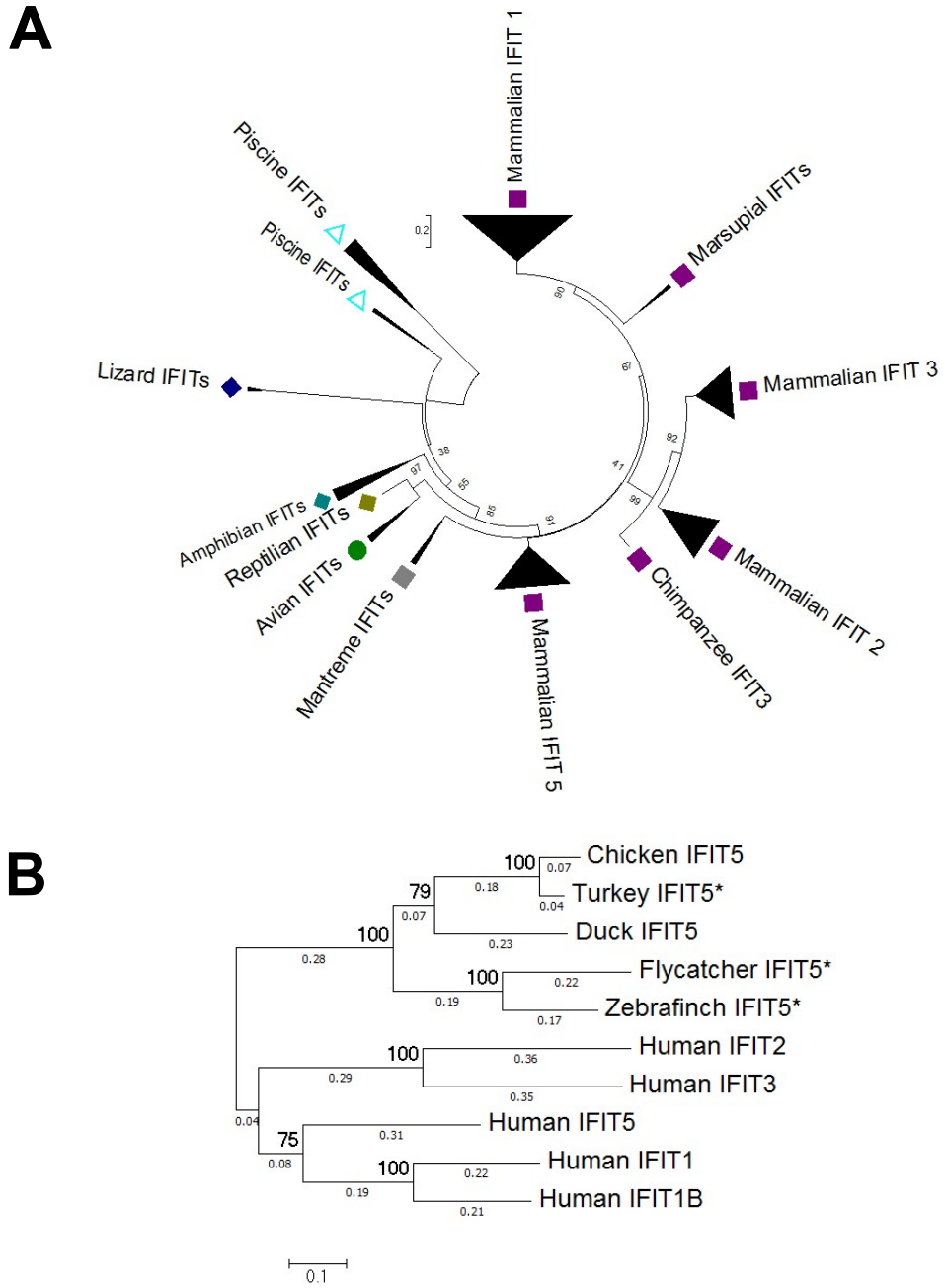

Figure 8-3 Phylogenetic analysis of IFIT5 proteins

(A) Phylogenetic tree of all IFIT published protein sequences using constructed the Neighbor-Joining method. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. (B) Predicted evolutionary history of the indicated human and bird IFIT proteins constructed using the Maximum Likelihood method. Bootstrap values are shown at the nodes as a percentage of 1000 replicates. The scale bar represents 0.1 aa substitutions per site.

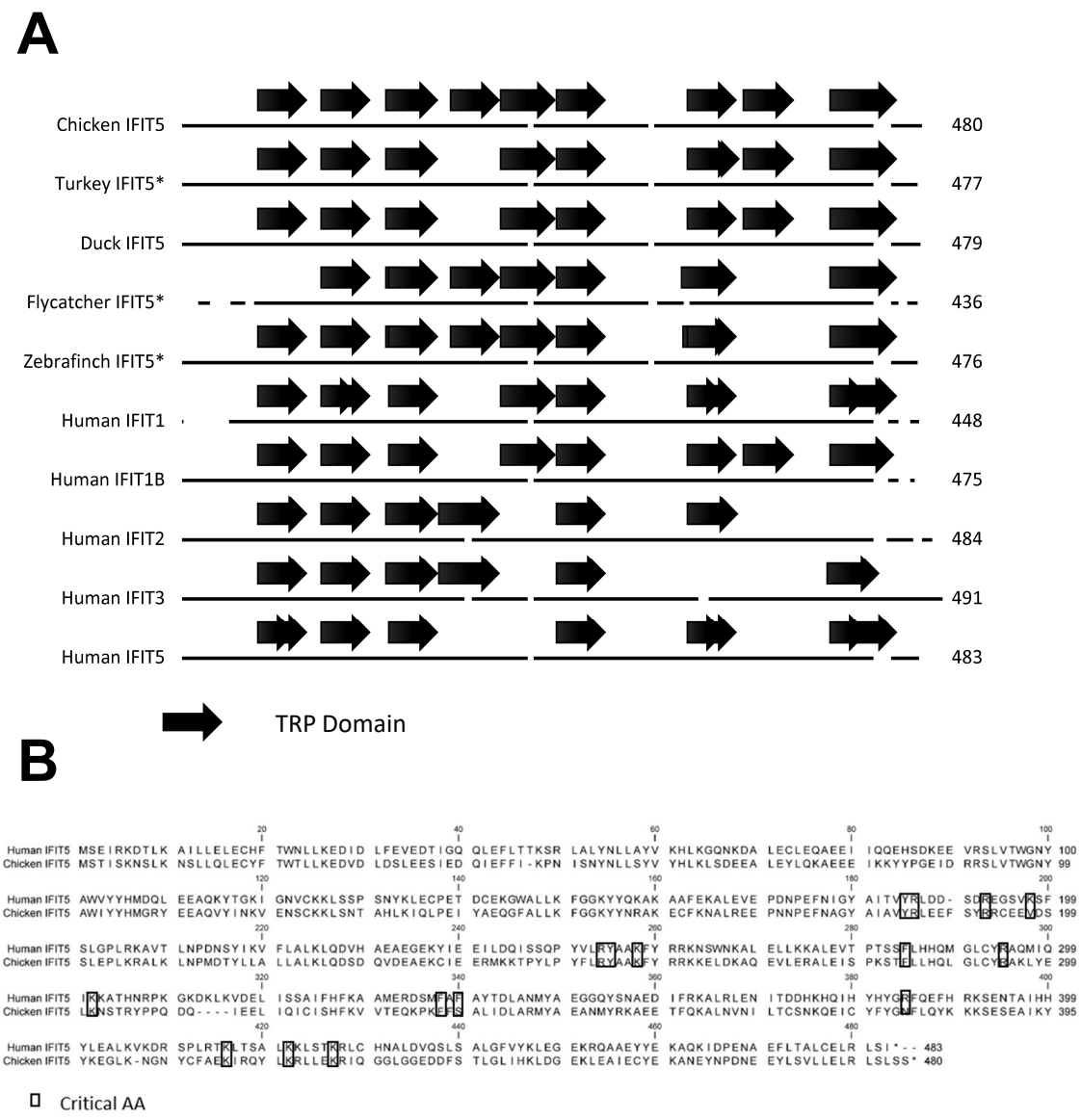

Figure 8-4 Domain analysis of human and avian IFITs

(A) Schematic representation of the predicted Tetratricopeptide repeats (TRP) domains within the indicated IFIT protein sequences (* denotes predicted sequences). Numbers on at the end of each sequence indicates protein length. (B) Alignment of chicken and human IFIT5 sequences. Numbers above the sequence denote bp from the start of the aligned sequence, dashes mark gaps in the alignment, and stars indicate the end of sequence. Residues identified as functionally critical in humans are highlighted

8.2.3 Expression of chicken IFIT5 following immune stimulation

To further characterize the chicken IFIT5 gene, its expression in response to immune stimuli was investigated. Some ISGs can be induced by TLR pathways independently of IFN. To assess this chicken splenocytes were treated with 50 µg/mL poly (I:C) (**Figure 8-5A**) and 10 µg/mL LPS (**Figure 8-5B**) and the expression of IFIT5 and the well-known chicken Myxovirus resistance protein 1 (Mx1) gene quantified. The poly (I:C) treatment up-regulated both IFIT5 and Mx1 expression, which peaked at 6 h post stimulation at ~90- and 100-fold, respectively. LPS stimulation induced a 15-fold increase in IFIT5 expression from 6-24 h post stimulation followed by a rapid decline to undetectable levels at 48 h, with a similar pattern of Mx1 induction observed.

The interferon inducible nature of mammalian IFITs has been well characterized. To confirm that IFIT5 is an ISG, splenocytes from SPF chickens were treated with recombinant chicken IFNα (**Figure 8-6A**) and IFNλ (**Figure 8-6B**). Following IFNα treatment, IFIT5 was rapidly induced reaching significant levels at 1.5 h with peak expression at 3 h at 75-fold, Mx1 induction was delayed, peaking at 6 h at 45-fold. Both IFIT5 and Mx1 expression remained significantly above base-line until 24 h followed by a decrease at 48 h. Following IFNλ stimulation there was a steady increase in IFIT5 expression with the highest increase in expression of around 25-fold at 48 h post treatment. Mx1 behaved similarly although the fold induction was lower with an approximate 10-fold increase in mRNA levels at 3 h.

8.2.4 Expression of chicken IFIT5 following viral infection

Since IFIT5 is considered an antiviral gene it was important to also characterize the gene expression levels in response to acute infection *in vivo*. Therefore, the expression of IFIT5 and Mx1 was measured in spleen (**Figure 8-7A**) and lung (**Figure 8-7B**) of H5N6 virus-infected SPF chickens. Both IFIT5 and Mx1 were upregulated approximately 400-fold in the lungs and 60-fold in the spleen of infected birds compared to healthy age-matched chickens.

Since eggs are universally used to amplify influenza virus the expression of these genes was also assessed in eggs infected with an H1N1 Influenza A virus (**Figure 8-8**). IFIT5 was upregulated at 24 h post infection reaching significance at 48 h in the CAM, while in muscle and brain no expression was detected at 24 h but expression was observed at 48 h. Mx1 behaved similarly in the CAM although did not reach statistical significance. Mx1 expression in the muscle was upregulated at 24 h with a significant increase at 48 h, while the brain showed no induction at 24 h but increased Mx1 mRNA was detected at 48 h post infection.

