Health & Medicine | Lancaster University

PhD Thesis in Biomedical and Life Sciences

By

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Title

The Impact of Interferon-Stimulated Genes in the Pathobiology of Influenza A Viruses

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January 2025

Declaration of Originality

I declare that the content of this thesis is my own work and any sections of this thesis which have been published have been clearly identified.

Emma Louise Gardiner

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Acknowledgements

My sincerest thanks to Prof. Muhammad Munir for giving me the opportunity and privilege to study under his guidance and for providing the highest support over the last 4 years. Studying for my PhD has been life-changing for me, and I couldn't have done it without Munir.

I would also like to thank my secondary supervisor, Dr. Leonie Unterholzner, for her support and guidance throughout my supervisory meetings and input on my writing skills.

I would also like to thank the Pro-Vice-Chancellor for Education, Wendy Robinson, and University Academic Dean, Alisdair Gillespie, for their support whilst working and studying at Lancaster University and their guidance for how to achieve high in my studies.

I would like to thank my family, particularly my partner Ellis and my best friends Lauren and Emily. Their support over the last 4 years has been invaluable, and I wouldn't be the person I am today without them.

Finally, I would like to thank all the staff within the BLS department at Lancaster University. Their teaching and guidance throughout my three degrees at this institution have been amazing and have made me love my specialised subject even more.

Abstract

Innate immunity defines the first line of defence against viral infection and is mainly attributed to cytokines, chemokines, interferons, and interferon-stimulated and regulated genes. Amongst myriads of interferons-stimulated genes, interferon-inducible transmembrane (IFITM) proteins are responsive to virus-induced transcriptional activation and display dynamic roles in regulating viruses with diverse genetic backgrounds. IFITM proteins are genetically and functionally characterised in humans against several medically important viruses, including influenza; however, the nature and functional insights into the roles of IFITM proteins in chickens remain less characterised. In this thesis, a range of approaches were applied to investigate the roles of chicken IFITM proteins against avian influenza viruses, ranging from stable and transient expression, cellular distributions, *in vivo* investigation, and structural and mechanistic insights into different domains of IFITM proteins, which form the basis of the antiviral potential of IFITM proteins. Using transcriptomic datasets, it was identified that chicken IFITM genes are profoundly activated in chickens in response to influenza viral infections. Next, deploying transient expression system we mapped the cellular distribution of chIFITM1 and chIFITM3 in chicken fibroblasts (DF-1 cells) and concluded functionally important residues which retain IFITM proteins in the plasma membrane. Additionally, residues that define the antiviral roles of IFITM proteins (IFITM1 and IFITM3) against influenza viruses were mapped. Extensive mutation and deletion mapping catalogued the C-terminus amphipathic helix and hydrophobic domain residues, which defined the antiviral roles of IFITM1 and IFITM3 against influenza viruses. Next, efforts were made to establish a basis for structural modelling and functional characterisation of chicken IFITM1 and IFITM3. Finally, by applying an avian sarcoma-leucosis virus (RCAS)-based gene transfer system, transgenic chickens constitutively expressing IFITM3 showed resistance against highly pathogenic avian influenza virus (HPAIV) subtype H5N1. These findings establish solid foundations to characterise chIFITM1 and chIFITM3 in regulating influenza viruses both *in vitro* and *in vivo*.

Chapter 1

Introduction

1.1 The Pathobiology of Influenza Viruses

Influenza Viruses (IV) belong to a family known as *Orthomyxoviridae* viruses which is made up of four known subtypes: A, B, C and D (IAV, IBV, ICV and IDV) (Park and Ryu, 2018). Influenza A and B are recognised for inducing seasonal epidemics of illness, commonly known as the flu. Influenza C virus causes the least severe infections, leading to moderate sickness. They are not believed to induce human outbreaks or epidemics, while influenza D viruses are linked to cattle and exhibit minimal transmission to other animals (Hutchinson, 2018). Although influenza viruses have been widely studied, there is no direct cure, and we rely solely on the need for a yearly vaccination of four prevalent strains.

IV pandemics result from the emergence of an IAV that is novel for the human population. The common threat of IV outbreaks is the reassortment of subtypes that can occur in avian and transfer to human hosts, which occurred in 1957 and 1968. This allowed the creation of new viruses that possessed novel surface antigens with the potential to spread within the human population. The influenza pandemic of 1918-1919 resulted in an estimated 20-50 million fatalities all over the world. This pandemic's nature led to an abnormally elevated mortality rate, especially among healthy adults aged 15 to 34 years (Glezen, 1996). Historical pandemics have been marked by a transition in virus subtypes; thus, there is a potential for the virus to instigate a pandemic if it interacts with avian influenza or another subtype of human influenza virus, leading to reassortment (Kapoor and Dhama, 2014). In addition to pandemics of IV, multiple annual winter outbreaks occur. Each year, the World Health Organisation (WHO) estimates the global attack rate of IV to be between 5 to 10% in adults and 20 to 30% in children (WHO, 2024). Another ailment, termed respiratory disease, accounts for around 650,000 additional fatalities each year due to its correlation with seasonal influenza (WHO, 2024).

The principal method of influenza transmission is by the inhalation of infectious respiratory droplets expelled by an infected individual during coughing or sneezing. There is also a large amount of evidence of airborne and fomite transmission through small particles

being transmitted through talking or exhalation (Xu et al., 2019). Patients are typically infectious one or two days before the onset of symptoms, then a further five to seven days afterwards. Symptoms present within humans following infection include fever, headache, sneezing, chills, cough and muscle pain. Elderly people (noted as over 65 years old) possess a greater risk of infection. Furthermore, persons with respiratory ailments such as asthma and chronic obstructive pulmonary disease (COPD), as well as those with other medical disorders including diabetes, cancer, or compromised immune systems (Keilman, 2019), are at an elevated risk of infection.

Type A influenza viruses are those of the most concern for a future pandemic (**Fig. 1.1**). Influenza A viruses are categorised as zoonotic pathogens that can infect a wide range of hosts; however, avian species have become the primary host, with most maintained in wild aquatic bird populations. Sporadic transmission also occurs to other hosts (Kim, Webster, and Webby, 2018). The IAV genome comprises eight individual, negative-stranded RNAs. These encode essential viral structural proteins, including membrane protein (M), nucleoprotein (NP), and non-structural proteins. Type A viruses are made up of many different combinations of hemagglutinin (HA) and neuraminidase proteins (NA) and can be divided into 18 HA subtypes and 11 NA subtypes. The main subtypes that cause infection within humans are H1N1 and H3N2 (CDC, 2024).

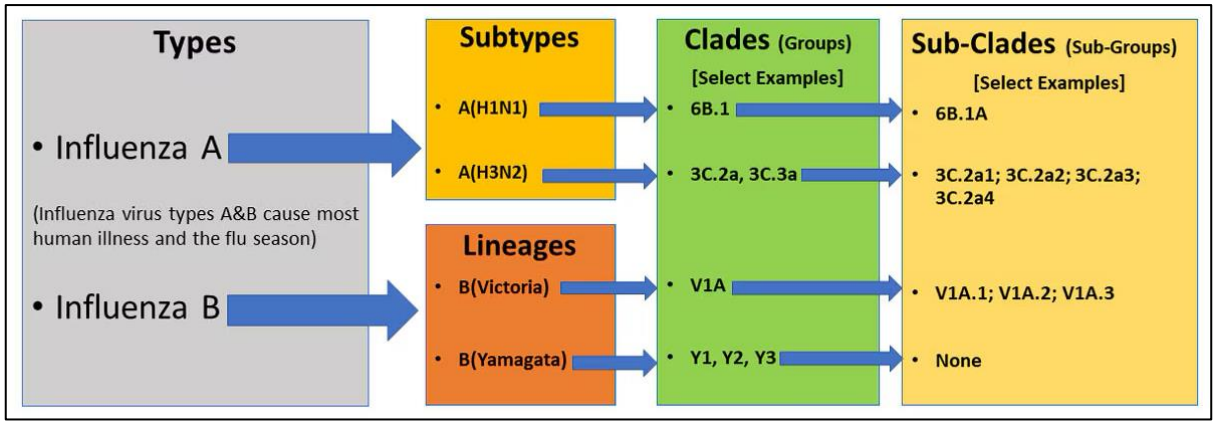


Figure 1.1: Graphic detailing the two types of influenza viruses (A and B) that cause the greatest illness in humans. Influenza A viruses are categorised into two subtypes: H1N1 or H3N2. Influenza B viruses are

categorised into two lineages: B/Yamagata and B/Victoria. Influenza A and B are subsequently categorised into clades and subclades. (Centres for Disease Control and Prevention, 2024).

Due to the unique structure of the influenza virus and the genetic variabilities it has, infection returns each year. The virion of influenza contains an envelope that is derived from a lipid membrane. This lipid membrane forms from the plasma membrane of host cells. This membrane safeguards the viral components of influenza viruses from environmental factors while the virus resides within the host cell. A matrix layer exists beneath the membrane envelope, which encases the viral genome comprised of eight segments of nucleoprotein-encased single-stranded RNA (Hutchinson, 2018).

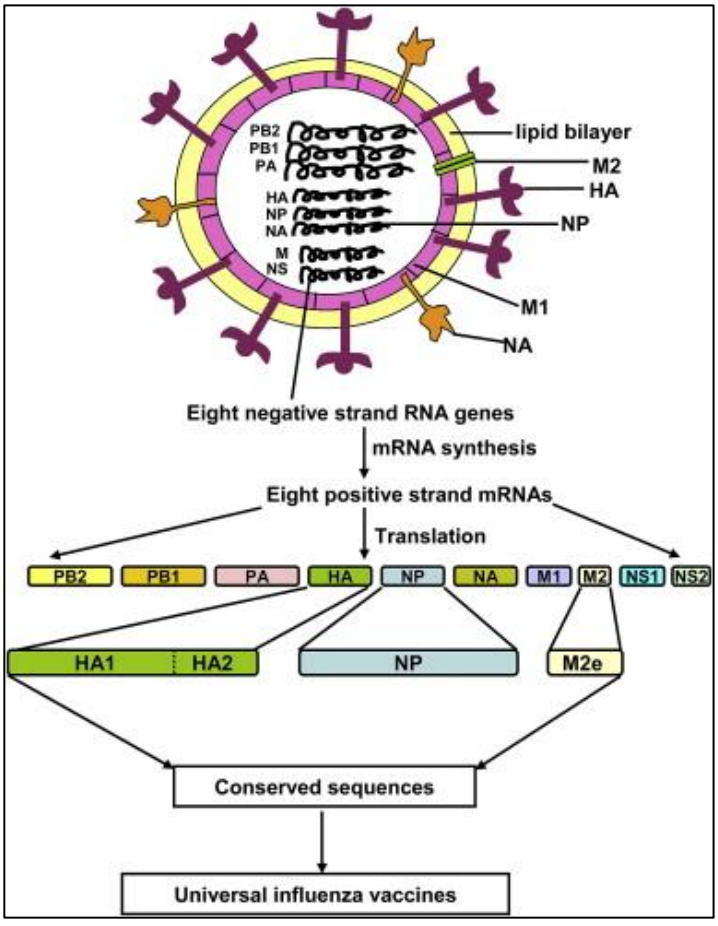


Figure 1.2: Schematic diagram of influenza A virus and key proteins as the groundwork for design of a universal vaccination. The viral genome is composed of eight single, negative-stranded RNA segments associated with nucleoprotein and three polymerase proteins designated as PB2, PB1, and PA. These RNAs are then transcribed into positive strand mRNAs, serving as the template for protein synthesis. Three structural

proteins, hemagglutinin (HA), neuraminidase (NA), and membrane protein 2 (M2), are integrated into the lipid bilayer of the virion's surface. Image sourced from Du, Zhou, and Jiang, 2010.

Influenza viruses are categorised according to the antigenic characteristics of their surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), enabling their classification into subtypes, clades, and sub-clades (**Fig. 1.2**). When mutations occur that alter the epitope, antigenic drift is driven to the host immunity. Antigenic shift results in an exchange of HA and/or NA genes among different influenza viruses, resulting in an influenza mutant to which the population has not gained immunity. Thus, resulting in increased transmission and therefore higher infection rates (Du, Zhou, and Jiang, 2010). The HA glycoprotein facilitates receptor binding and membrane fusion by identifying host proteins that possess sialic acid on their surface. This activates viral and host membrane fusion following the initiation of endocytosis, facilitating the entry of viral RNA into the cytoplasm. The virus is then released through the removal of sialic acid from the host cell, and this is facilitated by NA proteins (Watts, 2009). The HA proteins naturally form trimers, and the NA proteins naturally form tetramers.

The M protein is another important structural protein in IAV and encodes two proteins. The M1 is a capsid protein and an essential component of viral particles, necessary for viral assembly and budding. The M2 protein is an ion-channel integral membrane protein that safeguards the maturation and structural integrity of HA. M2 comprises an ectodomain at the N-terminus, a solitary membrane-spanning domain, and a C-terminal cytoplasmic tail (Pinto and Lamb, 2006). The regulation of RNA synthesis is governed by non-structural protein 1 (NS1). NS1 attaches to the viral genome and acts as an intermediary between the virus and host cells, serving as a regulatory element (Ng et al., 2008). Additionally, NS1 is one of the most characterised viral proteins with a profound impact on innate immune antagonism and contribution to the pathobiology of influenza viruses.

Due to the large amount of vRNA and mRNA that exists in influenza viruses, there is the possibility that antigenic drift and antigenic shift can present themselves (Webster and Gavorkova, 2014). Antigenic drift involves mutations or deletions in the genomes of influenza viruses, resulting in alterations to the surface proteins HA and NA, hence generating distinct subtypes of Influenza A viruses. Minor alterations linked to antigenic drift can accumulate over time, leading to viruses that exhibit antigenic divergence (Kim et al., 2018). When this occurs, the body's immune system may not recognise the virus as well in a second infection and may not be able to prevent sickness caused by the newer influenza virus (Altman et al., 2018). Antigenic drift is an important factor contributing to multiple infections in its host annually and is also the primary reason why the composition of flu vaccines is reviewed annually to generate new subtype vaccines. This can happen when an influenza virus from a non-human mammalian or avian population gains the ability to transmit to a human population. Antigenic shift is less common than antigenic drift, but when it occurs, it results in an influenza pandemic; consequently, there have only been four influenza epidemics within the last 100 years.