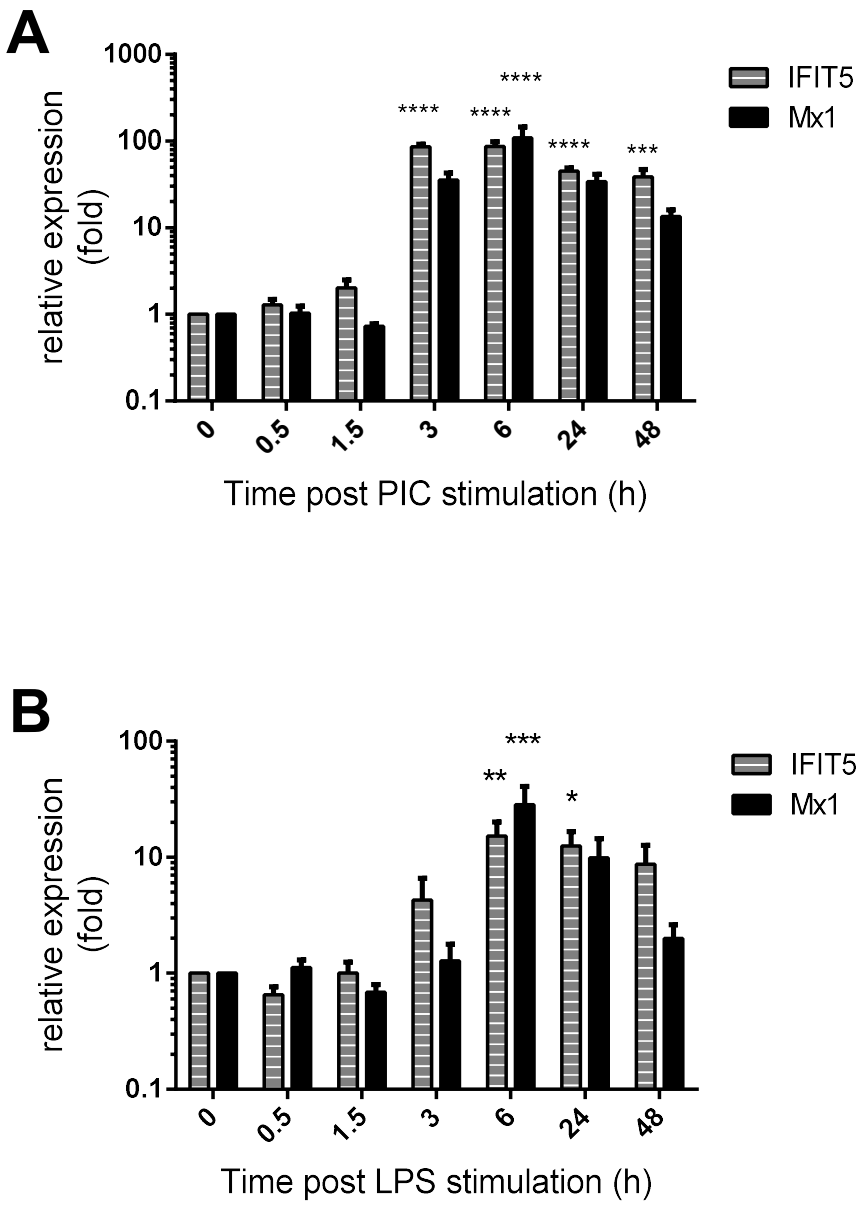

Figure 8-5 Expression of IFIT5 and Mx1 in response to TLR agonists

Expression of IFIT5 and Mx1 mRNA in splenocytes from SPF chickens stimulated with 50 µg/mL poly (I:C) (A) and 10 µg/mL LPS (B). This is shown as mean fold change with standard error of the mean (SEM) compared to the untreated sample, normalized against the housekeeping gene GAPDH (* p value < 0.05, ** p value < 0.005, *** p value < 0.001 **** p value < 0.0001 compared to the 0 h time point using a one-way ANOVA test with uncorrected Fischer LSD post-test). The Mx1 data is a duplicate of that already shown in 6.5A/B and serves as a comparison.

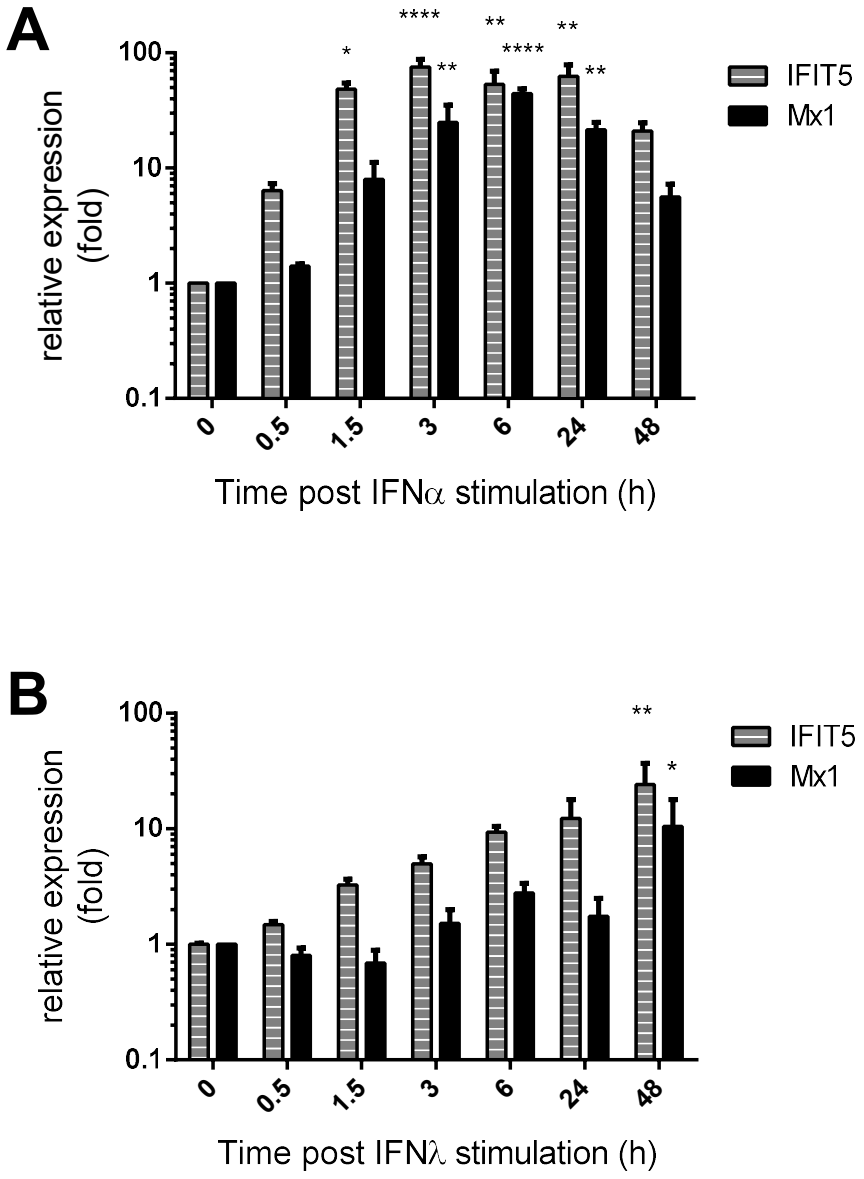

Figure 8-6 Expression of IFIT5 and Mx1 in response to IFN stimulation

Expression of IFIT5 and Mx1 mRNA in splenocytes from SPF chickens stimulated with 500 ng/mL IFNα (A), 50 µg/mL IFNλ (B). This is shown as mean fold change with standard error of the mean (SEM) compared to the untreated sample, normalized against the housekeeping gene GAPDH (* p value < 0.05, ** p value < 0.005, *** p value < 0.001 **** p value < 0.001 using a one-way ANOVA test with uncorrected Fischer LSD post-test). The Mx1 data is a duplicate of that already shown in 6.6A/B and serves as a comparison.

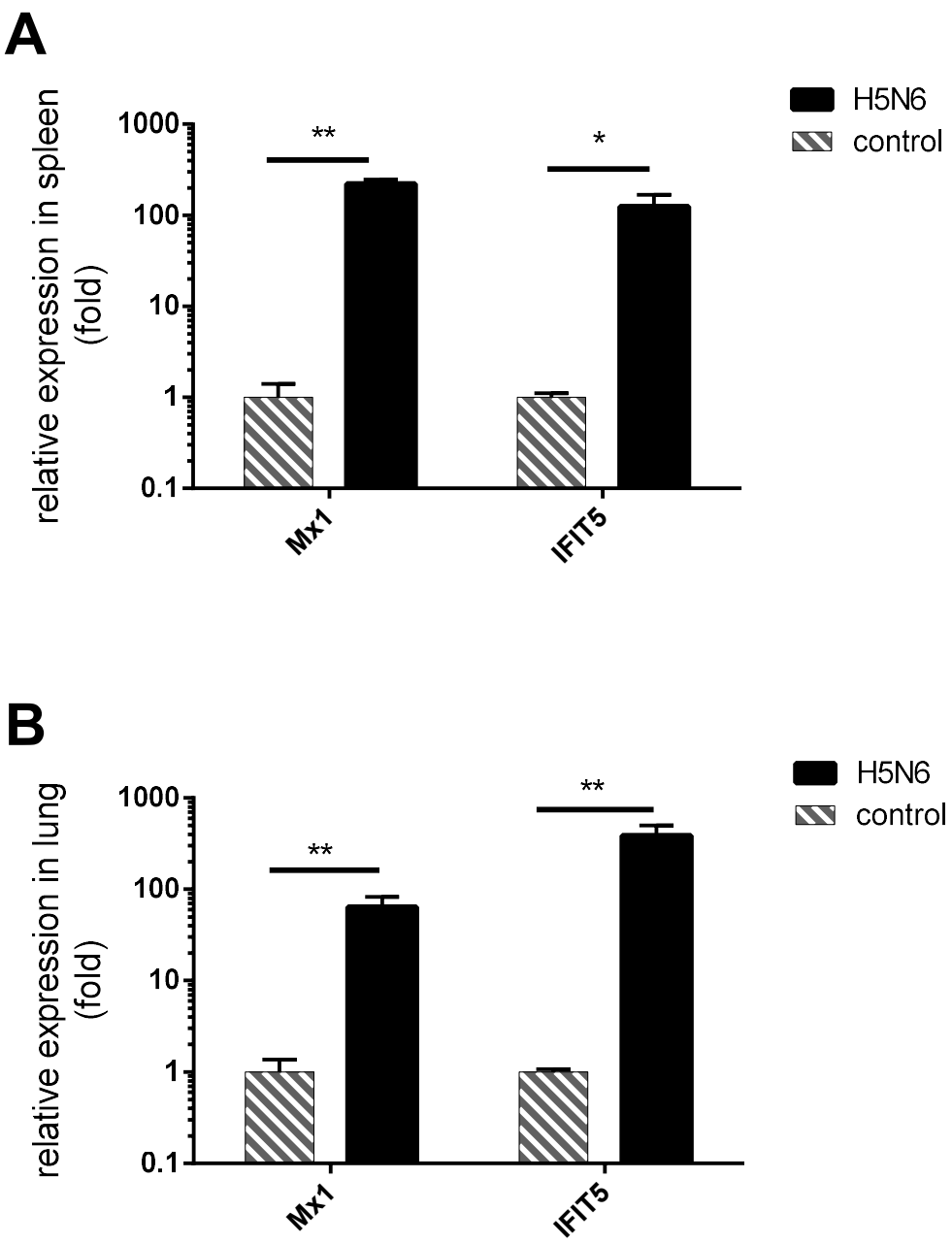

Figure 8-7 Expression of IFIT5 and Mx1 in response to viral infections *in vivo*

Expression of IFIT5 and Mx1 mRNA in six 5-week old SPF chicken spleen (A) and lung (B) at 22 h post infection with Influenza virus A/duck/Laos/XBY004/2014 (H5N6). Bars represent the mean fold change of mRNA expression with SEM compared to the same tissue of the uninfected birds, normalized against the housekeeping gene GAPDH (* p value < 0.05 using the Students *t* test with Mann Whitney U test). The Mx1 data is a duplicate of that already shown in 6.8A/B and serves as a comparison.

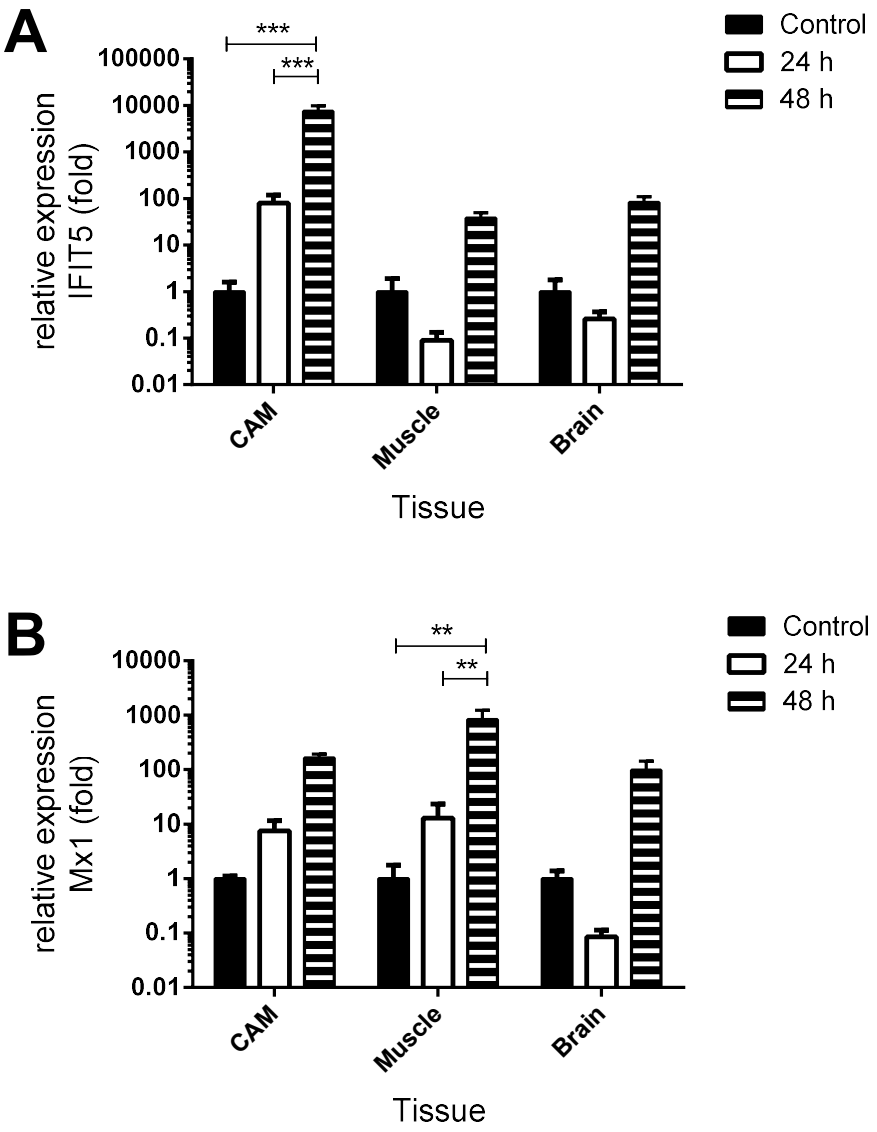

Figure 8-8 Expression of IFIT5 and Mx1 in response to viral infections *in ovo*

Expression of IFIT5 (A) and Mx1 (B) mRNA in D10 embryonated chicken eggs 24 h and 48 h post infection with Influenza virus A/Puerto Rico/8/1934 (H1N1). Bars represent the mean fold change of mRNA expression with SEM compared to the same tissue of uninfected eggs, normalized against the housekeeping gene GAPDH (* p value < 0.05, ** p value < 0.005, using a one-way ANOVA test with uncorrected Fischer LSD post-test).

8.3 Discussion

The IFIT family of proteins is found across diverse species [211,299]. However, the mode of action of these proteins is not well understood. In avian species, even less is known, with only limited recent transcriptome studies characterizing their expression profiles [231,300,301]. The identification and characterization of chicken IFIT5 allows for further in-depth investigation of its role in avian immunity, including in response to virus. Bioinformatic analysis was used to identify the genes and determine its location and structure. The chicken IFIT5 gene was located on chromosome 6, flanked by the CH25H and LIPA genes upstream and SLC16A and PANK1 downstream, which revealed conserved synteny with human and other species [211]. It also consisted of 2 exons and 1 intron like in other species, which strongly suggests common derivation. The chicken IFIT5 was shown by RT-PCR to be 1440 bp in length, encoding a protein of 470 amino acids, which is consistent with mammalian and other avian IFIT proteins investigated. Ten SNPs were identified (Figure 11-2, Appendix) of which five were silent and five were missense mutations (Figure 11-3, Appendix). SNPs in the IFIT5 gene have been described in humans and other species [211] and could be explained by genetic diversity, in this case variations between individual birds, which may be even greater between chicken breeds [302].

Phylogenetic analysis showed that the bird sequences clustered together but were related to mammalian IFITs. These findings followed the general trend for IFIT genes to cluster with their own class rather than paralogues in other classes, which makes definitive identification of orthology difficult based on sequence alone. Further analysis of the chicken IFIT5 protein sequence indicated it was closely related to other bird IFITs and amongst the human IFIT family was most similar to human IFIT5 with 0.89 aa substitutions per site. Protein sequence alignment and domain prediction analysis revealed conservation of the Tetratricopeptide domains that are the hallmarks of IFITs compared to other bird and human IFITs [211,299].

The crystal structure of the human IFIT5 protein has been solved and aa critical to the function of IFIT5 identified by non-conserved site-directed mutagenesis [228]. Of the 16 critical aa identified, 13 were conserved in the chicken sequence. Amongst the three non-conserved critical aa only one was associated with a decrease in binding affinity to nucleic acids in the human protein, the other two only showed a decrease in tandem with another mutation site. Importantly, the sites that determined substrate specificity to ssRNA or dsDNA (F284 and R294 in humans) were found to be conserved in the chicken sequence, suggesting that IFIT5 can bind to both substrates, although possibly with reduced binding affinity.

The robust induction of IFIT genes in response to immune stimulation and concomitant antiviral function is well established in mammalian systems [303]. A similar strong induction of IFIT5 was observed following stimulation of chicken splenocytes with the TLR agonist poly (I:C). Upregulation of mRNA expression started at 3 h and peaked at 6 h post stimulation, which is consistent with observations made in human cells [304], and with similar kinetics to the Mx1 gene, one of the best characterized ISGs in chicken [220]. The TLR4 ligand, bacteria-derived LPS, also induced significant increase for IFIT5 and Mx1 mRNA after 6 h treatment. The induction of IFIT5 started at 3 h and was still significant at 24 h and elevated at 48 h consistent with previously reports observing in human macrophages and neutrophils [278,279]. Whether this effect was due to direct transcriptional stimulation or indirectly via the IFN pathway is unclear at this point although it has been proposed that IFITs can be induced directly via PRRs [303]. Treatment of splenocytes with IFNα led to a significant upregulation of IFIT5 after 1.5 h with a peak at 3 h that remained high until 24 h, whereas Mx1 was more delayed and reached significant upregulation at 3 h peaking at 6 h post stimulation followed by a decline. IFNλ treatment led to a much slower increase, peaking at 48 h post treatment. Although different in kinetics, the ability of both type I and type III IFNs to induce IFITs clearly demonstrated the interferon stimulated nature of the chicken IFIT5 gene.

During viral infection, IFITs bind to viral RNA and host factors needed for viral replication and thereby limit viral growth [211,303]. In chickens, some of the most devastating infections are caused by highly pathogenic avian influenza A viruses of the H5 and H7 subtypes. To determine if the identified IFIT homolog was potentially involved in the host response to this agent lung and spleen samples were analyzed from birds infected with HPAI virus (H5N6). These tissues were selected as representatives of the primary site of infection (lung) and the subsequent immune response (spleen). A significant upregulation of IFIT5 mRNA was observed in both the lung and spleen of infected birds compared to uninfected controls. Little is known about the antiviral defense in chicken eggs despite their wide use as virus substrate and so this was investigated.

Both HPAI viruses A/duck/Laos/XBY004/2014 (H5N6) and A/Vietnam/1194/2004 (H5N1) resulted in the death of the embryo within 24 h of infection, this made sampling of the tissues impossible and a low pathogenic strain was chosen to give more consistent results. The H1N1 infection of eggs led to the induction of IFIT5 in the CAM after 24 and 48 h while significant induction of Mx1 was observed in the muscle. This confirms that IFIT5 expression is highly upregulated during active infection in chicken and might have a critical role.

9 General Discussion

HPAI represents a serious problem, that impacts us on multiple levels. These viruses can lead to severe economic losses, potential bottlenecks in vaccine production and food shortages. Some of the H5 and H7 strains can also infect humans through bird-human transmission and cause high mortality rates with the potential for pandemics [28,30].

Vaccination is the most efficient way to prevent viral spread through populations, although the genetic instability of Influenza viruses makes this a continuous effort with new vaccines annually based on circulating virus strains [305-307]. There vaccines provide a good level of protection, but they are not always efficient against emerging viruses [242]. Vaccination chicken populations with seasonal as well as emerging HPAI strains has so far proven successful in keeping the burden of disease down but is not enough to prevent further spill over [308]. Moreover, the key reservoir, the wild aquatic bird population, cannot be vaccinated [308].

Another strategy to counter HPAIs are antiviral therapies which have been successfully deployed in acute cases of human Influenza infection. However, despite multimillion dollar compound screening enterprises [309-312]. The treatment of birds remains problematic and would no doubt lead to resistance of these viruses and negate any beneficial effect for humans in the long run as seen with Relenza in recent years [313-315].

An alternative strategy to combat HPAI viruses and so protect the human population and the food and vaccine supply, is to focus on disease resistance in the transmission host. In most human HPAI infection the source of infections can be traced back to chickens, which have severe reactions and, in case of some HPAI strains, die within 24-48 h [316]. The timeframe suggests that these birds are not able to contain the viruses in the first stages of infection, indicating inappropriate innate immune response. In order to develop strategies making chickens resistant to these HPAI strains, it is necessary to understand chicken innate immunity in order to identify in which part of the immune response fails to respond in an appropriate manner.

The first line of defence against viruses are the IFNs, which were incidentally discovered in chicken eggs 60 years ago [60]. Greater understanding of these important mediators of innate (and adaptive) immunity could lead to insight into the issues around the high mortality rates and severe disease outcomes in birds. Although all IFN types contribute to the antiviral immune response only type I and type III are directly antiviral [59]. Due to the very short

window between infection, diseases onset and death the focus was on IFNα, the oldest and best characterized antiviral IFN [60], and IFNλ [119,120] , the newest member of the IFN family. IFNαs has been well characterized in all model species as well as the chicken, while information on IFNλ in mammals is rapidly emerging. Nonetheless it was of considerable importance to study chicken IFNλ function in more detail as mice and human data are not directly applicable due to their multiple variants, IFNλ1,2,3 and 4 showing distinctive functions as reported by a myriad of studies. Which of these are shared by chicken IFNλ remains unclear, although sequence comparison suggests IFNλ2 [201] is the most highly related. This study has identified similarities between IFNα and IFNλ but also distinct differences, which highlights important non-redundant roles of IFNλ in the antiviral immune response in chickens.

Host receptors such as TLRs [44], RLRs [317] and NLRs [318] detect the presence of pathogens to trigger the interferon-mediated immune response. Compared to their mammalian TLR counterparts, chicken TLR8 is non-functional and TLR9 and RIG-1 are absent. However, TLR3, the most important receptor in dsRNA detection, appears to be present and functional [261,296,297]. Furthermore, there is evidence to suggest that chicken MDA5 is also a functional analogue of mammalian RIG-1 [255]. Chicken cells were found to respond to the foreign RNA mimic PIC with significant IFNα and IFNλ upregulation even at low doses. IFNλ showed a greater fold induction, but this is probably best explained as a consequence of the relatively high baseline expression of IFNα, which makes the fold change comparably low. In fact, the high baseline IFNα expression has been linked to eliciting an efficient response to viral triggers [319]. PIC-dependent IFNα induction was more consistent whereas IFNλ induction was highly variable. Despite this, the expression of both genes followed the same kinetics, peaking after 3-6 h post stimulation with PICs, which is comparable to mammalian systems [257,271]. LPS, on the other hand, did not upregulate IFNα or IFNλ at any time point. Human studies suggest that only IFNλ1 can respond to LPS stimulation but not IFNλ2 or IFNλ3. Collectively, this data suggests a common PRR mediated induction pathway with these IFNs. In contrast, IFN mediated induction of the IFNs was markedly different. IFNα induced IFNα expression shortly after stimulation, but not IFNλ. IFNα also induced peak ISG expression after 6 h, although whether that is a direct result of PIC stimulation or indirect through the IFNs produced is unknown. In contrast, the IFNλ mediated ISG induction took place at 48 h and so likely indirect, which was also observed in DF1 cells (data not shown) and reportet in human cells [320]. The altered ISG expression kinetics is suggestive of a non-redundant role in immune regulation.