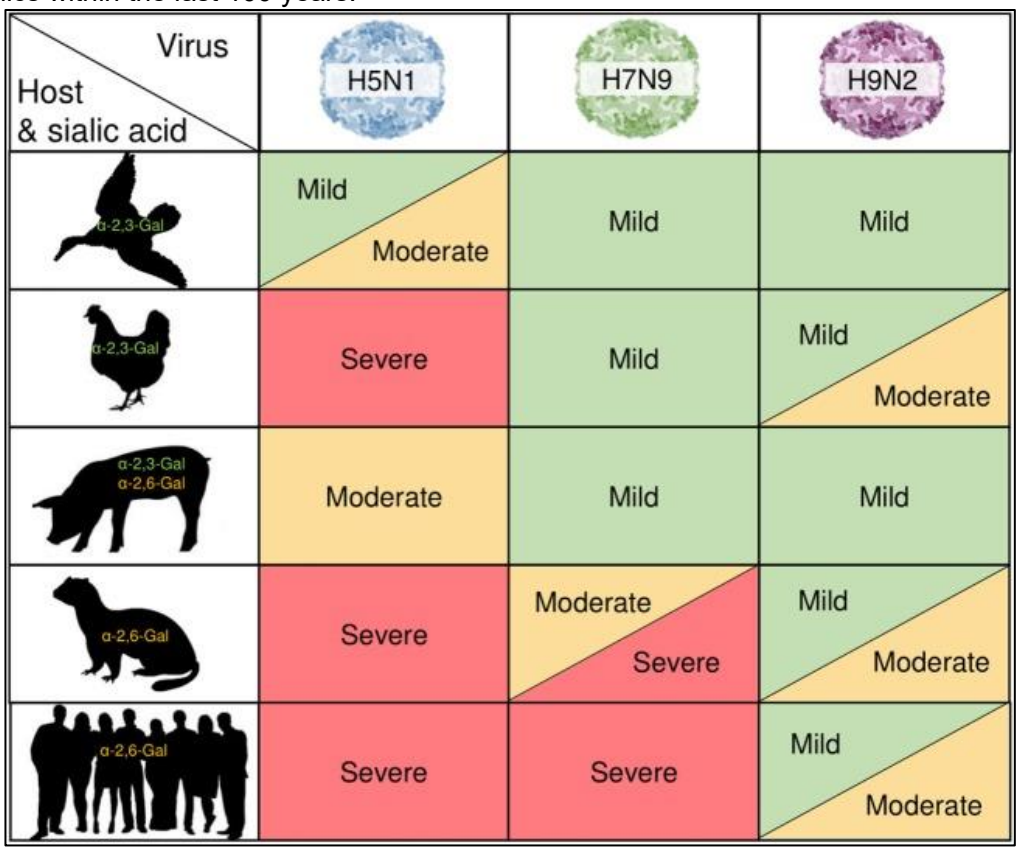


Figure 1.3: Zoonotic influenza pathology from multiple subtypes is linked to host biology. The picture illustrates the H5N1, H7N9, and H9N2 avian influenza viruses together with their differing levels of clinical symptoms. This is demonstrated across several species/hosts and elucidates their sialic acid characteristics. The

clinical manifestations indicate viral fitness and may offer insights into the mechanisms that determine illness severity and host susceptibility. Image sourced from Horman et al., 2018.

The disease intensity seen within different hosts of different subtypes of IAV varies dramatically. H5N1 is the subtype that causes the most severe response in several hosts, including humans, as seen in **Fig. 1.3**. The sialic acid glycosidic bond composition is also seen to be a factor contributing to disease severity, and this links back to the NA protein and its ability to remove sialic acid from the host cell. Host proteases contain a viral HA receptor envelope that is cleaved and activated, resulting in the initiation of IAV infection. The HA receptor binds to sialylated proteins on the cell's surface, instigating endocytosis of the viral particles (Mercer, Schelhaas, and Helenius, 2010). Endocytosed viruses are then transported through early and late endosomes triggering a conformational change in HA resulting in the membrane fusion of the virus. The process of membrane fusion occurs from the movement of a hemifusion intermediate into a fusion pore where eight viral ribonucleoproteins (vRNPs) enter the cytosol and subsequently then move to the nucleus. Once in the nucleus, viral RNA-dependent RNA polymerase synthesises viral genomes and mRNAs. This results in the production of viral progeny once they are exported to the cytosol (Feely et al., 2011). Imaging studies have revealed that IAV can utilise clathrin and non-clathrin entry routes in parallel (Lou, 2011). The entry process of a virus follows a specific pathway with several cellular factors involved (**Fig. 1.4**). The amalgamation of viral membranes with lysosomes is initiated by the acidification of late endosomes, resulting in the release of the viral RNA-dependent RNA polymerase-associated nucleocapsid (RNP) into the nucleus, where viral transcription commences.

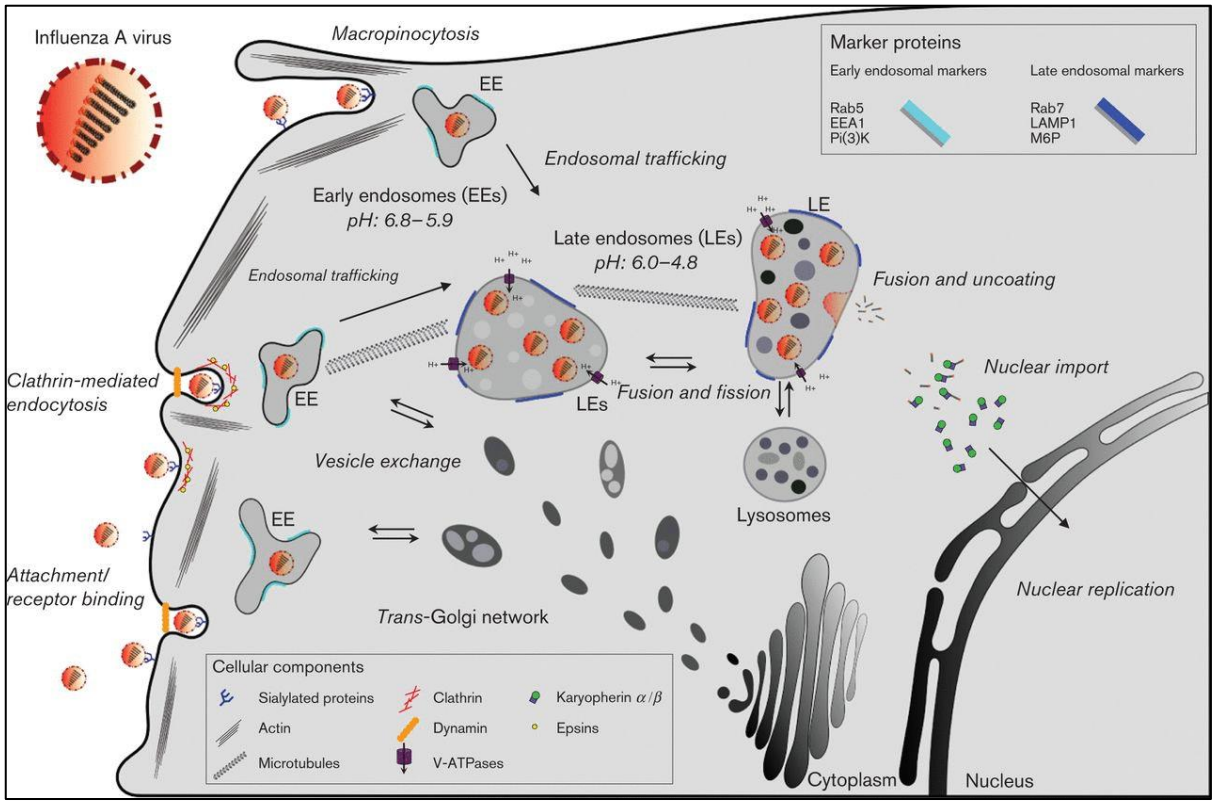


Figure 1.4: Schematic diagram of Influenza virus entry into host cell. The influenza virus infiltrates the body and attaches to sialic acid residues on mucins via hemagglutinin proteins on the virion. NA proteins liberate the encapsulated virus by cleaving the terminal sialic acid residues. This facilitates the virus's penetration of the mucosal fluid to access the target cells. The virus binds to sialylated host cell receptors and is subsequently internalised through endocytosis. The acidified endosome of the target cell subsequently initiates HA-mediated fusion of the endosomal and viral membranes. The viral genome is subsequently released into the nucleus, where viral RNA synthesises mRNA, leading to protein expression (figure derived from Hartshorn, 2020).

1.2 Chickens as a host for Influenza A Virus

IAV infection manifests in a diverse array of avian species. Both turkeys and chickens are encompassed in this classification, and both species belong to the order *Galliformes*. The predominant species for IAV infection is domesticated birds in Europe and America, with a production total in 2019 of around 0.4 million turkeys and 8 million chickens (FAO, 2021). Human cases of avian influenza (AI) infection have become more common since the late 1990s, when there was a large outbreak of H5N1 (Pinsent et al., 2017). A subsequent outbreak of H7N9 in China has demonstrated that these viruses are transmitted to individuals

who are in close proximity to live poultry markets. Live poultry markets are environments where avian influenza viruses in chickens may be prevalent yet generally undetected (Huo et al., 2017) due to the dense concentration of chicks in confined areas. There are three AI subtypes that have the greatest cause for concern regarding transmission to humans. These are the H5Nx, H7Nx, and H9Nx viruses. Therefore, targeting these viruses in their avian hosts is critical for combating human infections (Kalaiyarasu et al., 2016).

The H3 subtype of IAV exhibits a broad host range, encompassing transmission among wild birds and poultry. In addition to these animals, it is also recognised for infecting many mammals. The initial report of a novel recombinant H3N2 influenza virus strain occurred in 1968 in Hong Kong. This resulted in the third recognised human influenza pandemic (Yang et al., 2022). The transmission of low pathogenic (LP) IAV of H5 and H7 subtypes to highly pathogenic (HP) IAV in chickens occurs owing to alterations in the HA monobasic cleavage site, which transforms into a polybasic motif. The majority of IAVs exhibit traits of a low pathogenicity pathotype in chickens, presenting with symptoms that range from absent to mild disease. Certain LPIAV H5 and H7 viruses can evolve into HP viruses, potentially resulting in significant morbidity and mortality (Blaurock et al., 2022), thereby greatly impacting the chicken industry.

Avian influenza can have devastating repercussions for the poultry industry, impacting farmers' livelihoods, interrupting international trade, and compromising the overall health of wild birds, hence generating ethical problems. During outbreaks in domestic avian populations, it is typically mandated to slaughter all chickens, regardless of their health status. This aims to control the dissemination of avian influenza and prevent its transmission to further avian populations. This leads to significant economic losses for farmers and a prolonged effect on their livelihoods. Wild migratory birds serve as a natural reservoir for avian influenza viruses. Waterfowl are a specific species that significantly facilitate the dissemination of AI across extensive geographical regions while simultaneously succumbing to AI, resulting in a decline in their overall population (World Organisation for Animal Health, 2024). As of March

2024, the UK has no reported outbreaks of HPAI in poultry or other captive avians (**Table 1.1**). However, in Jan 2025, the influenza has over 20 outbreaks in England and Scotland, and a report of influenza has been documented in humans (UKSA, 2025). With this, extensive guidance has now been issued to keepers of domestic birds, ensuring that they are registered with the Department for Food, Rural, and Agriculture in the UK. Further to this, a more recent report has mapped out the cases of influenza HPAI H5N1 across Europe in poultry and non-poultry/wild birds (**Fig. 1.5**).

Table 1.1: Table to detail the number of cases of HPAI H5N1 in each year of the last outbreak, within England, Scotland, Wales, and Northern Ireland.

	1 October 2021 to 30 September 2022	**1 October 2022 to 30 September 2023**	**1 October 2023 to 28 March 2024**	**Total**
England	134	160	4	298
Scotland	11	38	2	51
Wales	7	8	0	15
Northern Ireland	6	1	0	7

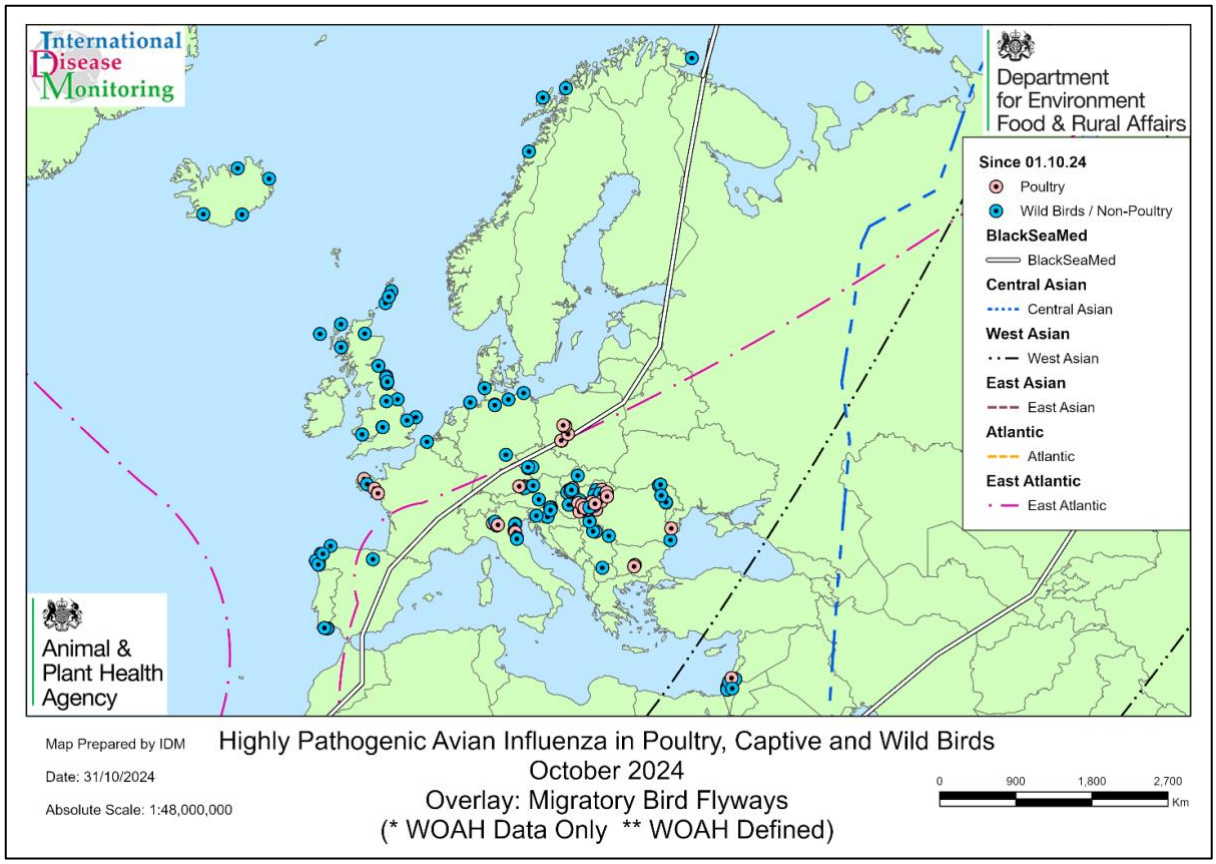


Figure 1.5: Map showing geographical HPAI outbreaks in domestic poultry and wild birds in Europe reported by WOAH between 1st October and 30th October 2024. Cases and outbreaks can be seen across Europe. Red indicates poultry and blue indicates wild birds/non-poultry. (Figure taken from APHA Publications, UK Government, 2024, and WOAH, 2024).

1.3 Host responses against Influenza A virus

There are several factors that contribute to the pathology of highly pathogenic avian influenza (HPAI). The first is the cytokine response, which is known as hypercytokinemia. Hyper-cytokinemia is the build-up of cytokines and results in an inflammatory environment at the site of IAV infection, which leads to immune cell filtration. A depletion of leukocytes occurs at the infection site, resulting in significant pathophysiology associated with these infections (To et al., 2001). Recent research indicates that, post-infection with H5N1 *in vitro*, chicken lung cells exhibit elevated expression of pro-inflammatory cytokines, specifically interleukin (IL)-6 and IL-8. This was contrasted with cells infected with low pathogenic avian influenza

(LPAI), indicating that IL-6 and IL-8 may be crucial regulators contributing to the more severe pathology observed in HPAI infections (Kuchipudi et al., 2014).

1.3.1 Influenza-induced immunity

There are two key pathways that are activated in response to IAV infection. The vaccine-induced humoral immunity provides protection against influenza viruses. The licensed IAV vaccines (existing as either inactivated, live attenuated or recombinant HA/NA) is mediated by the induction of antibodies directed toward the HA and NA subtypes of that specific strain (Jansen et al., 2019). The neutralising antibodies target the head domain of the HA on the virus. More specifically, they target the epitopes located in or around the receptor-binding site (Krammer and Palese, 2013). The antibodies provide sterilising immunity when the vaccine strain matches the strain, resulting in the most infections from the previous year. Once this is achieved, the antibodies block the binding of IAV to its sialic acid receptors on its host respiratory epithelial cells. Antibodies that are specific to the stalk (more conserved region of HA) or NA may also contribute to protective immunity, but their specific mechanism remains unknown (Tan et al., 2016). Antibodies that are specific to NA inhibit the enzymatic activity of NA, therefore limiting further viral spread. In contrast, HA stalk antibodies facilitate the elimination of IAV-infected cells by regulating antibody-dependent cell cytotoxicity (Hayward et al., 2015). Alternative mechanisms include HA0 cleavage (the uncleaved variant of the HA protein), suppression of membrane fusion, and viral egress from infected cells (Christensen et al., 2019). Many antibodies generated by the humoral response aim to inhibit viral particle fusion, preventing the virus from entering the cell entirely.

The second measure of immunity is cell-mediated and is more cross-reactive, therefore protecting against infection of IV of various subtypes and drift variants (van de Sandt et al., 2013). This type of immunity occurs when the virus particles have entered the host cells. Thus, further internal studies at a molecular level are needed to investigate this within the infected cell and what specifically occurs once the virus has entered the cell and what host antiviral responses are present. Viral peptides are associated with the major histocompatibility

complex (MHC) found on the surface of infected or antigen-presenting cells (APCs) and are identified by T lymphocytes. Co-stimulation and engagement of T cell receptors cause further stimulation resulting in the secretion of cytokines and the activation and proliferation of naïve T cells, which result in the killing of infected cells (**Fig. 1.7**). T cells can recognise the viral epitopes of specific strains. This T cell response has been shown to demonstrate multiple mechanisms of protection underlying what is known as 'heterosubtypic' immunity (**Fig. 1.6**). Virus-specific CD8+ T cells facilitate heterosubtypic immunity by identifying epitopes within internal antigens, including M1 (Hilsch et al., 2014), NP (Biswas, Boutz, and Nayak, 1998), and polymerases (Voeten et al., 2000). This subset of virus-specific CD8+ T lymphocytes persists in the host, establishing enduring memory populations that confer protection against subsequent infections. The numbers of T lymphocytes are modulated by inflammatory chemokine signalling (Kohlmeier et al., 2011). After an IAV infection in mice and humans, virus-specific CD8+ T cells can be identified for several months post-infection (Jozwik et al., 2015), facilitating the monitoring of viral dissemination.

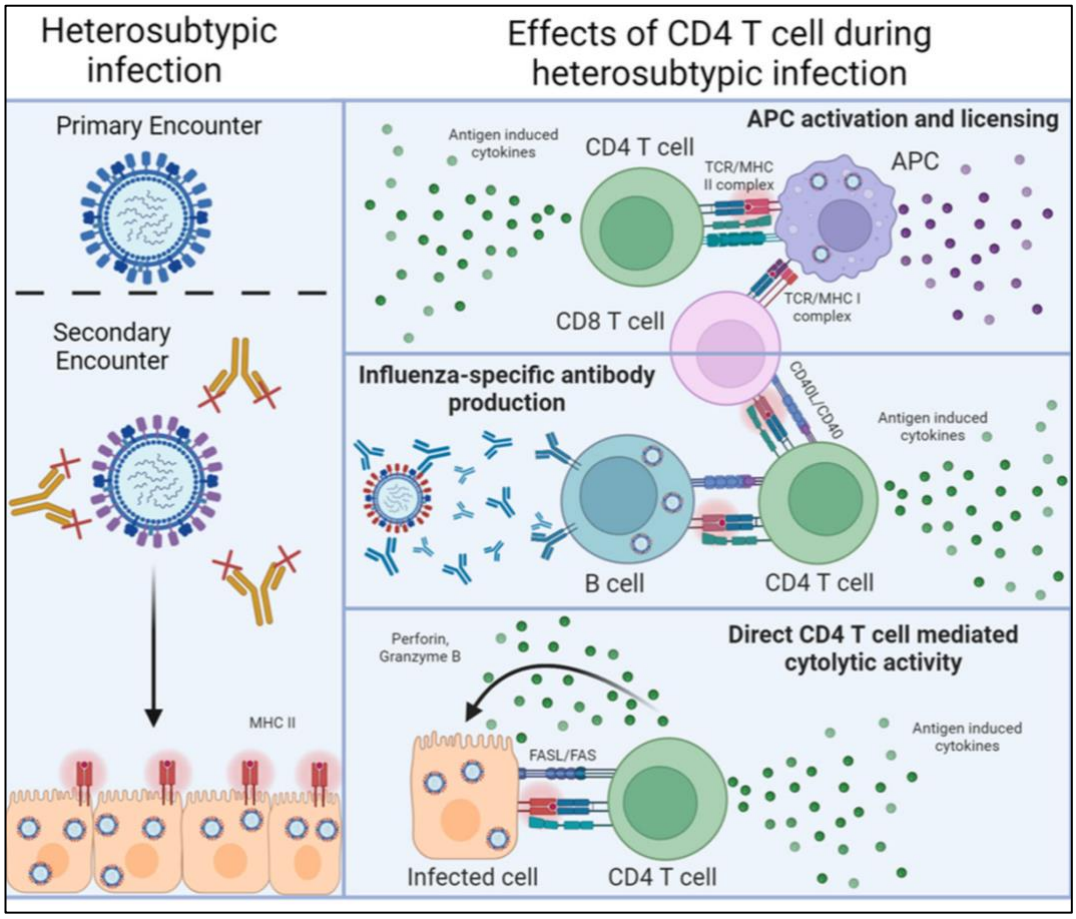


Figure 1.6: Heterosubtypic immunity in IAV infection. The figure displays the interactions that occur through CD4 T cells and how this contributes to heterosubtypic immunity after the viral antigens presented by MHC-II are recognised. (Figure derived from Finn and McKinstry, 2024).

After the infection is established most of the T cells that have differentiated die by apoptosis and the remaining cells then form a pool of memory T cells. These memory T cells stay dormant, ready to respond to a secondary infection, existing as CD4+ and CD8+ T cells (Mueller et al., 2013). The memory T cells are divided into two groups, central memory (T_{CM}) and effector memory cells. Which group they form is dictated by the expression of several surface markers. T_{CM} express high levels of CD62 L and CCR7, which are present in the lymph nodes and tonsils (Sallusto et al., 1999). T_{EM} is characterised by low surface expression of CD62 L and CCR7, and these cells can migrate to peripheral tissues and exert a more potent effector function (Hufford et al., 2014). The contribution of these T cells to influenza infection and the protective immunity has been researched significantly.

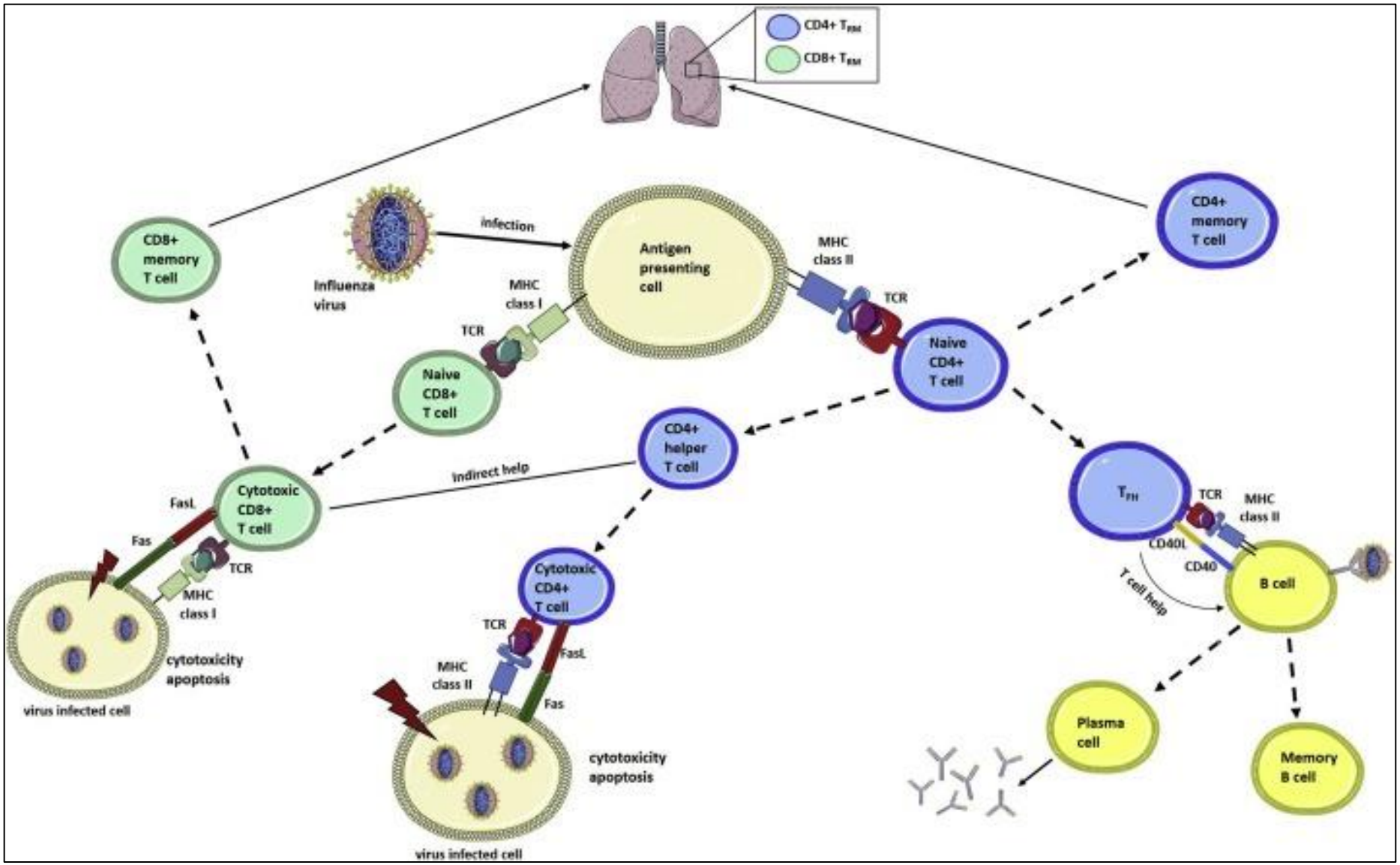

CD4+ T_{RM}
CD8+ T_{RM}
CD8+ memory T cell
Influenza virus
infection
Antigen presenting cell
MHC class II
TCR
CD4+ memory T cell
MHC class I
TCR
Naive CD8+ T cell
Naive CD4+ T cell
CD4+ helper T cell
indirect help
T_{FH}
TCR
MHC class II
CD40L
CD40
T cell help
B cell
FasL
Fas
Cytotoxic CD8+ T cell
TCR
MHC class I
cytotoxicity apoptosis
virus infected cell
Cytotoxic CD4+ T cell
TCR
FasL
MHC class II
Fas
cytotoxicity apoptosis
virus infected cell
Plasma cell
Memory B cell

Figure 1.7: Schematic diagram representing the cellular and humoral responses that are induced by IV infection. IV infection of antigen-presenting cells (APCs) by the virus leads to the intracellular breakdown of viral proteins. These antigen-presenting cells capture viral particles and fragments from infected cells without being infected themselves. These generate peptide fragments that are displayed on MHC class I and II molecules, subsequently identified by CD8+ and CD4+ naïve T lymphocytes. These cells proliferate and develop into effector and memory T cells, aiding in viral clearance by eliminating virus-infected cells and secreting pro-inflammatory cytokines. This facilitates the effective activation of memory T cells after a secondary infection (figure taken from Mueller et al., 2013).

1.3.2 Influenza-induced Adaptive immunity

The adaptive immune response occurs because of acquired immunity and is specific to the virus that is presented. It usually becomes activated due to exposure to the virus and develops from the production of memory B cells, as well as learning more about the virus and enhancing the immune response. This means that the adaptive immune response can provide longer-lasting protection to the virus. The initial function of the adaptive response is to destroy invading viral particles present in host cells. A fundamental feature of this response is that it needs to recognise what is foreign and what is host due to its destructive nature (Bahadoran et al., 2016). The adaptive immune response can be divided into the humoral and cellular immune response pathways.