The high level of variance observed in experiments examining IFN and ISG expression levels led to the investigation of possible causes. Differences between the male and female mammalian immune systems have been a field of discussion in scientific literature [289,290,321-324]. This is of particular relevance in chickens since the type I IFNs are located on the sex chromosome (Z). Moreover, in contrast to sex determination in mammals, male chickens are determined but two copies of this chromosome (ZZ) and females by one (ZW). This means that males have additional copies of these genes. Despite this, PIC stimulation of purified splenocytes resulted in a significantly higher expression of type I IFNs in females compared to age-matched males. IFNλ expression, on the other hand, was significantly higher in males than females. In mature chickens both type I and type III IFNs showed higher expression levels in female, although only IFNλ reached significance. Higher expression of IFNα in female DCs has previously been reported and was linked to the relative expression levels of IRF5 [324]. The influence of estrogen was investigated as a possible explanation but no direct correlation between estrogen levels and IFN expression was observed in individual birds although, the estradiol levels were, as expected, significantly higher females than in males. These data do, however, confirm that female and male birds differ significantly in the magnitude of antiviral response, which provides one explanation for the fluctuations in IFN and ISG induction observed. More research into whether gender differences and other potential sources of variance between birds observed are warranted. This could have important implications for the chicken industry as layers are naturally female and broilers universally male. Understanding the differences in immune response could lead to alternative strategies to prevent or treat infection between those populations.

Receptor expression represents one major control point in tailoring IFN signaling to specific cells [325] as well as the magnitude of the signal relayed [118]. A chicken IFNλR1 candidate was characterized and found to cluster with other birds and reptiles, while mammalian sequences clustered together and those from amphibians and piscine species clustered further away. A similar result was found for IL-10R2. This suggests a functional IFNλR complex was present before avian/mammalian divergence, and has been maintained in both lineages, indicative of important conserved functions in both lineages.

The tissue distribution of IFNλR complex components was investigated, with a focus on immune relevant tissues. In mammals it is primarily found in epithelial cells but recently evidence has emerged that IFNλ acts on endothelial cells of the blood-brain barrier [180]. Chickens are anatomically very different from mammals with regard to immunity. Chickens lack lymph nodes and so the spleen is an especially important organ involved in pathogen

defence. The thymus is the main organ for T(hymus) [326] cell development while the Bursa of Fabricius hold the B(ursa) [327] cell compartment. All tissues investigated expressed the IL-10R2 chain in relatively high levels, with the ratios of IFNλR1 and IL-10R2 always heavily skewed in favor of IL10R2, which suggests that IFNλR1 levels are the limiting factor. However, recently the potentially variable expression of GAPDH across the tissues has been documented could lead to false conclusions [328]. The highest expression of the IFNλR1 chain was observed in PBMCs followed by the spleen, bursa, thymus and the bone marrow, while brain tissue had the lowest expression. Purified chicken splenocytes showed higher IFNλR1 expression relative to the whole tissue. This data suggests that immune reactive tissues tend to express IFNλR1, while immune privileged tissues like the brain do not. An IFNλ-mediated effect on immune cells is therefore highly likely.

To provide evidence that the putative IFNλR1 and IL-10R2 constitute a functional chicken IFNλR complex, the expression of each chain was knocked down using siRNA. This demonstrated that both chains were needed for IFNλ-mediated STAT activation and ISG upregulation. Treatment with Ruxolitinib, a selective JAK1 inhibitor, also blocked IFNλ mediated responses. This collectively demonstrates an IFNλR1/IL-10R2-JAK-STAT-ISG pathway in chickens. Confirming the functionality of this central signaling cascade also opens up possibilities to repurpose drugs for pro-viral treatment. Ruxolitinib, which has FDA approval for used in myelofibrosis [329] treatment in humans, could be used to inhibit interferon signaling and enhance viral amplification for increasing yield for vaccine production [330,331] and sensitivity for diagnostic applications [249].

The use of embryonated eggs for amplification of viruses and the virus-host interaction has been characterized extensively in the past 60 years [332-346] but little to no work on the characterization of the egg immune system has been done since the discovery of IFNs in chicken egg [347]. To gain insight into the developing immune response and potential factors inhibiting or failing to inhibit viral growth, chicken eggs were infected with Influenza A according to the same protocols used by the vaccine production industry and diagnostic laboratories[348]. The results suggest that IFNα is inhibited by the virus, which can be explained by the agonistic effect of the NS1 protein in H1N1 that is well documented [349]. However, though IFN expression is repressed or delayed, ISGs are expressed at high levels in a tissue dependent fashion. The inoculation of the virus into the allantoic fluid [348] brings the virus into close contact with the inner layer the Chorio-allantoic membrane and the outer layer of embryonic tissue. By 24 h the virus replicates to very high levels in the CAM and by 48 h the virus has spread to every tissue. This data shows that even in the absence of IFNα

the ISGs respond by 24 h to viral invasion, but this is not sufficient to control viral replication as by 48 h the virus has spread to every tissue of the embryo including the brain. This work gives insights into the localisation of viral replication as well as the mounting innate immune response, but more work needs to be done to dissect the various virus sensing and immune response inducing pathways in the developing chicken embryo. The data collected here suggests that the IFN system is well developed although unable to deal with a substantial viral infection in an appropriate manner.

Limited knowledge around the effectors of the IFN-mediated antiviral response, the ISGs, hinders he progress in avian immunology. Compared to mammals the avian ISGs are very poorly characterized, with few genes analyzed. The first human ISG cloned was the interferon-induced proteins with Tetratricopeptide repeats (IFITs) 1 [350]. Considerable research has been performed on IFIT family members identifying a key role in anti-viral host defense by inhibiting virus replication through binding and regulating the functions of cellular and viral proteins and RNAs [351]. The IFIT family consists of four members in human and three in mouse. All identified IFITs have multiple copies of Tetratricopeptide repeats (TRPs), their distinct tertiary structures enable them to bind different partners and affect host-virus interactions differently. Human IFIT1 and IFIT2 are thought interfere with the viral RNA translation by binding to the EIF3 [352]. Viral pppRNA is also recognized by IFIT1 which leads to sequestering by an IFIT1, IFIT2 and IFIT3 complex, although only IFIT1 is directly able to interact with ppp RNA. IFIT5 has been shown to also interact with ppp RNA but not with IFIT2 or IFIT3, indicating a non-redundant role in antiviral defense [227,353].

A chicken IFIT homolog has been mentioned in several publications [231,284,300,301], but no in depth characterization of the gene has been performed. A single potential chicken IFIT gene candidate was identified, amplified by RT-PCR and sequenced. The encoded chicken IFIT protein showed greatest homology to the human IFIT5 and was thus named chicken IFIT5. The chicken IFIT5 was most closely related to other bird IFITs and possessed nine TRP domains are distributed over its length similar to other bird and human IFITs. The crystal structure of the human IFIT5 was recently solved with extensive mutation studies identifying residues critical for function [228]. The chicken IFIT5 sequence showed conservation of 12 out of 16 residues identified as important for substrate binding specificity. Collectively, this points towards a functionally conserved IFIT gene in chickens.

The expression of chicken IFIT5 was analyzed in response to different immune stimuli, including infection in vivo and in ovo. IFIT5 was highly induced in chicken cells after

treatment with PIC and LPS, while PIC triggered a significant increase in expression after 3 h with relatively stable expression up until 48 h. In contrast, LPS stimulation induced a peak expression after 6 h followed by a rapid decline. The IFIT5 gene was also induced in response to chIFNs like IFNα and IFNλ with similar kinetics to other ISGs observed, although induction by IFNα reached significant levels after 1.5 h and stayed significantly induced until 24 h. Following viral infection IFIT5 was highly induced in the lung and spleen of chickens infected with H5N6 HPAI and in the chorio-allantoic membrane and the muscle of infected embryonated eggs. This collectively suggests that chicken IFIT5 represents a bone fide ISG, with applicability to studies on chicken innate immune system and contribute to the understanding of HPAI pathogenesis in chickens.

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11 Appendix

Table 2 Accession Numbers of interferon sequences

Accession Number	Sign	Species	type		
NP990758	a	chicken	interferon	alpha	
AAX83679.1	b	chicken	interferon	beta	
AAU10091.1	g	chicken	interferon	gamma	
ABU82742	l	chicken	interferon	lambda	
ADU04501.1	o	chicken	interferon	omega	
AAC41702	b	human	interferon	beta	
AAI00873	e	human	interferon	epsilon	
CAA41626	o	human	interferon	omega	
CAA44325	g	human	interferon	gamma	
CAA72532	a2	human	interferon	alpha	2
EAW56869	l3	human	interferon	lambda	3
EAW56870	l2	human	interferon	lambda	2
EAW56871	l1	human	interferon	lambda	1
EAW58609	a1	human	interferon	alpha	1
EAW58610	a8	human	interferon	alpha	8
EAW58612	a13	human	interferon	alpha	13
EAW58613	a6	human	interferon	alpha	6
EAW58615	a5	human	interferon	alpha	5
EAW58616	a14	human	interferon	alpha	14
EAW58617	a17	human	interferon	alpha	17
EAW58618	a16	human	interferon	alpha	16
EAW58619	a10	human	interferon	alpha	10
EAW58620	a7	human	interferon	alpha	7
EAW58621	a4	human	interferon	alpha	4
EAW58623	a21	human	interferon	alpha	21
EAW58563.1	k	human	interferon	kappa	
NP482513660	l4	human	interferon	lambda	4
AAA37888	a7	mouse	interferon	alpha	7
AAA37891	b	mouse	interferon	beta	
AAH99376	k	mouse	interferon	kappa	
AAI04353	a12	mouse	interferon	alpha	12
AAI04378	e	mouse	interferon	epsilon	
AAI16873	a11	mouse	interferon	alpha	11
AAI19352	a4	mouse	interferon	alpha	4
AAI20725	a13	mouse	interferon	alpha	13
AAI20912	a5	mouse	interferon	alpha	5
AAI25322	a14	mouse	interferon	alpha	14
AAX58714	l2	mouse	interferon	lambda	
AAX58715	l3	mouse	interferon	lambda	

ACR22510	g	mouse	interferon	gamma	
EDL30963	z	mouse	interferon	zeta	

Table 3 Accession numbers of IFNλR1 sequences

Accession Number		Species	type			
XP011227661.1	Predicted	Ailuropoda melanoleuca	Interferon	lambda	receptor	1
KYO25772.1		Alligator mississippiensis	Interferon	lambda	receptor	1
XP006019447.1	Predicted	Alligator sinensis	Interferon	lambda	receptor	1
AHY86485.1		Anolis carolinensis	Interferon	lambda	receptor	1
AJD08473.1		Anser cygnoides	Interferon	lambda	receptor	1
XP012295126.1	Predicted	Aotus nancymaae	Interferon	lambda	receptor	1
XP009866512.1	Predicted	Apaloderma vittatum	Interferon	lambda	receptor	1
XP009274512.1	Predicted	Aptenodytes forsteri	Interferon	lambda	receptor	1
XP011582322.1	Predicted	Aquila chrysaetos canadensis	Interferon	lambda	receptor	1
XP007175194.1	Predicted	Balaenoptera acutorostrata scammoni	Interferon	lambda	receptor	1
XP010299251.1	Predicted	Balearica regulorum gibbericeps	Interferon	lambda	receptor	1
XP010828357.1	Predicted	Bison bison bison	Interferon	lambda	receptor	1
XP010132822.1	Predicted	Buceros rhinoceros silvestris	Interferon	lambda	receptor	1
XP014804126.1	Predicted	Calidris pugnax	Interferon	lambda	receptor	1
XP008998890.1	Predicted	Callithrix jacchus	Interferon	lambda	receptor	1
XP010955647.1	Predicted	Camelus bactrianus	Interferon	lambda	receptor	1
XP010988103.1	Predicted	Camelus dromedarius	Interferon	lambda	receptor	1
XP014423818.1	Predicted	Camelus ferus	Interferon	lambda	receptor	1
XP013829748.1	Predicted	Capra hircus	Interferon	lambda	receptor	1
XP010170252.1	Predicted	Caprimulgus carolinensis	Interferon	lambda	receptor	1
XP009707716.1	Predicted	Cariama cristata	Interferon	lambda	receptor	1
XP013009632.1	Predicted	Cavia porcellus	Interferon	lambda	receptor	1
XP004425789.1	Predicted	Ceratotherium simum simum	Interferon	lambda	receptor	1
XP009998894.1	Predicted	Chaetura pelagica	Interferon	lambda	receptor	1
XP009880511.1	Predicted	Charadrius vociferus	Interferon	lambda	receptor	1
XP007068110.1	Predicted	Chelonia mydas	Interferon	lambda	receptor	1
XP010128944.1	Predicted	Chlamydotis macqueenii	Interferon	lambda	receptor	1
XP005302269.1	Predicted	Chrysemys picta bellii	Interferon	lambda	receptor	1
XP010199749.1	Predicted	Colius striatus	Interferon	lambda	receptor	1