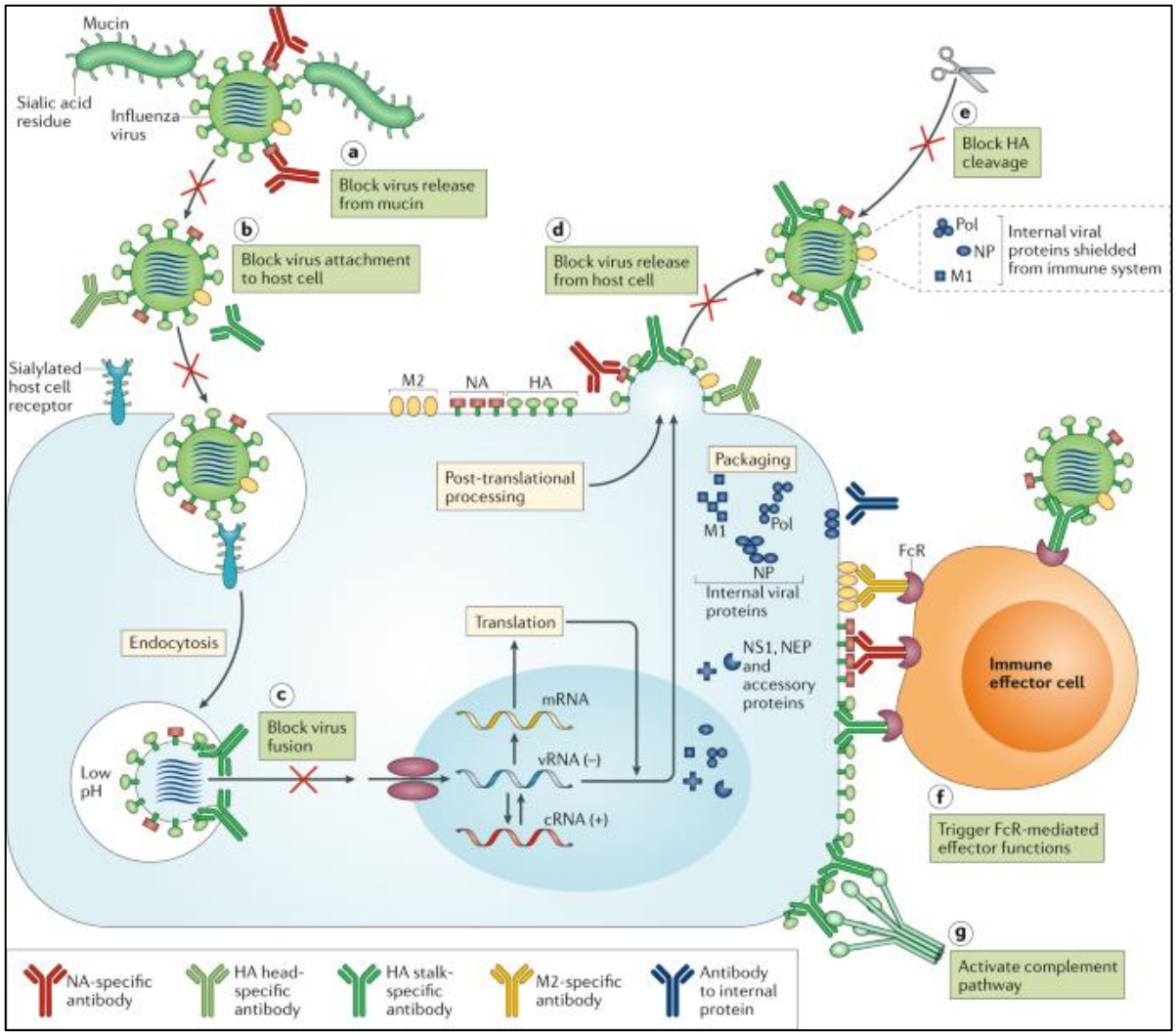


Figure 1.8: Mechanism of action of antibodies against IV. Antibodies disrupt many phases of the viral life cycle. A) Neuraminidase activity, which liberates incoming virions from decoy receptors on mucins, can be inhibited by neuraminidase antibodies. B) Antibodies that attach to the HA head domain can obstruct the interaction between HA and sialylated host cell receptors. C) The fusing of viral and endosomal membranes can be inhibited by antibodies targeting the HA stalk, which prevent it from transitioning to a post-fusion conformation. D) The activity of neuraminidase (NA) for viral release from the host cell is inhibited by the direct binding of antibodies to NA or by steric hindrance. E) HA stalk-specific antibodies attach near the cleavage site of HA, obstructing the cleavage of HA1 and HA2 subunits essential for generating infectious viral particles. Antibodies targeting nucleoprotein (NP) are produced and localised at this location, however their function remains ambiguous. They may induce antibody-dependent cell-mediated cytotoxicity and antibody-dependent cellular phagocytosis. G) Antibodies targeting the HA stalk also activate the complement system, resulting in the destruction of infected cells (Figure derived from Pichlmair and Reise Sousa, 2007).

The main entrance for IV entry to the body is through mucosal tissues, and therefore IgA and IgM are the main antibodies that neutralise mucosal viruses and prevent their entry and replication into host cells. The primary response of the adaptive immune response occurs in organised lymphoid tissues, and the secondary response occurs in the peripheral tissues (van de Sandt, Kreijtz, and Rimmelzwann, 2012).

The cellular response of the adaptive immune response uses CD4+ and CD8+ T cells, and these are induced upon influenza infection. CD4+ cells, commonly referred to as T helper cells, orchestrate the immune response by activating other immune cells. These may include macrophages, B lymphocytes, and CD8+ lymphocytes, which serve to combat infection (Tubo and Jenkins, 2014). CD8+ cells, or cytotoxic T cells, induce death in target cells, leading to the presentation of the corresponding antigen on MHC class I (Zhang and Bevan, 2011). Upon activation of CD8+ cells alongside both Th1 and Th2 cells, these identify virus-derived MHC class II-associated peptide cells that present antigens. CD4+ T cells produce various co-stimulatory ligands that enhance B cell activity and antibody production, notably the CD40 ligand, which is recognised for its significance (Swain, Mckinstry, and Strutt, 2012). Th1 effector CD4+ cells secrete antiviral cytokines, including TNF and IL-2, and stimulate alveolar macrophages. Regulatory T cells (Tregs) govern the responses of both CD8+ T cells and T helper cells following the onset of infection. Interleukin-35, released by Tregs, functions as an inhibitor of inflammatory responses to mitigate infection symptoms (Chen et al., 2016). Th1 cells secrete IFN-β and IL-2 and primarily participate in the cellular immune response. Th2 cells secrete IL-4 and IL-13, which are recognised for their ability to enhance B cell responses.

Cytotoxic T cells targeting viral HA have been demonstrated to induce lung injury via the secretion of TNF-α. The secretion of this protein has demonstrated minimal to no antiviral defence, suggesting that its inhibition may be advantageous in certain instances of severe infection (Xu et al., 2004).

The complete eradication of viruses relies on adaptive immunity, which encompasses the development of a robust CD8 response and a neutralising antibody

response. This protection is effective but not enduring due to the fluctuating characteristics of IAV infection resulting from antigenic drift and antigenic shift (Hartshorn, 2020).

1.3.3 Influenza-induced Intrinsic and Innate immunity

The innate immune system refers to the capacity to identify pathogens or tissue injury through specialised receptors. The activation of the innate immune system has both beneficial and harmful effects during influenza infection. This system is the sole mechanism by which the host can inhibit or attenuate viral replication during the initial stages of infection. The adaptive immune system requires up to 5 days to activate following infection; thus, this period is critical for the innate immune system to generate immediate defensive responses to viral infection and initiate the subsequent production of antibodies and T cell responses (Olive, 2012). Elevated cytokine production by the innate immune system might result in collateral damage to the host. This may manifest as heightened inflammation or aberrant tissue repair mechanisms, especially in the lungs (Flerlage et al., 2021). The principal aim of IV research is to clarify the mechanisms underlying the dual roles of the innate immune system and to ascertain whether defensive responses can be dissociated from the detrimental inflammatory consequences that result in morbidity and occasionally mortality (Fukuyama and Kawaoka, 2011). The innate immune system is conserved and made up of proteins capable of recognising molecules frequently found in pathogens, called pattern recognition receptors (PRRs), to detect pathogen-associated molecular patterns (PAMPs) (Malik and Zhou, 2020). The viral RNA is the main PAMP and is targeted by the system to detect the presence of IV. PAMPs that are presented in the innate immune response are a key feature of the IFN induction cascade to become initiated. These PAMPs primarily characterise viral RNA and are typically absent in cellular RNA. The previously mentioned PRRs are also involved in the induction of IFNs by IVs (Killip, Fodor, and Randall, 2015). Virus-induced lysis results in the release of damage-associated molecular patterns (DAMPs). Pattern recognition receptors (PRRs) can detect viral components, and their activation results in the release of effector chemicals that initiate inflammation, such as pro-inflammatory cytokines and chemokines (Samy and Lim, 2015). This results in the accumulation of additional cells involved in the host

defences. A further anti-viral response is the production of interferon (Gazit et al., 2016). There are three subsets of PRRs that are important within IV infection. These are Toll-like receptors (TLRs), RIG-I-like receptors (RLRs), and NOD-like receptors (NLRs). Research on mice deficient in specific receptors and downstream signalling transduction components has enhanced the understanding of innate immune detection and signalling. Sakaniwa et al. (2023) indicate that TLR3 identifies double-stranded RNA, TLR7 and TLR8 detect single-stranded RNA, and RIG-I recognises 5'-triphosphate RNA, thus demonstrating that these pattern recognition receptors (PRRs) can recognise several kinds of RNA viruses. RLRs are receptors crucial for the generation of interferon during RNA viral infections. Research indicates that mice with a faulty RLR signalling pathway exhibit heightened susceptibility to RNA viruses while possessing a functional TLR system (Kato et al., 2006). NLRs directly identify viral products and DAMPS, thereby initiating processes that lead to the creation of huge complexes known as inflammasomes (Strindhall et al., 2007). These inflammasomes detect IAV infection, which is crucial for the innate immune response (Ichinohe et al., 2009).

There have been key discussions as to whether the innate immune response may result in some particularly harmful outcomes. Reactive nitrogen intermediates (RNIs) and reactive oxygen species (ROSs) have been shown to promote viral mutagenesis and play a role in the production of new viral strains of IV (Akaike, 2001). TLR3 recognises IV RNA on respiratory epithelial cells, which triggers the recruitment of neutrophils and inflammatory responses. Mice deficient in TLR3 had a less inflammatory response and enhanced survival, despite elevated virus titers, indicating that TLR3 may adversely affect host cells.

Intrinsic immunity (**Fig. 1.9**) refers to the capacity of infected and bystander cells to limit infection prior to the activation of innate or adaptive immune cells. Intrinsic antiviral immunity directly inhibits viral multiplication and assembly. This categorises a cell as nonpermissive to a particular class or species of viruses. Intrinsic viral restriction factors identify specific viral components and directly impede viral reproduction, in contrast to pattern recognition receptors (PRRs), which hinder indirectly by stimulating interferons and other

antiviral agents. This is carried out as many cell-intrinsic restriction factors are further up-regulated by type I interferons. The intrinsic response often occurs before the induction of interferons and uses pre-existing antiviral factors of certain cell types. Key antiviral host factors in lung and respiratory tract cells may provide intrinsic immunity. The TRIpartite Motif (TRIM) proteins constitute a family that has several roles in host protection against viral infections. By analysis of a cell line of lung origin, it has been reported that expression of TRIM22 can restrict several viruses, including IAV and similar viruses, as part of the intrinsic immune response (Biondo et al., 2019). Other research has also studied the use of TRIMs and TRIM5, TRIM5α, which have been shown to target the capsids of incoming IV particles. But the specific mechanism of action of these proteins is yet to be determined (Pichlmair and Russell, 2017). The focus of research moving forward is to determine whether the intrinsic and innate immune systems can function without developing excessive inflammatory reactions, which can lead to morbidity and lethality (Li et al., 2018), as well as determining the direct pathway that intrinsic immunity is achieved when an IV infection is present.

1.4 Interferons and ISGs

Interferons (IFNs) are proteins synthesised by many cells during the inflammatory response to infections (Graber and Dhib-Jalbut, 2014). They are a class of cytokines that are responsible for the initiation of a cascade of immune responses against pathogens (Negishi et al., 2017) and consist of three different types, namely type I, type II and type III (White et al., 2008). They are produced by lymphocytes, especially T cells, NK cells, and K cells, as well as macrophages, in response to the presence of pathogens or cytokines. Upon activation, they initiate many molecular pathways that influence cellular responses, encompassing cell proliferation and inflammation, among others. The three types of IFN all signal through distinct complexes on the cell surface that result in the production of IFN-stimulated response

elements and gamma-activated sequence promoter elements. The addition of these elements contributes to the production of antiviral genes (**Fig. 1.10**).

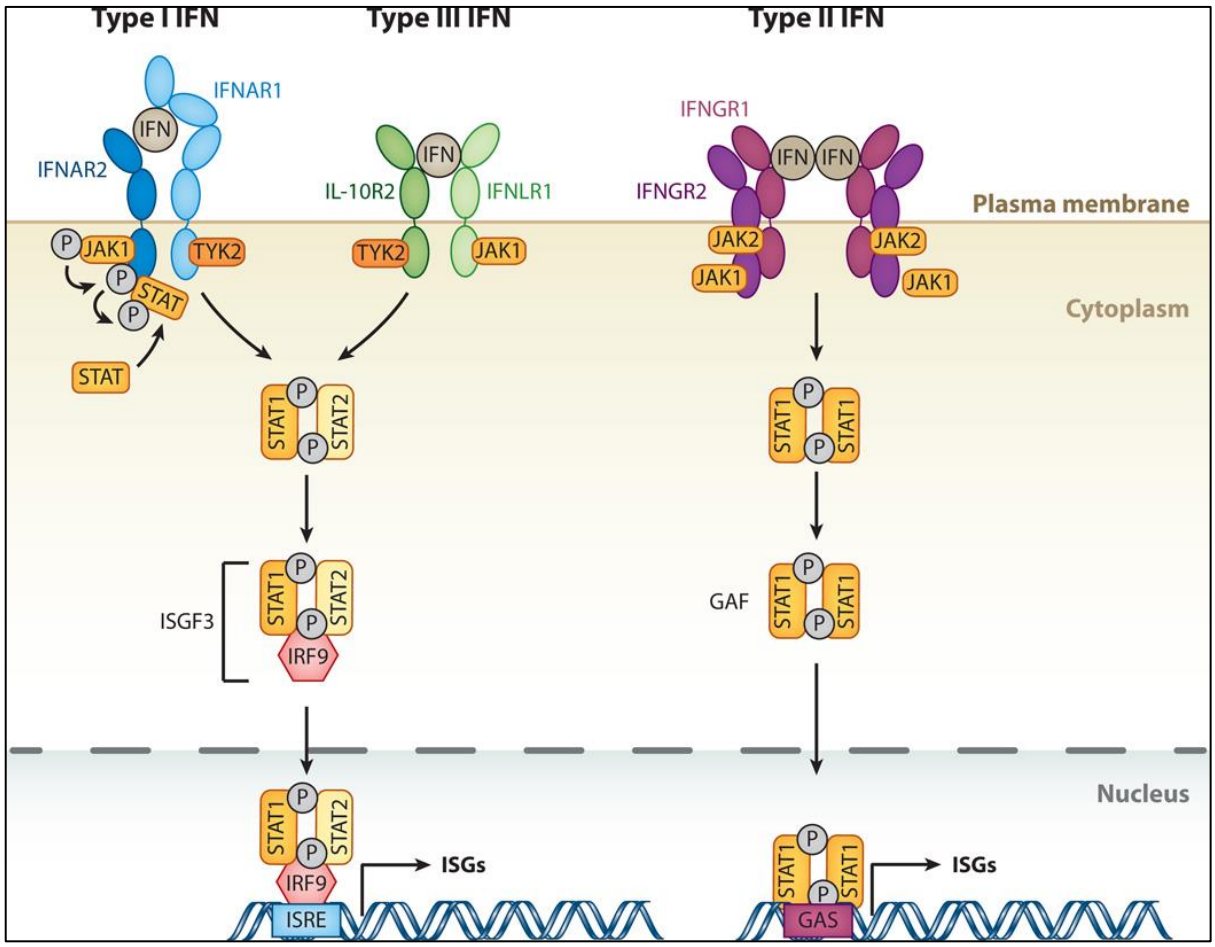


Figure 1.10: Schematic diagram of the interferon-signalling cascade. The three types of IFNs signal through distinct receptor complexes on the cell surface. Type I IFNs act through IFN-α receptor 1 and 2 heterodimers. Type II IFNs act through heterodimers of IFN-γ receptors 1 and 2. Type III IFNs act through interleukin-10 receptor 2 and IFN-λ receptor 1. The binding of type I and type III interferons to their complexes initiates the phosphorylation of pre-associated Janus Kinase 1 and Tyrosine Kinase 2. These then phosphorylate the receptors at designated intracellular tyrosine residues. This results in the recruitment and phosphorylation of signal transducers and activators of transcription 1 and 2 (STAT 1 and 2). STAT 1 and 2 dimerise to attract IFN-regulatory factor 9, thereby forming the IFN-stimulated gene factor 3. The binding of type II IFN dimers to its complex results in the phosphorylation of pre-associated JAK1 and JAK2 tyrosine kinases, while transphosphorylation of the receptor chains facilitates the recruitment and phosphorylation of STAT1. Phosphorylated STAT1 homodimers derived from the interferon-gamma activation factor (GAF). Both IFN-stimulated gene factor 3 and GAF migrate to the nucleus to activate genes governed by IFN-stimulated response elements and gamma-activated sequence promoter

elements. This leads to the expression of antiviral genes (Figure modified from Schneider, Chevillotte, and Rice, 2014).

The Mx family of GTPases plays a crucial role in the assembly of oligomeric rings around viral nucleocapsids to inhibit their nuclear import and/or replication. Viperin is also important in contributing to the inhibition of virus budding and therefore limiting the release of viral particles in infected cells (Gao et al., 2011).

Serine-threonine kinase protein R (PKR) is an interferon-stimulated gene product that plays a crucial role in regulating viral replication. It exists in the cytosol in an inactive form at low concentrations and then rises significantly upon IFN signalling. Upon binding to viral nucleic acids, it induces the phosphorylation of the alpha subunit of eukaryotic translation initiation factor 2 (eIF2α). This leads to the translation of viral and cellular mRNAs and suppresses viral proliferation. It additionally suppresses viral proliferation by enhancing autophagy and death in infected cells (Dauber et al., 2009). During the advanced phases of IV infection, PKR is activated by 5'-triphosphate-dsRNA structures seen in viral ribonucleoprotein complexes.

Type I interferons are recognised for their capacity to directly elicit an antiviral response in infected and adjacent cells by upregulating molecules that inhibit viral replication (Meurs et al., 1990). They are generated early in the course of infection and are crucial for initiating the innate immune response, including the effector capabilities of NK cells (Lee et al., 2017). The induction of both type I and type III interferons in virus-infected epithelial cells is thought to occur in response to the same viral ligands and via the same signalling components (Crotta et al., 2013). The signal from type I interferons occurs in an autocrine and paracrine manner to induce hundreds of interferon-stimulated genes (ISGs). ISGs demonstrate antiviral properties by obstructing phases in the viral life cycle (Zhang et al., 2019). The acute antiviral response is facilitated by a group of interferon-stimulated genes that confer cell-intrinsic immunity. Host vulnerability to viral infection is influenced by multiple factors, although the initial management of previously unencountered viruses is mostly governed by the ISG family.

The significance of ISGs in combating diverse infections is evidenced by the heightened illness severity linked to single nucleotide polymorphisms in genes that encode ISGs (Smith et al., 2019). The number of ISGs that are upregulated in the airway epithelial in response to type I and type III IFNs following IV infection shows an overlap (Fig. 1.9). IFN-induced transmembrane proteins (IFITMs) and protein kinase R are examples of ISGs. They obstruct viral entrance and impede the translation of some viral proteins by suppressing the eukaryotic translation initiation factor (EIF) 2a, respectively.

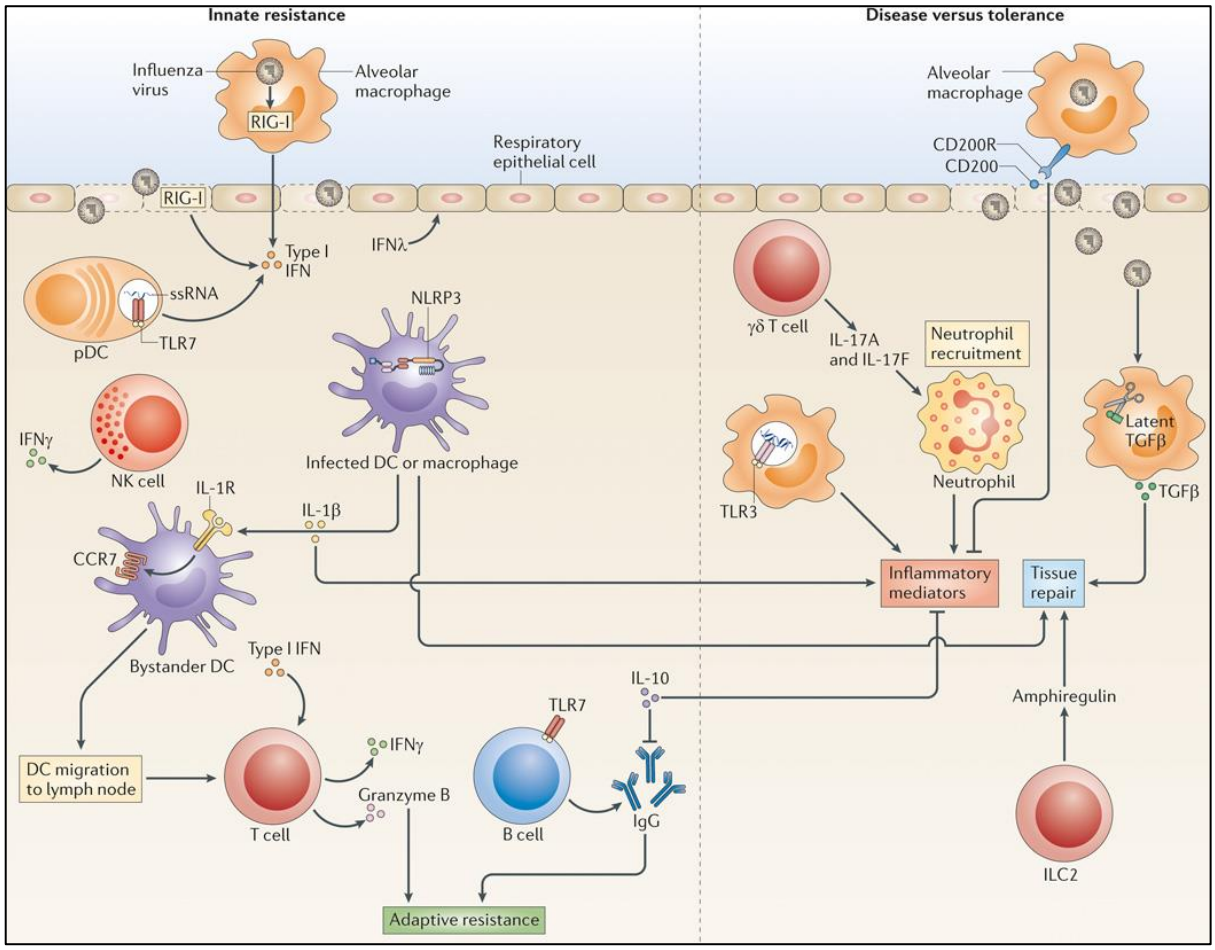


Figure 1.11: Mechanisms of resistance, disease, and tolerance. Innate resistance (left) is provided by type I and type III interferons, which are released upon the activation of retinoic acid-inducible gene I (RIG-I) in infected cells. TLR7 is released by plasmacytoid dendritic cells. Type I interferons influence many cells, while type III interferons specifically target epithelial cells to inhibit viral replication. Infected dendritic cells and macrophages release interleukins, prompting adjacent dendritic cells to enhance CC-chemokine receptor 7 expression and move to the draining lymph nodes to activate T lymphocytes. Inflammasomes enhance disease tolerance by facilitating tissue healing. T cells and natural killer cells release IFNγ to elicit an antiviral response or facilitate B cell-mediated

destruction of virus-infected cells. The production of cytokines induces a pathological alteration due to inflammation caused by interleukins and tumour necrosis factor (TNF). The equilibrium between negative and positive regulators dictates whether the host succumbs to sickness or achieves a condition of tolerance (right) (Figure modified from Li et al., 2020).

It is clear from the responses produced by the innate immune response that the aim is to generate type I IFN to target infection. Studies within mice investigating the deletion of key interferon signalling proteins or receptors have seen results of great mortality after infection with a highly virulent IV strain (Garcia-Sastre, 2006). IVs have developed a means to counteract the type I interferon response due to the NS1 protein of the virus being able to inhibit IFN-β production within the infected epithelial cells (Wang et al., 2000). This inhibition arises from the binding of dsRNA and interactions with NS1 and RIG-I (**Fig. 1.11**). Deletions of the NS1 protein have shown significant prevention in virus replication and through gene array analysis, have shown that this deletion also results in greater induction of IFNs, NF-κβ and other antiviral pathways. There is some contradictory research, as NS1 production has been shown to occur within a few hours of viral infection and therefore blocks the innate antiviral genes by directly binding with cellular DNA (Anastasina et al., 2016). Thus, showing that NS1 may be multi-functional.

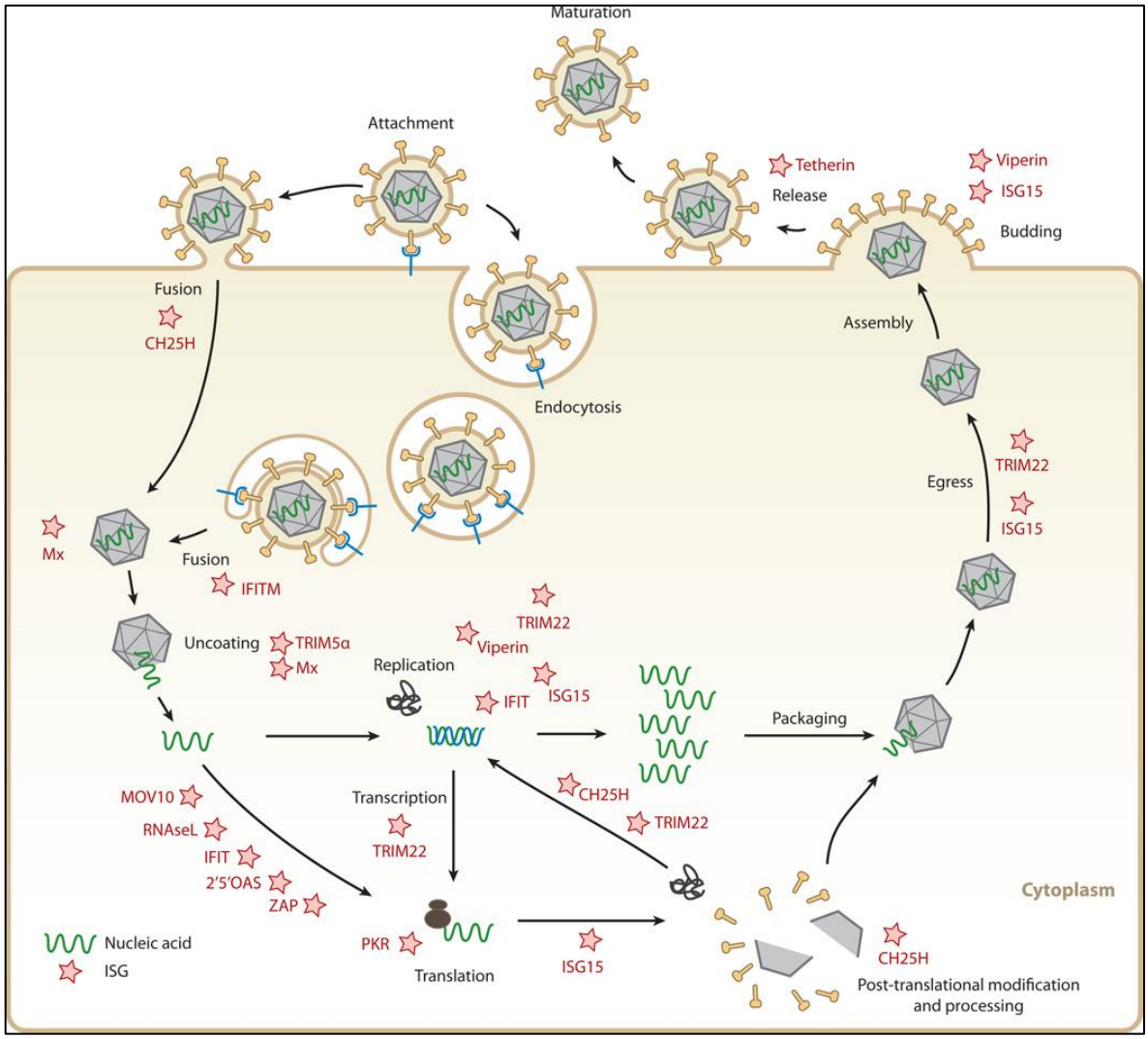


Figure 1.12: Targets for interferon (IFN)-stimulated proteins within viral life cycles. ISG products (shown by a star in the figure) disrupt various stages of the viral life cycle. Cholesterol-25-hydroxylase (CH25H) influences viruses during host membrane fusion events at the initial stage of the cycle. Members of the interferon-induced transmembrane (IFITM) proteins obstruct endocytic-fusion processes of a wide array of viruses. Tripartite motif protein 5 α suppresses human immunodeficiency virus 1 (HIV-1) by facilitating the uncoating of viral RNA. Myxoma resistance protein 1 (Mx1) impedes many viruses by obstructing the endocytic transport of incoming viral particles and the uncoating of ribonucleocapsids. Figure adapted from Schneider, Chevillotte, and Rice, 2014.