XP005513512.1	Predicted	Columba livia	Interferon	lambda	receptor	1
XP004679439.1	Predicted	Condylura cristata	Interferon	lambda	receptor	1
XP008633390.1	Predicted	Corvus brachyrhynchos	Interferon	lambda	receptor	1
XP010406501.1	Predicted	Corvus cornix cornix	Interferon	lambda	receptor	1
XP007617245.1	Predicted	Cricetulus griseus	Interferon	lambda	receptor	1
XP007643617.1	Predicted	Cricetulus griseus	Interferon	lambda	receptor	1
XP009560095.1	Predicted	Cuculus canorus	Interferon	lambda	receptor	1
XP004465448.1	Predicted	Dasypus novemcinctus	Interferon	lambda	receptor	1
XP012887869.1	Predicted	Dipodomys ordii	Interferon	lambda	receptor	1
XP004705411.1	Predicted	Echinops telfairi	Interferon	lambda	receptor	1
XP009646796.1	Predicted	Egretta garzetta	Interferon	lambda	receptor	1
XP006883717.1	Predicted	Elephantulus edwardii	Interferon	lambda	receptor	1
XP008146305.1	Predicted	Eptesicus fuscus	Interferon	lambda	receptor	1
XP007522647.1	Predicted	Erinaceus europaeus	Interferon	lambda	receptor	1
XP010904278.1	Predicted	Esox lucius	Interferon	lambda	receptor	1
XP010154970.1	Predicted	Eurypyga helias	Interferon	lambda	receptor	1
XP005433108.1	Predicted	Falco cherrug	Interferon	lambda	receptor	1
XP005234192.1	Predicted	Falco peregrinus	Interferon	lambda	receptor	1
XP003989777.1	Predicted	Felis catus	Interferon	lambda	receptor	1
XP016152229.1	Predicted	Ficedula albicollis	Interferon	lambda	receptor	1
XP005058494.1	Predicted	Ficedula albicollis	Interferon	lambda	receptor	1
XP010624247.1	Predicted	Fukomys damarensis	Interferon	lambda	receptor	1
XP009578178.1	Predicted	Fulmarus glacialis	Interferon	lambda	receptor	1
AHF20241.1		Gallus gallus	Interferon	lambda	receptor	1
XP009805197.1	Predicted	Gavia stellata	Interferon	lambda	receptor	1
XP015261062.1	Predicted	Gekko japonicus	Interferon	lambda	receptor	1
XP005427215.2	Predicted	Geospiza fortis	Interferon	lambda	receptor	1
XP009917474.1	Predicted	Haliaeetus albicilla	Interferon	lambda	receptor	1
XP010574400.1	Predicted	Haliaeetus leucocephalus	Interferon	lambda	receptor	1
XP012929689.1	Predicted	Heterocephalus glaber	Interferon	lambda	receptor	1
AAI40873.1		Homo sapiens	Interferon	lambda	receptor	1
XP005318127.1	Predicted	Ictidomys tridecemlineatus	Interferon	lambda	receptor	1
XP012803978.1	Predicted	Jaculus jaculus	Interferon	lambda	receptor	1
XP009951551.1	Predicted	Leptosomus discolor	Interferon	lambda	receptor	1

XP010593387.1	Predicted	Loxodonta africana	Interferon	lambda	receptor	1
XP014986729.1	Predicted	Macaca mulatta	Interferon	lambda	receptor	1
XP011761158.1	Predicted	Macaca nemestrina	Interferon	lambda	receptor	1
XP008918805.1	Predicted	Manacus vitellinus	Interferon	lambda	receptor	1
XP011833300.1	Predicted	Mandrillus leucophaeus	Interferon	lambda	receptor	1
XP010721813.1	Predicted	Meleagris gallopavo	Interferon	lambda	receptor	1
XP012986518.1	Predicted	Melopsittacus undulatus	Interferon	lambda	receptor	1
XP008933896.1	Predicted	Merops nubicus	Interferon	lambda	receptor	1
XP012975582.1	Predicted	Mesocricetus auratus	Interferon	lambda	receptor	1
XP012605910.1	Predicted	Microcebus murinus	Interferon	lambda	receptor	1
XP005353444.1	Predicted	Microtus ochrogaster	Interferon	lambda	receptor	1
XP001365104.2	Predicted	Monodelphis domestica	Interferon	lambda	receptor	1
XP005875259.2	Predicted	Myotis brandtii	Interferon	lambda	receptor	1
XP015412682.1	Predicted	Myotis davidii	Interferon	lambda	receptor	1
XP010021868.1	Predicted	Nestor notabilis	Interferon	lambda	receptor	1
XP009469068.1	Predicted	Nipponia nippon	Interferon	lambda	receptor	1
XP004592432.1	Predicted	Ochotona princeps	Interferon	lambda	receptor	1
XP004637786.1	Predicted	Octodon degus	Interferon	lambda	receptor	1
XP012415878.1	Predicted	Odobenus rosmarus divergens	Interferon	lambda	receptor	1
XP002716066.1	Predicted	Oryctolagus cuniculus	Interferon	lambda	receptor	1
XP015393611.1	Predicted	Panthera tigris altaica	Interferon	lambda	receptor	1
XP005954133.1	Predicted	Pantholops hodgsonii	Interferon	lambda	receptor	1
XP003891375.2	Predicted	Papio anubis	Interferon	lambda	receptor	1
XP015504532.1	Predicted	Parus major	Interferon	lambda	receptor	1
XP009486303.1	Predicted	Pelecanus crispus	Interferon	lambda	receptor	1
XP010281772.1	Predicted	Phaethon lepturus	Interferon	lambda	receptor	1
XP009513439.1	Predicted	Phalacrocorax carbo	Interferon	lambda	receptor	1
XP007121168.1	Predicted	Physeter catodon	Interferon	lambda	receptor	1
XP009906845.1	Predicted	Picoides pubescens	Interferon	lambda	receptor	1
XP014113903.1	Predicted	Pseudopodoces humilis	Interferon	lambda	receptor	1
XP010080597.1	Predicted	Pterocles gutturalis	Interferon	lambda	receptor	1
AEQ38017.1		Pteropus alecto	Interferon	lambda	receptor	1
XP009330413.1	Predicted	Pygoscelis adeliae	Interferon	lambda	receptor	1
XP012400962.1	Predicted	Sarcophilus harrisii	Interferon	lambda	receptor	1

XP009096915.1	Predicted	Serinus canaria	Interferon	lambda	receptor	1
XP016309571.1	Predicted	Sinocyclocheilus anshuiensis	Interferon	lambda	receptor	1
XP012787040.1	Predicted	Sorex araneus	Interferon	lambda	receptor	1
XP009666548.1	Predicted	Struthio camelus australis	Interferon	lambda	receptor	1
XP014741053.1	Predicted	Sturnus vulgaris	Interferon	lambda	receptor	1
XP004174443.1	Predicted	Taeniopygia guttata	Interferon	lambda	receptor	1
XP008060367.1	Predicted	Tarsius syrichta	Interferon	lambda	receptor	1
XP009987356.1	Predicted	Tauraco erythrolophus	Interferon	lambda	receptor	1
XP010223088.1	Predicted	Tinamus guttatus	Interferon	lambda	receptor	1
XP014447446.1	Predicted	Tupaia chinensis	Interferon	lambda	receptor	1
XP009975065.1	Predicted	Tyto alba	Interferon	lambda	receptor	1
XP015093173.1	Predicted	Vicugna pacos	Interferon	lambda	receptor	1
ACV32138.1	transcript variant 1	Xenopus tropicalis	Interferon	lambda	receptor	1
ACV32139.1	transcript variant 2	Xenopus tropicalis	Interferon	lambda	receptor	1
ACV32140.1	transcript variant 3	Xenopus tropicalis	Interferon	lambda	receptor	1
XP005494786.2	Predicted	Zonotrichia albicollis	Interferon	lambda	receptor	1
NP001184131.1	Precurser	Danio rerio	Interferon	lambda	receptor	1

Table 4 Accession Numbers of IFNAR1 sequences

Accession Number		Species	type			
XP014918068.1	Predicted	Acinonyx jubatus	Interferon	alpha/beta	receptor	alpha
XP002919632.2	Predicted	Ailuropoda melanoleuca	Interferon	alpha/beta	receptor	alpha
KYO22780.1		Alligator mississippiensis	Interferon	alpha/beta	receptor	alpha
XP008120201.1	Predicted	Anolis carolinensis	Interferon	alpha/beta	receptor	alpha
AJD38996.1		Anser cygnoides	Interferon	alpha/beta	receptor	alpha
XP012302648.1	Predicted	Aotus nancymaae	Interferon	alpha/beta	receptor	alpha
XP009868282.1	Predicted	Apaloderma vittatum	Interferon	alpha/beta	receptor	alpha
XP009281347.1	Predicted	Aptenodytes forsteri	Interferon	alpha/beta	receptor	alpha
XP013810825.1	Predicted	Apteryx australis mantelli	Interferon	alpha/beta	receptor	alpha
XP010860591.1	Predicted	Bison bison bison	Interferon	alpha/beta	receptor	alpha
XP005893409.1	Predicted	Bos mutus	Interferon	alpha/beta	receptor	alpha
XP006048305.1	Predicted	Bubalus bubalis	Interferon	alpha/beta	receptor	alpha
XP010143146.1	Predicted	Buceros rhinoceros silvestris	Interferon	alpha/beta	receptor	alpha
XP014793775.1	Predicted	Calidris pugnax	Interferon	alpha/beta	receptor	alpha
XP002761441.1	Predicted	Callithrix jacchus	Interferon	alpha/beta	receptor	alpha
XP008494921.1	Predicted	Calypte anna	Interferon	alpha/beta	receptor	alpha
XP010954868.1	Predicted	Camelus bactrianus	Interferon	alpha/beta	receptor	alpha
XP013819137.1	Predicted	Capra hircus	Interferon	alpha/beta	receptor	alpha