Overall, many ISGs pose an antiviral response. It is therefore worth investigating this aspect with regard to IAV infections to determine if the ISG IFITMs can display significant enough antiviral responses to counteract the IAV infection. This is due to the nature that these

ISGs have in respect to their influence on the innate immune system and the ability to strengthen its response (Crosse et al., 2017).

1.5 Interferon-Induced Transmembrane Proteins (IFITMs)

Alongside the virus's capacity for functional enhancement via mutation, recent years have seen a growing emphasis on the influence of host genetic variables in altering resistance or susceptibility to IAV. A variety of factors have been recognised in the prevention of IAV infection, with a novel emphasis on the interferon-induced transmembrane (IFITM) protein family, which, in contrast to other elements like MxA, is primarily regulated by the host rather than the virus in determining the virus's capacity for replication. The IFITM genes were identified in the early 1980s by the laboratories of Stark and Kerr (Friedman et al., 1984). Extensive research has concentrated on investigating their function in obstructing viral infections by inhibiting viral entry into the cell. Human IFITMs are now well known to repress replication of IAV, flaviviruses (dengue virus, tick-borne encephalitis), and HIV-1. IFITM1, 2, and 3 are ubiquitous among several taxa, encompassing amphibians, fish, birds, and mammals. They are characterised by their roles in immune cell signalling and adhesion, oncology, germ cell physiology, and bone mineralisation (Lange et al., 2003).

The human IFITM family comprises five proteins. IFITM1, IFITM2, and IFITM3 are concurrently expressed in many locations and are inducible by type I, type II, and type III interferons. The inclusion of interferon response elements (ISREs) and gamma-activated sequences (GASs) in their promoter accounts for this (Forero et al., 2019). The human IFITM gene cluster resides on chromosome 11 (Zhang et al., 2012). All IFITM proteins encode two transmembrane domains (TM1 and TM2), which are separated by a conserved intracellular loop (CIL). Both terminal domains are present either extracellularly or intravascularly (Smith et al., 2006). TM1 and CIL exhibit significant conservation among the IFITM proteins.

IFITM3 is a broadly expressed interferon-stimulated gene that is recognised for its role in suppressing the reproduction of pathogenic viruses, particularly the influenza A virus

(Diamond and Farzan, 2012). Human IFITM3 was initially discovered by two genome-wide RNAi screens and yeast two-hybrid assays as a host restriction factor for the human influenza A virus. IFITM3 is thought to inhibit the fusion of the IAV virus with target cells during the hemifusion and fusion pore creation stages. This is achieved by diminishing membrane fluidity or by enhancing the spontaneous positive curvature of the outer membrane. IFITM3 is a 15 kDa single-domain transmembrane protein localised inside the endosomal-lysosomal system. It consists of two amphipathic helices orientated towards the cytosolic side of the endosomal membrane, which are crucial for its antiviral effects (Chesarino et al., 2017).

Fluorescence microscopy-based live-cell imaging has demonstrated that lipid mixing between the viral and endosomal membranes can still occur, despite IFITM3 potentially inhibiting the release of the viral genome into the cytoplasm (Desai et al., 2014). Human patients possessing a non-functional allele of huIFITM3 or a mutation in the promoter region of huIFITM3 exhibit an elevated risk for IAV infections, which can lead to severe cases. Furthermore, Ifitm3 mutant mice exhibited heightened vulnerability to IAV, underscoring the critical role of IFITM3 in influencing viral pathogenicity (Gorman et al., 2016). Posttranslational palmitoylation of conserved cysteine residues is essential for the membrane clustering of IFITM3 (McMichael et al., 2017).

Recent investigations have demonstrated that the integration of IFITM3 into IAV particles diminishes the HA protein on the viral surface. This leads to increased sensitivity of the virus to neutralising antibodies (Lanz et al., 2021). During an IAV infection, IFITM3 clusters and localises to endosomal vesicles in primary airway epithelial cells, resulting in an elevated concentration of IFITM3 within these cells. This leads to the formation of vesicles coated with IFITM3 that effectively inhibit IAV infection (Suddala et al., 2019). Although human IFITM1 and IFITM3 have been thoroughly described both *in vitro* and *in vivo*, chicken IFITM1 and IFITM3 are inadequately characterised (Rohaim et al., 2023).

The localisation of the specific IFITM allows for different antiviral functions, and recently, extensive research has been carried out to determine where on the cell different IFITMs localise. IFITM2 and IFITM3 are predominantly found in late endosomes and lysosomes; this is due to the presence of an N-terminal endocytosis motif (Spence et al., 2019). Whereas IFITM1 is found at the cell periphery in the cellular membrane.

IFITM1 has been demonstrated to localise to the plasma membrane. The primary role of this transmembrane protein is to facilitate cellular infection through fusion with the plasma membrane. It has also been demonstrated to inhibit infection by an enveloped DNA virus that can penetrate through the plasma membrane. Research has examined the significance of plasma membrane localisation for IFITM1 functionality and determined that it obstructs the

amino acids located in the conserved intracellular loop (CIL) domain (Smith et al., 2019). This region modifies the subcellular localisation of the protein and diminishes antiviral activity. IFITM1 is induced by type I and type II interferons and is proposed to facilitate the antiproliferative impact of type II interferons. It is most accurately described by contrasting it with B cells, as it lowers the threshold of B cell receptor interaction required for cellular activation (Wilkins et al. 2013).

There is little to no research that has been carried out on the role of IFITM1 that is specific to influenza virus infections. Previous research has looked at virus infection but focused on the aspects of HIV specifically. The mechanisms of action shown in HIV infections from IFITM1 may well be cross-referenced to influenza infections due to the viruses being known for functioning in a similar manner. A previous study has shown that IFITM1 can restrict the influenza virus, and these expression patterns are predicted to be an independent determinant of viral tropism (Huang et al., 2011). In other viral infections, such as hepatitis C, IFITM1 has been proven to inhibit its entry and has been determined as a tight junction protein. They found that the IFITM interacted with the coreceptors of hepatitis C and disrupted the process of viral entry.

DOMAIN	FUNCTION	REFERENCE
N-terminal	Modulates antiviral activity by regulating cellular localisation.	Jia et al., 2012.
Amphipathic helix	Critical role in anti-viral function, through affecting physical properties.	Chesarino et al., 2017.
Intracellular loop	Influences subcellular localisation of protein to reduce viral activity.	Smith et al., 2019.
Transmembrane	Contains two phenylalanine residues (F75/F78) required for IFITM/IFITM interactions for inhibition of viral entry.	Winkler et al., 2019

C-terminal	The sorting signal for IFITM1 and contains multiple motifs that regulate the subcellular localisation and antiviral functions.	Zhao et al., 2018.

Table 1.2: IFITM1 domains and their respective functions.

As noted in **Table 1.2**, the C-terminal domain functions as the sorting signal for IFITM1 and therefore regulates the subcellular localisation and antiviral functions. There is little known information with regards to the influenza A virus, but efforts have been made to investigate the role of IFITM1 in HIV virus infection. A study showed that when deleting amino acids 117-125 of the C-terminal of IFITM1, the NL4-3 strain of HIV-1 was profoundly inhibited. This was shown in contrast to IFITM1 WT, which did not affect HIV-1 virus entry. The investigation concluded that the 117-125 mutant diminished HIV-1 NL3-4 virus entry by 3-fold. Thus, showing the great antiviral properties that the IFITM1 C-terminal domain holds (Jia et al., 2015). Other studies have investigated the role of IFITM1 in hepatitis C virus (HCV) infection. They have concluded that this protein interacted with the HCV coreceptors and subsequently disrupted the process of viral entry, which is like the effects we see with other IFITMs (Wilkins et al., 2012).

As much as these studies have concluded an antiviral property of IFITM1, there is a clear lack of information regarding the pathways in which the inhibition of viral entry occurs. The only comparisons that can currently be made are to the research that focuses on other IFITMs and investigating their known functions with the unknown functions of IFITM1. This will allow us to compare functions and implications that this IFITM has on viral infections.

ChIFITM3 has been demonstrated to limit cell infection by influenza A viruses in a manner analogous to the restriction of IAV infection by human IFITM3. A recent study demonstrated that chIFITM3 is operational in chicken cells, and a reduction in its expression in chicken fibroblast cells led to an increased susceptibility to influenza A virus infection. They demonstrated varying amounts of chIFITM3 within the cells and established a robust link

between expression levels and the percentage of infected cells (Bailey et al., 2012). Overall, chIFITM3 only shares a 42% amino acid identity with its human homologue, but the levels of viral restrictions still show a much greater similarity (Brass et al., 2009).

DOMAIN	FUNCTION	REFERENCE
N-terminal	Contains a motif that is an endocytic signal essential for endocytosis and localisation of IFITM3 to endocytic vesicles and lysosomes.	Jia et al., 2014
Amphipathic helix	Critical role in anti-viral function, through affecting physical properties.	Chesarino et al., 2017.
Intracellular loop	Essential for viral inhibition, pathway is unknown.	Wrensch et al., 2015
Transmembrane	Contains two phenylalanine residues (F75/F78) required for IFITM/IFITM interactions for inhibition of viral entry.	Winkler et al., 2019
C-terminal	Determines that it has a type II and functions for subcellular localisation.	Bailey et al., 2013

Table 1.3: IFITM3 domains and their respective functions.

1.6 Using transcriptomics to understand host-virus interactions

IFITM1 and IFITM3 have been concluded in recent research to be of a major focus in relation to blocking virus entry into the cell of numerous different virus infections. This research focuses on the use of these interferon-induced transmembrane proteins to block the entry of influenza A virus. The research has focused on using chicken IFITMs in chicken fibroblast cells (DF-1) due to the significant similarity shown between the locus architecture of human and chicken IFITMs (**Fig. 1.14**). The difference between human (hu) and chicken (ch) is the chromosome that they are located on, but this is expected due to the different chromosome formations of the two species. Both huIFITMs and chIFITMs are comprised of two exons and

an intro-exon boundary that is conserved across all the IFITMs genes. The IFITMs loci contain four putative IFITM genes. It has previously been reported that both IFITM1-WT and IFITM3-WT show anti-viral properties for viral entry inhibition (Wellington et al., 2019). A comparison of chIFITM1-WT and chIFITM3-WT was carried out using blast sequence analysis, which found that the two proteins were of 77% similarity. Mutations were made within the chIFITM1-WT sequence of the intracellular loop domain of the protein. These mutations consisted of deleting the proteins sequentially in sections of six and replacing them with alanine's. The mutations of deletions and replacements with alanine were within the intracellular loop domain of IFITM1 alongside the two AH mutants.

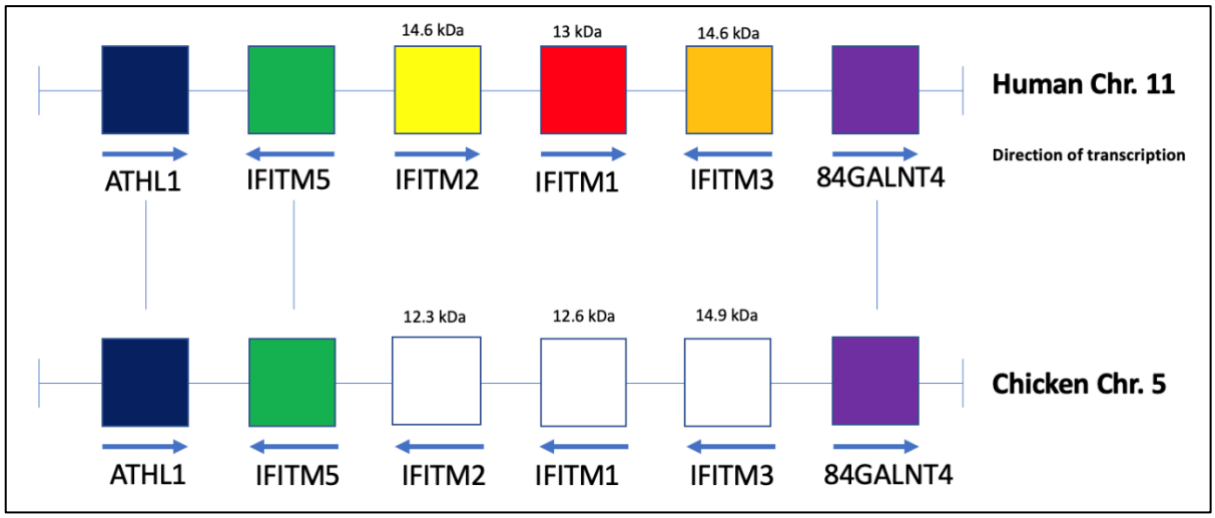


Figure 1.14: The locus architecture of chIFITM and huIFITM. The IFITM gene cluster in chickens is located on chromosome 5 of Gallus gallus. This is bordered by the genes ATHL1 and 84GALNT4. The region is recognised as syntenic with the IFITM gene cluster located on human chromosome 11. All chIFITMs, similar to huIFITMs, consist of two exons, and the position of the intron-exon boundary is constant among all chIFITM genes.

1.7 Conclusions

Major efforts are underway to combat the unmet clinical needs of IAVs, but the seasonal flu pandemic still largely exists. With a need to implement a seasonal flu vaccine still very much active, it shows how far behind the research is on targeting and combatting an effective and efficient treatment plan for the virus infections. As influenza is such a common

infection, there is a need to help the host cells provide a longer-lasting response and remove the collateral damage affects that occur throughout the infection. Interferons are a key part of the immune system, and as explained previously, individual ISGs have been shown to provide key antiviral properties in viral infection, particularly IFITMs. IFITM3 is the most researched and thus so far has shown to block viral entry and prevent viral membrane fusion. It has been noted that this type of IFITM is associated with cholesterol and influences the HA domain of the virus. Thus, preventing the virus from infecting host cells and initiating an infection. Another IFITM that has been noted to show antiviral properties is IFITM1, but this IFITM lacks research that explains its mode of action. The current issues with IFITMs are that on their own they are not sufficient to dramatically decrease virus entry and allow the host to enter a state of resilience. Due to the clear lack of research focusing on IFITMs, it is noted that this is an area that shows promising qualities as a potential target for preventing viral entry of IV. These proteins will become the focus of this research moving forward to determine the strategy that IFITM1 and IFITM3 possess when preventing IV infection. Therefore, more research is needed on the mode of action of these IFITMs that are initially showing antiviral properties to see what mechanisms can be targeted to influence viral membrane fusion at a higher efficacy.

1.8 Objectives

The primary objective of this project is to unravel key structural and functional implications of the chicken IFITM protein repertoire against IAV infection. Additionally, using chIFITM mutants, we aim to map the structural motifs directly influencing the viral entry steps in chickens, which is one of the most susceptible species for IAVs. Specifically,

- Investigate the level of transcriptional activation of chIFITM genes in transcriptomics datasets.
- By applying deep mutational mapping, determining the cellular localisation patterns of chIFITM1 and chIFITM3 in chicken embryo fibroblasts (DF-1 cells).

- Determine for the chIFITM mutants what endosomes they localise to (early, middle, late) resulting in the indications of the specific part of the viral fusion pathways these ISGs may interact with IAV.
- Providing structural and functional insights into the chIFITM1 and chIFITM3 in exerting anti-influenza roles in chicken embryo fibroblasts (DF-1 cells).
- Assessing the potential of transgenic chickens constitutively expressing chIFITM protein as a model to safeguard future avian-origin influenza pandemics.

Overall, these findings will help to underpin multiple aspects of the zoonotic bases of IVs at the point of entry.

Chapter 2

Materials and Methods

2.1 Cell Culture and antibodies

Chicken fibroblast cells (DF-1) were maintained in Dulbecco's modified eagle medium (DMEM), which was supplemented with 10% (vol/vol) fetal bovine serum (FBS) and 1% anti-anti. These were kept in a 5% CO_2 incubator at 37˚C. IAV (strain H9N2) was utilised for infection purposes. The primary monoclonal mouse antibody generated against flag-tagged IFITM proteins is known as rabbit anti-Flag and was purchased from Invitrogen. Secondary peroxidase-conjugated anti-rabbit was purchased from Invitrogen. Goat anti-rabbit Alexa Fluor 488-conjugated secondary antibodies were purchased from Invitrogen.

2.2 Influenza Viruses

Influenza A virus strain A/chicken/Egypt_128s_2012 (clade 2.2.1.2) HPAI-H5N1, UDL08 (H9N2) and PR8H1N1 were propagated in 9-day-old specific pathogen free (SPF) chicken eggs and the median egg infectious doses 50 (EID50) were determined in SPF eggs using the Reed and Muench method (Reed and Muench, 1938).

2.3 Reconstitution and propagation of plasmids

Different chicken IFITM plasmids tagged with flag-tag were firstly transformed into *E. coli*. This process consisted of using 30 µl DH5α cells, adding 1 µl of the individual plasmid, and leaving on ice for 20 mins. The culture was then heat shocked at 42˚C for 30 seconds and immediately placed on ice for 2-5 mins. The culture was then transferred to 100 µL of Lysogeny Broth and incubated at 37˚C for 1 hour in a shaking incubator at 200-220 RPM. The transformation mixture was then spread (80 µl) onto LB agar plates containing ampicillin and incubated overnight at 37˚C.

The next step was to multiply the bacteria in liquid culture. The culture was made up of 6 mL of LB broth and 24 µL of ampicillin. Several colonies were picked from the agar plate using a pipette tip, and this was kept inside the Falcon tube. The tube was shaken overnight at 37˚C

at 200-220 RPM. When using the midi-prep kit, a further step was carried out. An extra culture was made by taking 1 mL from the Falcon tube and adding it to a solution of 99 mL of LB broth and 400 µL of ampicillin. This was then left shaking overnight at 37˚C at 200-220RPM.

The next step was to purify the sample by either mini-prep or midi-prep kit.

The mini-prep process occurred as follows:

A total of 1 mL of the culture solution was transferred to a new Eppendorf and centrifuged at 8,000 RPM for 2 minutes. The supernatant was decanted, and the cells were resuspended in the resuspension solution. Lysis buffer was then added, and the solution was mixed by inverting until it appeared cloudy. Neutralisation solution was then added and immediately mixed by inverting and left on ice for 3-5mins. The Eppendorf was centrifuged for 10 min at 15,000 RPM, and the supernatant was transferred to the centre of a GeneJet spin column. The column was centrifuged for 1 min at 12,000 RPM, and the flow-through was discarded. The wash solution was then added and centrifuged twice, and the column was centrifuged once on its own to remove any residual wash buffer. Elution buffer was heated to 70˚C prior to transferring the centre of the column to a new Eppendorf. 25 µl of elution buffer was added to the column, incubated for 2 mins, and then centrifuged at 12,000 RPM for 2 min. The plasmid concentration was then measured using the NanoDrop and stored at -20˚C.

The midi-prep process occurred as follows:

The culture was centrifuged in a Falcon tube for 20 minutes at 4500 RPM. The supernatant was decanted, and the cells were resuspended in the resuspension solution. Lysis buffer was then added, and the solution was mixed by inverting until it appeared cloudy. At this point, it was left to incubate for 3 minutes at room temperature. The neutralisation solution was then added and immediately mixed by inverting and left on ice for 1 min. The solution was then centrifuged for 5 minutes at 4500 RPM, and 0.5 mL of endotoxin binding reagent was added and mixed immediately. A total of 96% ethanol was then added and mixed immediately, then centrifuged for 40mins at 4500RPM. The supernatant was decanted into a new Falcon tube

and centrifuged again for 10 minutes. 96% ethanol was added again, and the white precipitate was avoided. The solution was transferred to a new falcon containing a column and centrifuged for 3mins at 2000 RPM. Wash solution was added twice and centrifuged twice at 3000 RPM for 2 min. The empty column was centrifuged to remove any residual solution. The column was then transferred to a new collection tube, and 0.35 mL of elution buffer was added. This was incubated for 2 minutes at room temperature and then centrifuged for 5 minutes at 300 RPM. The solution in the collecting tube was then transferred to a new Eppendorf. The plasmid concentration was then measured using the NanoDrop and stored at -20˚C.

2.4 Immunofluorescent Assay

DF-1 (chicken fibroblast) cells were grown in DMEM growth media. Cells were transfected with IFITM1 and IFITM3 plasmids at a ratio of 1000 ng of DNA per well in Opti-MEM growth media. Cells were transfected with Viafect in a 1:3 ratio per DNA per well, concluding that 1000 ng of DNA and 3 µL of Viafect were used. Prior to transfection, the cells were incubated in paraformaldehyde for 1 hour on a rocker and then washed with PBS. Cells were then incubated in 0.1% Triton X100 for 10 mins and washed again with PBS. Cells were further incubated with 0.5% BSA for 1 hour on a rocker and then incubated for 1.5 hours in a solution containing BSA and the primary antibody at a concentration of 1500 ng of DNA per well. Cells were then washed three times with PBS and incubated for 1.5 hours with the secondary antibody at an aim concentration of 3000 ng of DNA per well. Cells were then washed twice with PBS and once with distilled water and then incubated with 1:1000 DAPI for 30 mins. The cover slips were then mounted onto a microscope slide using a small drop of Vectashield mounting medium. The slides were then left to dry and sealed with clear nail varnish and dried. The slides were then viewed using a confocal microscope. The slides were visualised using a (Zeiss LSM880) confocal laser scanning microscope, and procedures were performed according to manufacturer recommendations.

2.5 Western Blot

DF-1 (chicken fibroblast) cells were grown in DMEM growth media. Cells were transfected with IFITM1 and IFITM3 plasmids at a ratio of 1000 ng of DNA per well in Opti-MEM growth media. Cells were transfected with Viafect in a 1:3 ratio per DNA per well, concluding that 3000 ng of Viafect was used.

Cells were then washed with PBS and lifted with trypsin and then transferred to an Eppendorf. The Eppendorf was then centrifuged for 3 mins at 10,000 RPM, washed with ice-cold PBS, and re-centrifuged for 3 mins. The supernatant was removed, and the pellet was resuspended in 100 µl of NP40. The cells were lysed on ice for 30 mins, with vertexing every 10 mins. The supernatant was then aliquoted and stored at -80 degrees.

A 12.5% stacking gel was made, and 15 µl of each sample was run against a protein ladder. The gel was run for 1 hour at 50 volts and a further hour at 100 volts in SDS-running buffer. The gel was then transferred onto a membrane using a trans-blot apparatus. The apparatus was run for 45 minutes to transfer the gel to a PVDF membrane.

The membrane was then incubated in blocking buffer for 1 hour. The PVDF membrane was then washed with 1xPBS + 0.5% Tween 20. The membrane was then incubated in a solution containing the primary antibody overnight in a cold room. The membrane was washed again with 1xPBS + 0.5% Tween 20 and incubated with the secondary antibody overnight in a cold room. The membrane was then washed with 1xPBS + 0.5% Tween 20 and then incubated for 1 min in an ECL solution. The membrane was then viewed using the ChemiDoc.

2.6 Plaque Assay

DF-1 (chicken fibroblast) cells were grown in DMEM growth media. Cells were transfected with IFITM1 and IFITM3 plasmids at a ratio of 4000ng of DNA per well in Opti-MEM growth media and seeded into a 6-well plate. 24-hours post seeding the well were inoculated with 450ul of diluted virus sample and was rotated to cover the monolayer with inoculum with

shaking every 20 minutes. 1:1 2x plaque assay medium was mixed with a warm 1.6% agarose solution before solidification with the addition of 3 mL of agarose medium solution. The plates were incubated for 72 hours at 37°C, and then the agar plug was removed from each well. 4% paraformaldehyde was added to each well and incubated for 20–30 minutes at room temperature to fix the MDCK monolayer and inactivate the virus. The paraformaldehyde was removed, and 1 mL of crystal violet was added to each well and incubated for 20 minutes at room temperature to stain the DF-1 monolayer. The crystal violet was removed, and the wells were washed with 1-2 mL of water to remove any excess stain solution. The plates were left to dry, and the number of plaques was counted to determine the number of plaque-forming units for each well.

2.7 IFITM1 and IFITM3 Sequence Analysis

The protein sequences for chIFITM1, chIFITM3, huIFITM1, huIFITM3, and miIFITM1 were found via protein search using NCBI. The sequences were inserted into BioEdit software and aligned for comparison. Key features, including the domains and specific residues of interest, were labelled, including where the mutations occurred to form the mutants being analysed.

2.8 Transcriptional Analysis

DF-1 were infected with an MOI of 1.0 of influenza viruses in triplicate or were left uninfected (mock). Total RNA was extracted and subjected to poly-A enrichment, cDNA library preparation, and RNA sequencing using 150bp reading. The sequence data was used to normalise the reads against mock-treated cells, and total read counts were uploaded into the iDEP software, and heatmaps and volcano plots were generated in the online version of iDEP. iDEP software incorporates R-based coding in the background and provides a highly interactive and user-friendly interface for transcriptomic analysis. Initially, the reads were normalized, and input in the Excel sheet in numerical forms. and exported in the Excel sheet

as tab-delimited file. This is dataset was fed into iDEP against the Gallus gallus genome for differential gene expression and quantification purposes. The output of the heatmaps depicting differential gene expression at a network and gene clustering level were exported as image and used in the presentation of the study.

2.9 Bioinformatics analysis

The protein sequences for chIFITM1 (NP_001336988.1), chIFITM3 (NP_001336990.1), huIFITM1 (NP_003632.4), huIFITM3 (AFF60355.1), and miIFITM1 were found via protein search using NCBI. The sequences were inserted into BioEdit software and aligned for comparison. Key features, including the domains and specific residues of interest, were labelled, including where the mutations occurred to form the mutants being analysed. SDT software was used to plot the differences and similarities between proteins. The 3D structures were predicted using the I-Tasser server. The protein sequence for chIFITM1 was found via protein searching using NCBI. The file was uploaded to PyMOL software, where the structure of the protein was developed to form a schematic diagram. For the mutants of chIFITM1, the structure was labelled in blue, indicating where in the sequence the mutation occurred and within what domain of chIFITM1 the sequence was mutated. Transmembrane domains were predicted using the TMHMM 2.0 online tool.

2.10 Construction and Rescue of RCAS Viruses Expressing chIFITM3

The open reading frame of chIFITM3 was codon-optimised and chemically synthesised in fusion with a Flag tag, then subcloned into an enhanced version of RCASBP(A)-ΔF1 utilising ClaI and MluI restriction sites. This restriction digestion removed the src gene and substituted it with chIFITM3, preserving the splice acceptor signals. The new vector was developed as RCASBP(A)-chIFITM3. The GFP coding sequence was cloned between ClaI and MluI to create the reporter RCASBP(A) system, resulting in the plasmid designated as RCASBP(A)-eGFP. The sequence's integrity and orientation were validated by Sanger sequencing. We

employed previously established methods (Amini-Bavil-Olyaee et al., 2013) to isolate recombinant RCASBP(A) retroviruses. DF-1 cells were transfected with each plasmid using Lipofectamine 2000 in Opti-MEME at a specified optimum ratio of 1:3. The media were altered 6 hours after transfection and substituted with DMEM enriched with 5% FCS and 1% penicillin/streptomycin for 48 hours. Cells were proliferated until the target quantity of 10 million cells per egg was attained.

2.11 Confocal Microscopy

The expression of the report gene (GFP) was observed via fluorescence microscopy, while the replication efficiencies of chIFITM3-expressing retroviruses were evaluated by staining the gag protein of RCASBP(A) and the chIFITM3-Flag tag. DF-1 cells cultured on coverslips in 24-well plates were infected with retroviruses for a duration of 48 hours. Cells were subsequently fixed for 1 hour with 4% paraformaldehyde and permeabilized with 0.1% Triton-X100 prior to incubation with primary antibodies targeting either the flag tag, the gag protein of the retrovirus, or both. Subsequently, cells were treated with the appropriate secondary antibodies for 2 hours at ambient temperature. Cell nuclei were stained with 4’,6-diamidino-2-phenylindole (DAPI), and pictures were acquired using a Zeiss confocal laser scanning microscope. Confocal images were acquired using 40x and 63x high numerical aperture oil immersion objective lenses on an upright Zeiss LSM800 confocal microscope. The dimensions of the image were established at 1024 by 1024 pixels. Multitrack sequential acquisition settings were employed to eliminate inter-channel crosstalk. A 568 nm diode-pumped solid-state laser and an argon ion laser with a 488 nm line were utilised for excitation.

2.12 Generation of Transgenic Chickens and H5N1 Challenge

SPF eggs were procured from a local source in collaboration with the Department of Poultry Viral Vaccines at the Veterinary Serum and Vaccine Research Institute (VSVRI), Agriculture Research Centre (ARC), Egypt. Mosaic-transgenic chicken embryos were produced by inoculating 10 million RCASBP(A)-chIFITM3 or empty RCASBP(A)-WT infected

DF-1 cells into SPF chicken eggs via the intra-yolk sac using 24G needles on day 2 post-embryonation (ED2). Eggs were prepared for 2 hours post-inoculation before being incubated at 37°C with 60–80% humidity in a rotating incubator, with rotation occurring twice daily. Transgenic embryos were permitted to hatch spontaneously at 21 days of incubation (ED21). Each cohort of transgenic chickens was housed individually in containment level 3 isolators. Food and water were supplied ad libitum, and general animal care was administered by the animal house personnel as necessary.