XP010166276.1	Predicted	Caprimulgus carolinensis	Interferon	alpha/beta	receptor	alpha
XP008065125.1	Predicted	Carlito syrichta	Interferon	alpha/beta	receptor	alpha
XP010002987.1	Predicted	Chaetura pelagica	Interferon	alpha/beta	receptor	alpha
XP009893221.1	Predicted	Charadrius vociferus	Interferon	alpha/beta	receptor	alpha
XP006862663.1	Predicted	Chrysochloris asiatica	Interferon	alpha/beta	receptor	alpha
XP011815546.1	Predicted	Colobus angolensis palliatus	Interferon	alpha/beta	receptor	alpha
XP013225464.1	Predicted	Columba livia	Interferon	alpha/beta	receptor	alpha
XP008638917.1	Predicted	Corvus brachyrhynchos	Interferon	alpha/beta	receptor	alpha
XP007619344.2	Predicted	Cricetulus griseus	Interferon	alpha/beta	receptor	alpha
XP009565463.1	Predicted	Cuculus canorus	Interferon	alpha/beta	receptor	alpha
XP012373471.1	Predicted	Dasypus novemcinctus	Interferon	alpha/beta	receptor	alpha
XP012882888.1	Predicted	Dipodomys ordii	Interferon	alpha/beta	receptor	alpha
XP004711386.1	Predicted	Echinops telfairi	Interferon	alpha/beta	receptor	alpha
XP009639194.1	Predicted	Egretta garzetta	Interferon	alpha/beta	receptor	alpha
XP006886853.1	Predicted	Elephantulus edwardii	Interferon	alpha/beta	receptor	alpha
XP008144026.1	Predicted	Eptesicus fuscus	Interferon	alpha/beta	receptor	alpha
XP014699230.1	Predicted	Equus asinus	Interferon	alpha/beta	receptor	alpha
XP001494689.3	Predicted	Equus caballus	Interferon	alpha/beta	receptor	alpha
XP008515476.1	Predicted	Equus przewalskii	Interferon	alpha/beta	receptor	alpha
XP007519772.1	Predicted	Erinaceus europaeus	Interferon	alpha/beta	receptor	alpha
XP010157283.1	Predicted	Eurypyga helias	Interferon	alpha/beta	receptor	alpha
XP005438665.1	Predicted	Falco cherrug	Interferon	alpha/beta	receptor	alpha
XP005234444.1	Predicted	Falco peregrinus	Interferon	alpha/beta	receptor	alpha
XP011284053.1	Predicted	Felis catus	Interferon	alpha/beta	receptor	alpha
XP010607017.1	Predicted	Fukomys damarensis	Interferon	alpha/beta	receptor	alpha
AAD13669.1		Gallus gallus	Interferon	alpha/beta	receptor	alpha
XP014165295.1	Predicted	Geospiza fortis	Interferon	alpha/beta	receptor	alpha
XP004062764.1	Predicted	Gorilla gorilla gorilla	Interferon	alpha/beta	receptor	alpha
XP010583626.1	Predicted	Haliaeetus leucocephalus	Interferon	alpha/beta	receptor	alpha
XP004842365.1	Predicted	Heterocephalus glaber	Interferon	alpha/beta	receptor	alpha
AAT49100.1		Homo sapiens	Interferon	alpha/beta	receptor	alpha
XP012803362.1	Predicted	Jaculus jaculus	Interferon	alpha/beta	receptor	alpha
XP006012924.1	Predicted	Latimeria chalumnae	Interferon	alpha/beta	receptor	alpha
XP006749269.1	Predicted	Leptonychotes weddellii	Interferon	alpha/beta	receptor	alpha
XP009949621.1	Predicted	Leptosomus discolor	Interferon	alpha/beta	receptor	alpha
XP007447468.1	Predicted	Lipotes vexillifer	Interferon	alpha/beta	receptor	alpha
XP011724842.1	Predicted	Macaca nemestrina	Interferon	alpha/beta	receptor	alpha
XP008924472.1	Predicted	Manacus vitellinus	Interferon	alpha/beta	receptor	alpha
XP011854503.1	Predicted	Mandrillus leucophaeus	Interferon	alpha/beta	receptor	alpha
XP015334189.1	Predicted	Marmota marmota marmota	Interferon	alpha/beta	receptor	alpha
XP010721604.1	Predicted	Meleagris gallopavo	Interferon	alpha/beta	receptor	alpha
XP012971606.1	Predicted	Mesocricetus auratus	Interferon	alpha/beta	receptor	alpha
XP012602506.1	Predicted	Microcebus murinus	Interferon	alpha/beta	receptor	alpha
XP016069000.1	Predicted	Miniopterus natalensis	Interferon	alpha/beta	receptor	alpha

AAH52217.1		Mus musculus	Interferon	alpha/beta	receptor	alpha
XP004757568.1	Predicted	Mustela putorius furo	Interferon	alpha/beta	receptor	alpha
XP014390685.1	Predicted	Myotis brandtii	Interferon	alpha/beta	receptor	alpha
XP015415723.1	Predicted	Myotis davidii	Interferon	alpha/beta	receptor	alpha
XP010017322.1	Predicted	Nestor notabilis	Interferon	alpha/beta	receptor	alpha
XP009471830.1	Predicted	Nipponia nippon	Interferon	alpha/beta	receptor	alpha
XP012373317.1	Predicted	Octodon degus	Interferon	alpha/beta	receptor	alpha
XP004406365.1	Predicted	Odobenus rosmarus divergens	Interferon	alpha/beta	receptor	alpha
ETE57640.1		Ophiophagus hannah	Interferon	alpha/beta	receptor	alpha
XP009931683.1	Predicted	Opisthocomus hoazin	Interferon	alpha/beta	receptor	alpha
XP004264572.1	Predicted	Orcinus orca	Interferon	alpha/beta	receptor	alpha
XP007667937.1	Predicted	Ornithorhynchus anatinus	Interferon	alpha/beta	receptor	alpha
XP007939497.1	Predicted	Orycteropus afer afer	Interferon	alpha/beta	receptor	alpha
XP007090486.1	Predicted	Panthera tigris altaica	Interferon	alpha/beta	receptor	alpha
NP001162254.1		Papio anubis	Interferon	alpha/beta	receptor	alpha
XP006126098.1	Predicted	Pelodiscus sinensis	Interferon	alpha/beta	receptor	alpha
XP007115989.1	Predicted	Physeter catodon	Interferon	alpha/beta	receptor	alpha
XP009905697.1	Predicted	Picoides pubescens	Interferon	alpha/beta	receptor	alpha
XP002830697.2	Predicted	Pongo abelii	Interferon	alpha/beta	receptor	alpha
XP012512783.1	Predicted	Propithecus coquereli	Interferon	alpha/beta	receptor	alpha
XP015675028.1	Predicted	Protobothrops mucrosquamatus	Interferon	alpha/beta	receptor	alpha
XP006921038.1	Predicted	Pteropus alecto	Interferon	alpha/beta	receptor	alpha
XP011363988.1	Predicted	Pteropus vampyrus	Interferon	alpha/beta	receptor	alpha
XP009326292.1	Predicted	Pygoscelis adeliae	Interferon	alpha/beta	receptor	alpha
XP016017904.1	Predicted	Rousettus aegyptiacus	Interferon	alpha/beta	receptor	alpha
XP003927702.1	Predicted	Saimiri boliviensis boliviensis	Interferon	alpha/beta	receptor	alpha
XP009098361.1	Predicted	Serinus canaria	Interferon	alpha/beta	receptor	alpha
XP004620396.1	Predicted	Sorex araneus	Interferon	alpha/beta	receptor	alpha
XP009665899.1	Predicted	Struthio camelus australis	Interferon	alpha/beta	receptor	alpha
XP014745867.1	Predicted	Sturnus vulgaris	Interferon	alpha/beta	receptor	alpha
BAD06315.1		Sus scrofa	Interferon	alpha/beta	receptor	alpha
JAG69468.1		Sus scrofa domesticus	Interferon	alpha/beta	receptor	alpha
XP012424842.1	Predicted	Taeniopygia guttata	Interferon	alpha/beta	receptor	alpha
XP010211787.1	Predicted	Tinamus guttatus	Interferon	alpha/beta	receptor	alpha
XP006153941.1	Predicted	Tupaia chinensis	Interferon	alpha/beta	receptor	alpha
XP004317420.1	Predicted	Tursiops truncatus	Interferon	alpha/beta	receptor	alpha

Table 5 Accession Numbers of IL10R2 sequences

Accession Number		Species	type			
XP6032721	PREDICTED:	Alligator sinensis.	Interleukin	10	receptor	beta
AGC95877.1		Anas platyrhynchos	interleukin	10	receptor	beta
XP8120200	PREDICTED:	Anolis carolinensis.	interleukin	10	receptor	beta
XP013048351.1	PREDICTED:	Anser cygnoides domesticus	Interleukin	10	receptor	beta

XP009865443.1	PREDICTED:	Apaloderma vittatum	interleukin	10	receptor	beta
XP9865443	PREDICTED:	Apalodermavittatum.	interleukin	10	receptor	beta
XP009281346.1	PREDICTED:	Aptenodytes forsteri	interleukin	10	receptor	beta
XP9281346	PREDICTED:	Aptenodytesforsteri.	Interleukin	10	receptor	beta
XP011592119.1	PREDICTED:	Aquila chrysaetos canadensis	interleukin	10	receptor	beta
XP7183767	PREDICTED:	Balaenopteraacutorostrata scammoni.	Interleukin	10	receptor	beta
XP5893408	PREDICTED:	Bos mutus.	interleukin	10	receptor	beta
AAI23562		Bos taurus.	Interleukin	10	receptor	beta
XP6048306	PREDICTED:	Bubalusbubalis.	Interleukin	10	receptor	beta
XP014793464.1	PREDICTED:	Calidris pugnax	interleukin	10	receptor	beta
XP535581	PREDICTED:	Canis lupusfamiliaris.	interleukin	10	receptor	beta
XP5674740	PREDICTED:	Capra hircus.	interleukin	10	receptor	beta
XP3467317	PREDICTED:	Cavia porcellus.	interleukin	10	receptor	beta
XP4429624	PREDICTED:	Ceratotheriumsimum simum.	Interleukin	10	receptor	beta
XP010002989.1	PREDICTED:	Chaetura pelagica	interleukin	10	receptor	beta
XP10002989	PREDICTED:	Chaeturapelagica.	interleukin	10	receptor	beta
XP009893222.1	PREDICTED:	Charadrius vociferus	Interleukin	10	receptor	beta
XP9893222	PREDICTED:	Charadriusvociferus.	interleukin	10	receptor	beta
XP007063661.1	PREDICTED:	Chelonia mydas	Interleukin	10	receptor	beta
XP7063661	PREDICTED:	Chelonia mydas.	interleukin	10	receptor	beta
XP7962829	PREDICTED:	Chlorocebussabaeus.	interleukin	10	receptor	beta
XP6862662	PREDICTED:	Chrysochlorisasiatica.	interleukin	10	receptor	beta
XP005511441.1	PREDICTED:	Columba livia	interleukin	10	receptor	beta
XP5511441	PREDICTED:	Columba livia.	interleukin	10	receptor	beta
XP4675580	PREDICTED:	Condyluracristata.	interleukin	10	receptor	beta
XP008638918.1	PREDICTED:	Corvus brachyrhynchos	Interleukin	10	receptor	beta
XP8638918	PREDICTED:	Corvus brachyrhynchos.	interleukin	10	receptor	beta
XP009565422.1	PREDICTED:	Cuculus canorus	interleukin	10	receptor	beta
XP9565422	PREDICTED:	Cuculus canorus.	interleukin	10	receptor	beta
XP4474575	PREDICTED:	Dasypusnovemcinctus.	interleukin	10	receptor	beta
XP4711385	PREDICTED:	Echinopstelfairi.	interleukin	10	receptor	beta
XP009639193.1	PREDICTED:	Egretta garzetta	interleukin	10	receptor	beta
XP9639193	PREDICTED:	Egretta garzetta.	interleukin	10	receptor	beta
XP6886855	PREDICTED:	Elephantulusedwardii.	interleukin	10	receptor	beta
XP8144027	PREDICTED:	Eptesicus fuscus.	interleukin	10	receptor	beta
XP7519798	PREDICTED:	Erinaceuseuropaeus.	interleukin	10	receptor	beta
XP014134992.1	PREDICTED:	Falco cherrug	interleukin	10	receptor	beta
XP5438666	PREDICTED:	Falco cherrug.	interleukin	10	receptor	beta
XP013152260.1	PREDICTED:	Falco peregrinus	interleukin	10	receptor	beta
XP5234443	PREDICTED:	Falco peregrinus.	interleukin	10	receptor	beta
XP8565345	PREDICTED:	Galeopterus variegatus.	interleukin	10	receptor	beta
AAD13678		Gallus gallus.	interleukin	10	receptor	beta
XP5427850	PREDICTED:	Geospiza fortis.	interleukin	10	receptor	beta
XP4062758	PREDICTED:	gorilla gorilla.	interleukin	10	receptor	beta

XP010583627.1	PREDICTED:	Haliaeetus leucocephalus	interleukin	10	receptor	beta
AAH01903		Homo sapiens.	interleukin	10	receptor	beta
AHH37769.1		Ictalurus punctatus	interleukin	10	receptor	beta
XP5323582	PREDICTED:	Ictidomystridecemlineatus.	Interleukin	10	receptor	beta
XP4654632	PREDICTED:	Jaculus jaculus.	interleukin	10	receptor	beta
XP6749268	PREDICTED:	Leptonychotesweddellii.	interleukin	10	receptor	beta
XP008924473.1	PREDICTED:	Manacus vitellinus	interleukin	10	receptor	beta
XP8924473	PREDICTED:	Manacus vitellinus.	interleukin	10	receptor	beta
XP010721250.1	PREDICTED:	Meleagris gallopavo	interleukin	10	receptor	beta
XP5151846	PREDICTED:	Melopsittacusundulatus.	interleukin	10	receptor	beta
XP010182246.1	PREDICTED:	Mesitornis unicolor	interleukin	10	receptor	beta
XP10182246	PREDICTED:	Mesitornisunicolor.	Interleukin	10	receptor	beta
XP5345262	PREDICTED:	Microtusochrogaster.	Interleukin	10	receptor	beta
XP7493332	PREDICTED:	Monodelphisdomestica.	Interleukin	10	receptor	beta
AAI45792		Mus musculus.	Interleukin	10	receptor	beta
XP5860815	PREDICTED:	Myotis brandtii.	interleukin	10	receptor	beta
XP6753184	PREDICTED:	Myotis davidii.	interleukin	10	receptor	beta
XP010011567.1	PREDICTED:	Nestor notabilis	interleukin	10	receptor	beta
XP10011567	PREDICTED:	Nestor notabilis.	interleukin	10	receptor	beta
XP009471772.1	PREDICTED:	Nipponia nippon	interleukin	10	receptor	beta
XP9471772	PREDICTED:	Nipponia nippon.	Interleukin	10	receptor	beta
XP3263916	PREDICTED:	Nomascusleucogenys.	interleukin	10	receptor	beta
XP4588675	PREDICTED:	Ochotonaprinceps.	interleukin	10	receptor	beta
XP4645413	PREDICTED:	Octodon degus.	interleukin	10	receptor	beta
XP4406336	PREDICTED:	Odobenus rosmarusdivergens.	Interleukin	10	receptor	beta
XP009931682.1	PREDICTED:	Opisthocomus hoazin	Interleukin	10	receptor	beta
XP9931682	PREDICTED:	Opisthocomushoazin.	interleukin	10	receptor	beta
XP4264570	PREDICTED:	Orcinus orca.	interleukin	10	receptor	beta
XP1514079	PREDICTED:	Ornithorhynchusanatinus.	interleukin	10	receptor	beta
XP7939498	PREDICTED:	Orycteropus aferafer.	interleukin	10	receptor	beta
XP3797149	PREDICTED:	Otolemurgarnettii	interleukin	10	receptor	beta
JAA32934		Pan troglodytes	interleukin	10	receptor	beta
XP7090484	PREDICTED:	Panthera tigrisaltaica.	interleukin	10	receptor	beta
XP5954316	PREDICTED:	Pantholops hodgsonii.	interleukin	10	receptor	beta
XP006126099.1	PREDICTED:	Pelodiscus sinensis	interleukin	10	receptor	beta
XP6126099	PREDICTED:	Pelodiscus sinensis.	interleukin	10	receptor	beta
XP6983640	PREDICTED:	Peromyscusmaniculatus bairdii.	interleukin	10	receptor	beta
XP010292505.1	PREDICTED:	Phaethon lepturus	interleukin	10	receptor	beta
XP10292505	PREDICTED:	Phaethonlepturus.	interleukin	10	receptor	beta
XP5526692	PREDICTED:	Pseudopodoceshumilis.	interleukin	10	receptor	beta
AEQ38018		Pteropus alecto.	interleukin	10	receptor	beta
XP009326291.1	PREDICTED:	Pygoscelis adeliae	interleukin	10	receptor	beta
XP3927704	PREDICTED:	Saimiriboliviensis boliviensis.	interleukin	10	receptor	beta
XP009098360.1	PREDICTED:	Serinus canaria	interleukin	10	receptor	beta

XP9098360	PREDICTED:	Serinus canaria.	interleukin	10	receptor	beta
XP4620397	PREDICTED:	Sorex araneus.	interleukin	10	receptor	beta
XP009665944.1	PREDICTED:	Struthio camelus australis	interleukin	10	receptor	beta
XP9665944	PREDICTED:	Struthio camelusaustralis.	interleukin	10	receptor	beta
XP014745841.1	PREDICTED:	Sturnus vulgaris	interleukin	10	receptor	beta
BAD06316		Sus scrofa.	interleukin	10	receptor	beta
XP012424838.1	PREDICTED:	Taeniopygia guttata	Interleukin	10	receptor	beta
XP010211788.1	PREDICTED:	Tinamus guttatus	interleukin	10	receptor	beta
XP10211788	PREDICTED:	Tinamus guttatus.	Interleukin	10	receptor	beta
XP4317421	PREDICTED:	Tursiopstruncatus.	interleukin	10	receptor	beta
XP009967534.1	PREDICTED:	Tyto alba	interleukin	10	receptor	beta
XP9967534	PREDICTED:	Tyto alba.	interleukin	10	receptor	beta
XP6216099	PREDICTED:	Vicugna pacos.	interleukin	10	receptor	beta
NP1165294	precursor	Xenopus (Silurana)tropicalis.	interleukin	10	receptor	beta
NP1087014	precursor	Xenopus laevis.	interleukin	10	receptor	beta

Table 6 Accession Numbers of IFIT sequences

Accession Number		**Species**	**type**
ENSAMEG00000020101		Ailuropoda melanoleuca	IFIT2
ENSAMEG00000018958		Ailuropoda melanoleuca	IFIT5
ENSAMEG00000020102	predicted	Ailuropoda melanoleuca	IFIT
ENSAMEG00000020103	predicted	Ailuropoda melanoleuca	IFIT
KF956064		Anas platyrhynchos	IFIT
ENSACAG00000024133	predicted	Anolis carolinensis	IFIT
ENSACAG00000027620	predicted	Anolis carolinensis	IFIT
ENSBTAG00000007881		Bos taurus	IFIT1
ENSBTAG00000034918		Bos taurus	IFIT2
ENSBTAG00000009768		Bos taurus	IFIT3
ENSBTAG00000017367		Bos taurus	IFIT5
ENSCJAG00000005746		Callithrix jacchus	IFIT1
ENSCJAG00000005737		Callithrix jacchus	IFIT2
ENSCJAG00000005741		Callithrix jacchus	IFIT3
ENSCJAG00000011216		Callithrix jacchus	IFIT5
ENSCAFG00000009617		Canis familiaris	IFIT1
ENSCAFG00000009612		Canis familiaris	IFIT2
ENSCAFG00000031614		Canis familiaris	IFIT3
ENSCAFG00000007151	predicted	Canis familiaris	IFIT
ENSCAFG00000031100	predicted	Canis familiaris	IFIT
ENSCPOG00000009345		Cavia porcellus	IFIT1B
ENSCPOG00000008531		Cavia porcellus	IFIT5
ENSCHOG00000005879		Choloepus hoffmanni	IFIT2
ENSCHOG00000003530		Choloepus hoffmanni	IFIT3
ENSCHOG00000004669		Choloepus hoffmanni	IFIT5

ENSCHOG00000004664	predicted	Choloepus hoffmanni	IFIT
ENSCHOG00000004668	predicted	Choloepus hoffmanni	IFIT
ENSDNOG00000044243		Dasypus novemcinctus	IFIT2
ENSDNOG00000000986		Dasypus novemcinctus	IFIT3
ENSDNOG00000008825		Dasypus novemcinctus	IFIT5
ENSDNOG00000008816	predicted	Dasypus novemcinctus	IFIT
ENSDNOG00000045605	predicted	Dasypus novemcinctus	IFIT
ENSDORG00000003058	predicted	Dipodomys ordii	IFIT
ENSDORG00000012514	predicted	Dipodomys ordii	IFIT
ENSDORG00000012515	predicted	Dipodomys ordii	IFIT
ENSETEG00000014714		Echinops telfairi	IFIT5
ENSECAG00000004433		Equus caballus	IFIT1
ENSECAG00000010153		Equus caballus	IFIT4
ENSECAG00000004349		Equus caballus	IFIT5
ENSEEUG00000000242		Erinaceus europaeus	IFIT3
ENSEEUG00000002041		Erinaceus europaeus	IFIT5
ENSFCAG00000011708		Felis catus	IFIT2
ENSFCAG00000018779		Felis catus	IFIT3
ENSFCAG00000005933	predicted	Felis catus	IFIT
ENSFCAG00000007421	predicted	Felis catus	IFIT
ENSFCAG00000012538	predicted	Felis catus	IFIT
ENSFCAG00000031175	predicted	Felis catus	IFIT
ENSFALG00000007677	predicted	Ficedula albicollis	IFIT
ENSGGOG00000023234		Gorilla gorilla	IFIT1
ENSGGOG00000023376		Gorilla gorilla	IFIT1B
ENSGGOG00000010407		Gorilla gorilla	IFIT2
ENSGGOG00000012012		Gorilla gorilla	IFIT3
ENSGGOG00000003981		Gorilla gorilla	IFIT5
ENSGGOG00000022599	predicted	Gorilla gorilla	IFIT
ENSG00000185745		Homo sapiens	IFIT1
ENSG00000204010		Homo sapiens	IFIT1B
ENSG00000119922		Homo sapiens	IFIT2
ENSG00000119917		Homo sapiens	IFIT3
ENSG00000152778		Homo sapiens	IFIT5
ENSSTOG00000024733		Ictidomys tridecemlineatus	IFIT1
ENSSTOG00000022564		Ictidomys tridecemlineatus	IFIT2
ENSSTOG00000013100		Ictidomys tridecemlineatus	IFIT3
ENSSTOG00000028876		Ictidomys tridecemlineatus	IFIT5
ENSSTOG00000007101	predicted	Ictidomys tridecemlineatus	IFIT
ENSSTOG00000024810	predicted	Ictidomys tridecemlineatus	IFIT
ENSSTOG00000026318	predicted	Ictidomys tridecemlineatus	IFIT
ENSSTOG00000026437	predicted	Ictidomys tridecemlineatus	IFIT
ENSSTOG00000027912	predicted	Ictidomys tridecemlineatus	IFIT
ENSLACG00000004609	predicted	Latimeria chalumnae	IFIT

ENSLACG00000004753	predicted	Latimeria chalumnae	IFIT
ENSLAFG00000008209		Loxodonta africana	IFIT3
ENSLAFG00000011455		Loxodonta africana	IFIT5
ENSLAFG00000008208	predicted	Loxodonta africana	IFIT
ENSLAFG00000028666	predicted	Loxodonta africana	IFIT
ENSMEUG00000002401		Macropus eugenii	IFIT5
ENSMGAG00000015330	predicted	Meleagris gallopavo	IFIT
ENSMICG00000014624		Microcebus murinus	IFIT3
ENSMICG00000016819		Microcebus murinus	IFIT5
ENSMICG00000014633	predicted	Microcebus murinus	IFIT
ENSMICG00000014997	predicted	Microcebus murinus	IFIT
ENSMODG00000007358	predicted	Monodelphis domestica	IFIT
ENSMODG00000024881	predicted	Monodelphis domestica	IFIT
ENSMODG00000024882	predicted	Monodelphis domestica	IFIT
ENSMODG00000028143	predicted	Monodelphis domestica	IFIT
ENSMUSG00000034459		Mus musculus	IFIT1
ENSMUSG00000045932		Mus musculus	IFIT2
ENSMUSG00000074896		Mus musculus	IFIT3
ENSMPUG00000006489		Mustela putorius furo	IFIT2
ENSMPUG00000020198		Mustela putorius furo	IFIT3
ENSMPUG00000017274		Mustela putorius furo	IFIT5
ENSMPUG00000020197	predicted	Mustela putorius furo	IFIT
ENSMPUG00000006482	predicted	Mustela putorius furo	IFIT
ENSMLUG00000016645		Myotis lucifugus	IFIT2
ENSMLUG00000016653		Myotis lucifugus	IFIT3
ENSMLUG00000005766		Myotis lucifugus	IFIT5
ENSMLUG00000016659	predicted	Myotis lucifugus	IFIT
ENSMLUG00000024075	predicted	Myotis lucifugus	IFIT
ENSNLEG00000012308		Nomascus leucogenys	IFIT1
ENSNLEG00000018636		Nomascus leucogenys	IFIT1B
ENSNLEG00000018635		Nomascus leucogenys	IFIT2
ENSNLEG00000019258		Nomascus leucogenys	IFIT3
ENSNLEG00000019104		Nomascus leucogenys	IFIT5
ENSOPRG00000015105		Ochotona princeps	IFIT2
ENSOPRG00000015110		Ochotona princeps	IFIT3
ENSOPRG00000014668		Ochotona princeps	IFIT5
ENSOPRG00000000056	predicted	Ochotona princeps	IFIT
ENSOPRG00000015788	predicted	Ochotona princeps	IFIT
ENSOPRG00000015791	predicted	Ochotona princeps	IFIT
ENSOANG00000005322	predicted	Ornithorhynchus anatinus	IFIT
ENSOANG00000007390	predicted	Ornithorhynchus anatinus	IFIT
ENSOANG00000010184	predicted	Ornithorhynchus anatinus	IFIT
ENSOCUG00000024939		Oryctolagus cuniculus	IFIT2
ENSOCUG00000029154		Oryctolagus cuniculus	IFIT3

ENSOCUG00000024570		Oryctolagus cuniculus	IFIT5
ENSOCUG00000004197	predicted	Oryctolagus cuniculus	IFIT
ENSOCUG00000005438	predicted	Oryctolagus cuniculus	IFIT
ENSOCUG00000005445	predicted	Oryctolagus cuniculus	IFIT
ENSORLG00000008314	predicted	Oryzias latipes	IFIT
ENSORLG00000013161	predicted	Oryzias latipes	IFIT
ENSORLG00000013238	predicted	Oryzias latipes	IFIT
ENSORLG00000013495	predicted	Oryzias latipes	IFIT
ENSORLG00000013503	predicted	Oryzias latipes	IFIT
ENSORLG00000019695	predicted	Oryzias latipes	IFIT
ENSORLG00000020438	predicted	Oryzias latipes	IFIT
ENSORLG00000020439	predicted	Oryzias latipes	IFIT
ENSOGAG00000031058		Otolemur garnettii	IFIT1
ENSOGAG00000027677		Otolemur garnettii	IFIT2
ENSOGAG00000002215		Otolemur garnettii	IFIT3
ENSOGAG00000009660		Otolemur garnettii	IFIT5
ENSOARG00000015177		Ovis aries	IFIT1
ENSOARG00000015169		Ovis aries	IFIT2
ENSOARG00000014800		Ovis aries	IFIT3
ENSOARG00000014815		Ovis aries	IFIT5
ENSPTRG00000029833		Pan troglodytes	IFIT1B
ENSPTRG00000002733		Pan troglodytes	IFIT2
ENSPTRG00000029834		Pan troglodytes	IFIT3
ENSPTRG00000002736		Pan troglodytes	IFIT5
ENSPANG00000021700		Papio anubis	IFIT1
ENSPANG00000010650		Papio anubis	IFIT1B
ENSPANG00000021698		Papio anubis	IFIT2
ENSPANG00000021699		Papio anubis	IFIT3
ENSPANG00000021702		Papio anubis	IFIT5
ENSPSIG00000000678	predicted	Pelodiscus sinensis	IFIT
ENSPPYG00000002466		Pongo abelii	IFIT1
ENSPPYG00000002465		Pongo abelii	IFIT1B
ENSPPYG00000002463		Pongo abelii	IFIT2
ENSPPYG00000002464		Pongo abelii	IFIT3
ENSPPYG00000002467		Pongo abelii	IFIT5
ENSPCAG00000014616		Procavia capensis	IFIT5
ENSPVAG00000003647		Pteropus vampyrus	IFIT5
ENSRNOG00000019050		Rattus norvegicus	IFIT1
ENSRNOG00000036603		Rattus norvegicus	IFIT1lb
ENSRNOG00000036604		Rattus norvegicus	IFIT2
ENSRNOG00000022839		Rattus norvegicus	IFIT3
ENSSHAG00000001370	predicted	Sarcophilus harrisii	IFIT
ENSSHAG00000002618	predicted	Sarcophilus harrisii	IFIT
ENSSHAG00000014976	predicted	Sarcophilus harrisii	IFIT

ENSSARG00000001228		Sorex araneus	IFIT5
ENSSSCG00000010453		Sus scrofa	IFIT1
ENSSSCG00000010451		Sus scrofa	IFIT2
ENSSSCG00000010452		Sus scrofa	IFIT3
ENSSSCG00000010454		Sus scrofa	IFIT5
ENSTGUG00000008354	predicted	Taeniopygia guttata	IFIT
ENSTSYG00000010287		Tarsius syrichta	IFIT1B
ENSTSYG00000011715		Tarsius syrichta	IFIT5
ENSTSYG00000019338	predicted	Tarsius syrichta	IFIT
ENSTSYG00000019354	predicted	Tarsius syrichta	IFIT
ENSTNIG00000002787		Tetraodon nigroviridis	IFIT2
ENSTBEG00000002666		Tupaia belangeri	IFIT3
ENSTBEG00000002771	predicted	Tupaia belangeri	IFIT
ENSTBEG00000003552	predicted	Tupaia belangeri	IFIT
ENSTTRG00000015419		Tursiops truncatus	IFIT1
ENSTTRG00000015421		Tursiops truncatus	IFIT5
ENSVPAG00000007869		Vicugna pacos	IFIT3
ENSVPAG00000007871		Vicugna pacos	IFIT1
ENSVPAG00000007868		Vicugna pacos	IFIT2
ENSVPAG00000011508		Vicugna pacos	IFIT5
ENSXETG00000021822		Xenopus tropicalis	IFIT1
ENSXETG00000021818		Xenopus tropicalis	IFIT5
ENSXETG00000015254	predicted	Xenopus tropicalis	IFIT
ENSXETG00000016292	predicted	Xenopus tropicalis	IFIT
ENSXETG00000021819	predicted	Xenopus tropicalis	IFIT

Table 7 qRT-PCR Primer and Probe sequences

Target Gene	Forward Primer Seq.	Reverse Primer Seq.	Reporter 1 Sequence
ch GAPDH	CCCCAATGTCTCTGTTGTTGAC	CAGCCTTCACTACCCTCTTGAT	CTTGGCTGGTTTCTCC
ch IFNα	GGACATGGCTCCCACACTAC	TCCAGGATGGTGTCGTTGAAG	CAGCGCGTCTTGCTC
ch IFNβ	ACAACTTCCTACAGCACAACAACTA	GCCTGGAGGCGGACATG	TCCCAGGTACAAGCACTG
ch IFNλ	CATCGGAAGTGGGACATAGCT	CCTCCACCAGGGTGATTCG	TCAGGTACCGACAGCTC
ch IFNλR1	GGATCTCCACCAGATGTGTTGTAC	GGAACCTTTATCCATTTGTCCATACG	CTGTGAGGTATGAAAGCAA
ch IL-10R2	CGCAAAGGCAACCTAAGTTATACTG	TTGGTTGTCACATTGTTAAAATTCTGCTT	CCAGGCCAAAAGCATT
ch ISG IFIT5	CAGAATTTAATGCCGGCTATGC	TGCAAGTAAAGCCAAAAGATAAGTGT	TCTGAAGCGTGCACTGAAACTGAATCCAA
ch ISG MX	GTCCAAGAGGCTGAATAACAGAGAA	GGTCGGATCTTTCTGTCATATTGGT	CTGCTGCCTCATCCTT
ch ISG PKR	GCAGAAGTAAGAGTGAGGCAAATGA	GCCACCTTTACCAATAGGCTCTAT	CTGTGGATGAAAGGTTTC
ch ISG Viperin	CTGATCAGGGAACGGTGGTT	ACGTTGACTTCCTCATTAAAACTATCACA	CAAGAAGTATGGTGAATATTT
ch ISG ZAP	AAATTGAAAAAGCCTATTGTGACCCAAA	GGAGAGGGTCATTGTCTGGAAATT	CTGCTGCACTGCTGTTT

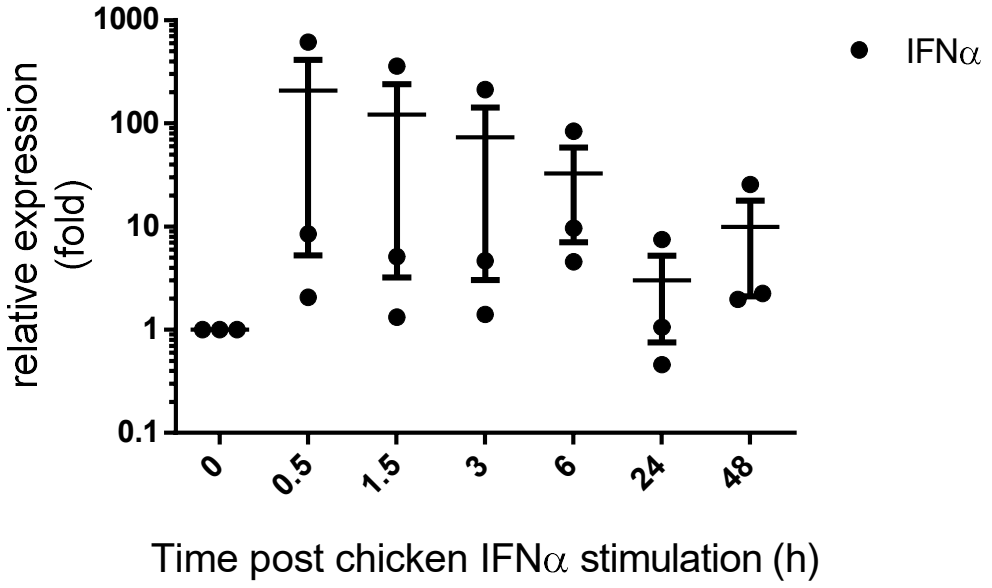

Figure 11-1 IFN alpha mRNA expression post IFN alpha stimulation

Expression of IFNα and IFNλ mRNA in purified splenocytes from SPF chickens stimulated with 500 ng/mL IFNα over the indicated time course. The bars represent the mean fold change of 3 chicken spleens with the standard error of the mean (SEM) compared to the untreated sample, normalized against the housekeeping gene GAPDH.

Figure 11-2 Alignment of th predicted and sequenced chicken IFIT5 sequence

Gene Alignment of the sequenced (KT180229.1) and the predicted sequence of IFIT5. Numbers on at the end of each sequence indicates gene length. Red indicates conserved base pairs and black indicated a SNP.

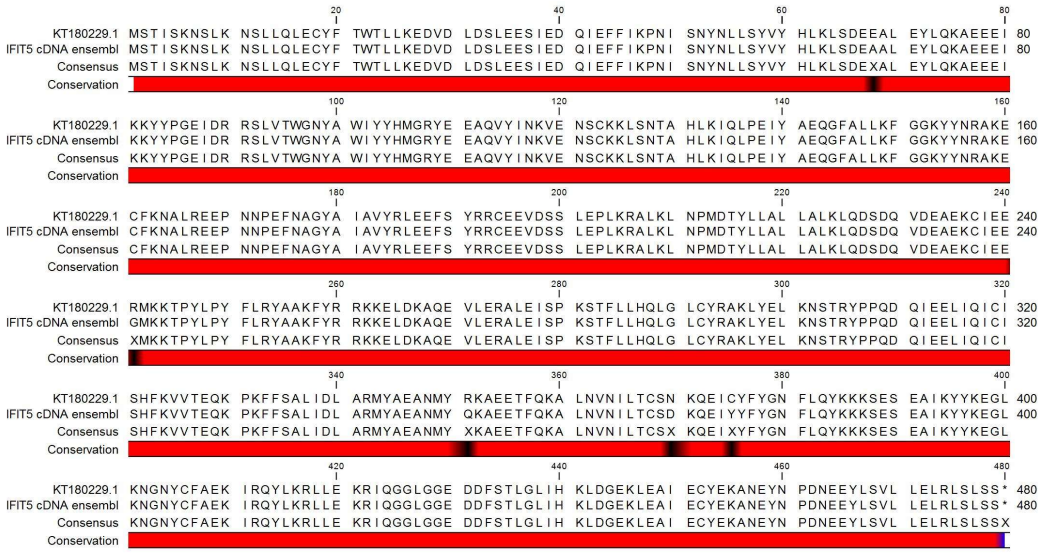

Figure 11-3 in silico translated protein sequence alignment of the predicted and sequenced chicken IFIT5 sequence

Protein alignment of the sequenced and in silico translated IFIT5(KT180229.1) and the predicted and translated sequence. Numbers on at the end of each sequence indicates protein length. Red indicates conserved amino acids and black indicated a SNP.