The optimisation of virus dosage (clinical and sub-lethal dosages) for H5N1 was performed in our prior study (Rohaim et al., 2018). Twenty RCASBP(A)-chIFITM3 transgenic chicks, twenty RCASBP(A)-WT transgenic chicks, and fifteen mock-inoculated chicks (positive control) were challenged with 100,000 EID50 H5N1 (clinical dose) twelve days post-hatching. Conversely, 10 chicks were maintained as a naïve negative control group (non-inoculated, non-challenged, inoculated with PBS). All avian subjects across all groups were observed for a duration of 15 days to assess the emergence of clinical symptoms, weight gain, and mortality rates in each group. The experiment concluded on day 35, and all remaining chicks were euthanised. Chick embryos were culled using Schedule 1 method and 14 days old embryos were killed by decapitation. All chicks or chickens were killed by an overdose of anaesthesia.

2.13 Confirmation of chIFITM3 Expression and Quantitative Assessment of the Chicken Antiviral Immune Response

Total RNA was isolated from the tracheas and lungs of transgenic (RCASBP(A)-chIFITM3) and non-transgenic (mock-treated negative control) chickens using TRIzol reagent. 150 ng of RNA was utilised in the PCR reactions employing the SuperScript III Platinum SYBR Green One-Step qRT-PCR Kit as specified. The quantity of chIFITM3 mRNA was assessed in relation to the 28S rRNA. The reactions were conducted with CFX96 Real-Time PCR equipment, and the data were analysed via the ddCt technique.

Total RNA was extracted using TRIzol reagents to assess the expression of innate immunity genes, as previously described. The Invitrogen SuperScript III Platinum One-Step

qRT-PCR Kit was utilised to quantify the mRNA levels of specific innate immune genes in the tracheas of transgenic chickens expressing RCASBP(A)-chIFITM3, non-transgenic (mock-treated positive controls) chicks challenged with H5N1, and negative control birds, relative to the corresponding 28S rRNA (housekeeping gene).

2.14 Virus Shedding and Histopathology

Oropharyngeal swabs were individually collected, placed in virus transport medium, filtered through a 0.2 µm filter, aliquoted, and stored at -70ºC until all samples were gathered for analysis via hemagglutination assay as previously outlined. Tissue samples, comprising trachea, lung, and spleen, were obtained and fixed at room temperature for 48 hours through immersion in 10% neutral buffered formalin, thereafter, undergoing paraffin wax embedding. The 5 µm tissue sections were stained with haematoxylin and eosin prior to examination under a light microscope for microscopic lesions.

2.15 Ethics Statement

All animal investigations and procedures were conducted in strict compliance with the regulations established by the Animal Ethics Committees of the Department of Poultry Viral Vaccines at the Veterinary Serum and Vaccine Research Institute (VSVRI), Agriculture Research Centre (ARC), Egypt. The research adhered to the protocols established by the Veterinary Serum and Vaccine Research Institute (VSVRI) and received approval from the Institutional Review Board. The research institution where the animal experiments were conducted is the national reference centre for biologicals in the country and high standards of ethics were maintained to execute the research. The research was conducted by trained staff in highly enriched and ethically well-managed isolators approved by the local body. The academics and employee of the Lancaster University have not directly worked on the animals and have provided the research material required to conduct the experiments. The digital data was transferred to researchers of this study and the data was analysed and presented in this study.

2.16 Statistical Analysis

Student’s t-test was utilised for pairwise comparisons between the challenged and control groups (both positive and negative). The Kaplan-Meier analysis was conducted to determine the survival rates. A two-tailed Student's t-test and one-way analysis of variance (ANOVA) were employed to assess group differences. Statistical significance is indicated by P values less than 0.05. All data were expressed as the mean ± standard deviation (SD). Statistical analyses were performed utilising GraphPad Prism 7.

Chapter 3

Structural Insights into the Antiviral Potential of Chicken IFITM1

3.1 Introduction

Virus infections are becoming more and more prevalent due to new pathways being formed, allowing the susceptibility for transmission into the human population. According to the World Health Organisation (WHO), there is an increasing worry that current strains of the influenza virus that are prevalent in mammals may cause a pandemic within the human population globally. When a virus infects a host cell, the innate immune system becomes activated in response. Pattern recognition receptors (PRRs) and inflammasomes are integral components of the antiviral innate immune response. This is due to them detecting conserved viral pathogen-associated molecular patterns (PAMPs) (Carty, Guy, and Bowie, 2021). The innate immune system is a robust mechanism utilised by hosts, wherein the signalling pathways engaged by pattern recognition receptors (PRRs) subsequently induce the release of pro-inflammatory cytokines to attract immune cells. Alongside immune cells, type I and type II interferons are expressed, resulting in the induction of interferon-stimulated genes (ISGs) (Koyama et al., 2008). Under the umbrella of ISGs are a family of proteins known as Interferon-induced transmembrane (IFITM) proteins. The family contains 5 members, including IFITM1 and IFITM3, which have shown key anti-viral functions (Smith et al., 2015). While IFITMs have been well characterised in humans and mice, there is a shortage of functional evidence regarding this ISG family in other species. But as avian influenza infections pose more of a threat to humans, chickens have become a key model for investigating the antiviral functions of IFITMs (Taubenberger and Kash, 2010). Chicken IFITM (chIFITM) is clustered onto chromosome 5 and contains five genes (chIFITM1, 2, 3, 5, 10) (Smith et al., 2013). Both human IFITMs and chIFITMs are comprised of two exons and an intro-exon boundary that is conserved across all the genes.

IFITM1 is of particular importance and has been shown to restrict the entrance of enveloped viruses that enter through the plasma membrane (Kim, Won, and Jeong., 2021). IFITM1 has a N- and C-termini, two transmembrane domains, and a conserved cytoplasmic domain (Siegrist, Ebling and Certa., 2011). The N-terminal region of IFITM1 is shorter than

other IFITMs and is found on the periphery of cells in early endosomes (Huang et al., 2011). Although the length of IFITM1 varies between different species, they all have the same domains that are present in the same areas of the cell. The C-terminal domain is present in the lumen, whereas the intracellular loop and the N-terminal domain are present in the cytosol. The amphipathic helix as well as the transmembrane domain sit in the plasma membrane of the cell (Zhao et al., 2019). This also confirms the research of the localisation location of IFITM1 to the plasma membrane.

chIFITM1 is a homologue of huIFITM1 which is located on chromosome 11 and is around 3,956 bases in length (Meischel et al., 2021). The topology of huIFITM1 and chIFITM1 has the same topology although both homologues vary in overall length. The specific functions of each domain within chIFITM1 are still under investigation, but speculations have been made due to the development of mutations within the chIFITM1 sequence. The N-terminal domain is currently known to modulate the anti-viral activity of chIFITM1 by regulating cellular localisation and resulting in the localisation to the plasma membrane to provide antiviral activity (Jai et al., 2012). The amphipathic helix domain is essential for the antiviral action of chIFITM1 by influencing the physical characteristics of the cell (Chesarino et al., 2017). The intracellular loops also influence the subcellular localisation of the chIFITM1 to reduce viral activity (Smith et al., 2019), and the C-terminal domain provides the sorting signal for chIFITM1 and contains multiple motifs that regulate the subcellular localisation and antiviral functions (Zhao et al., 2018). The transmembrane domain contains two key phenylalanine residues (F75/F78) required for IFITM/IFITM interactions for inhibition of viral entry (Winkler et al., 2019). chIFITM1 is only expressed in the bursa of Fabricius, gastrointestinal tract, caecal tonsil, and trachea. In comparison to other chIFITMs that are known for directly blocking viral entry, chIFITM1 has up to now only shown antiviral activity with no specific pathway being determined. A few other viruses have been investigated with respect to chIFITM1, and *in vitro*, the knockdown of chIFITM1 has resulted in enhanced replication of the avian Tembusu virus

(Chen et al., 2017) compared to wild-type chIFITM1 that impaired the replication. This showed the importance of chIFITM1 with respect to viral replication inhibition.

Previous studies have reported that *in vitro* expression of chIFITM1 has been shown to increase the resistance of avian cells to Influenza A (IAV) infection (Smith et al., 2013). The research focusing on this protein with respect to IAV is weak, due to the comparison of chIFITM3 providing more antiviral activity. Current research indicates that transgenic chicks expressing chIFITM1 stably via an avian sarcoma-leucosis virus (RCAS)-based gene transfer system demonstrated complete protection and considerable infection tolerance when exposed to a clinical dose of highly pathogenic avian influenza virus (HPAIV) subtype H5N1 (Rohaim et al., 2021). This study demonstrated that the transgenic chicks exhibited 60% protection against a sub-lethal dosage challenge, resulting in delayed clinical signs and decreased virus shedding. There is a clear lack of research involving the specific pathways surrounding the antiviral aspects of chIFITM1. Studies have projected that certain domains have functions, but this is yet to be backed up by significant investigation, and which part of specific domains results in the antiviral function.

The aims of this study are to investigate mutations of chIFITM1 in different domains of the sequence to determine their involvement in the overall antiviral function of the protein. This will allow key amino acids to be identified as detrimental to the protein's function as well as amino acids in the sequence that do not contribute to antiviral activity.

3.2 Results

An in-depth comparison between CHITM1 and other orthologs.

Studies have previously noted the domains of chIFITM1 but not the comparison to other orthologs. Given these previous findings, we sought to extend them and compare chIFITM1 to other species. A phylogenetic analysis was carried out detailing a variety of species of birds, fish, and mammals (**Fig. 3.1a**), with chickens' closest relation being the

Senegal Parrot. The data for these species was then further analysed to develop a complex identity percent plot, detailing the pairwise identity (up to 100%) for each of these (**Fig. 3.1b**). Due to the nature of IFITM1 and its antiviral properties, it is important to always compare it to its human ortholog. Therefore, the domains of chIFITM1 were compared to huIFITM1 (**Fig. 3.1c**) along with their amino acid lengths for each domain. HuIFITM1 is 125 amino acids long due to its N-terminal domain being 2 amino acids longer in length than chIFITM1 and the C-terminal domain being 18 amino acids long rather than 9 amino acids long in chIFITM1. The amino acid sequence alignment was then performed and compared between chIFITM1 and huIFITM1 (**Fig. 3.1d**). The comparison showed the insertions where huIFITM1 has longer domains and where amino acids are present in huIFITM1 and lacking in chIFITM1.

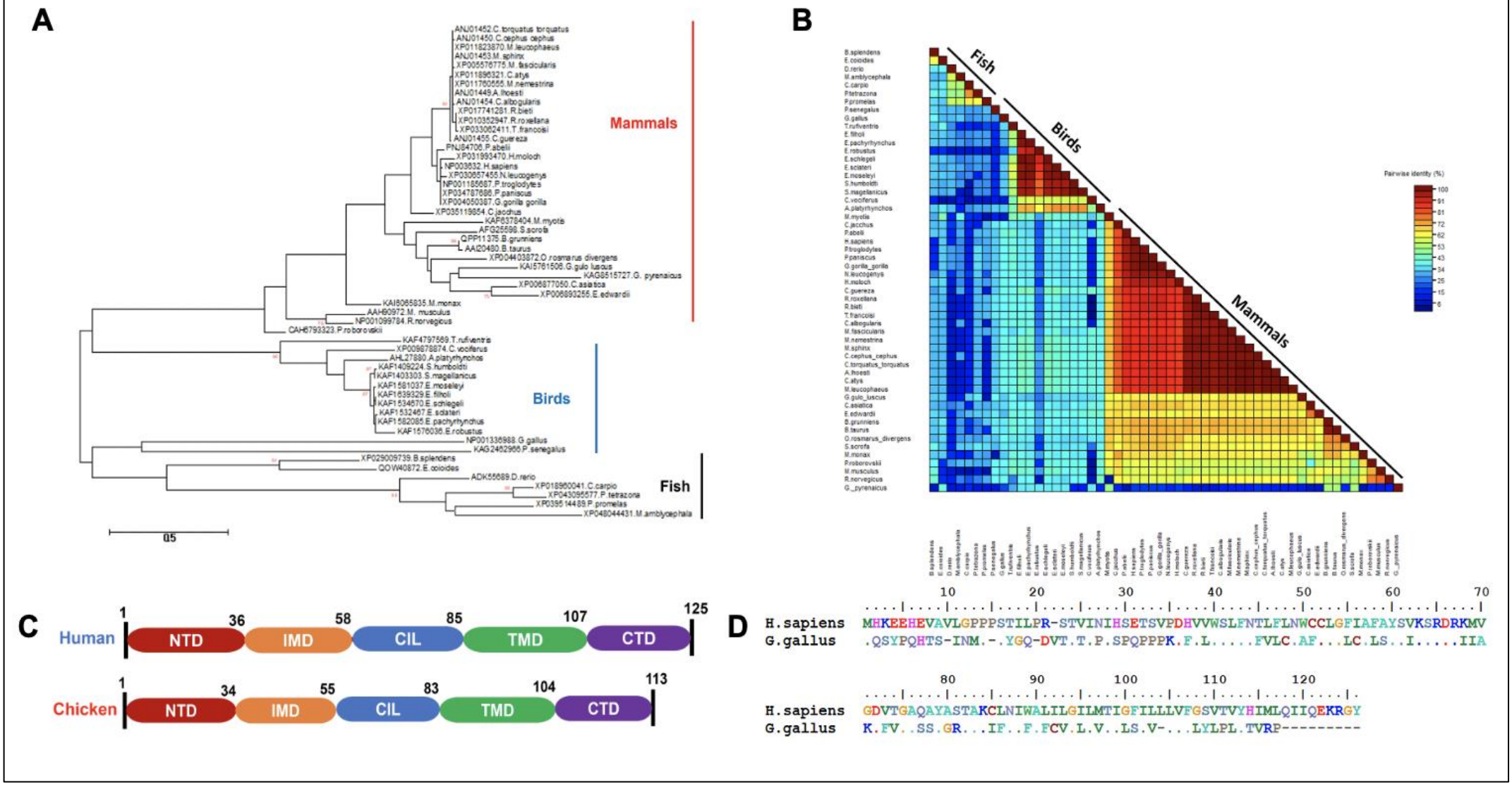

A
Mammals
Birds
Fish
0.5
B
Fish
Birds
Mammals
Pairwise identity (%)
C
Human
NTD
IMD
CIL
TMD
CTD
Chicken
D
H.sapiens MHKEEHEVAVLGPPPSTILPR-STVINIHSETSVPDHVVWSLFNTLFLNWCCLGFIAFAYSVKSRDRKMV
G.gallus .QSYPQHTS-INM.-.YGQ-DVT.T.P.SPQPPPK.F.L.....FVLC.AF...LC.LS..I.....IIA
H.sapiens GDVTGAQAYASTAKCLNIWALILGILMTIGFILLLVFGSVTVYHIMLQIIQEKRGY
G.gallus K.FV..SS.GR...IF..F.FCV.L.V..LS.V-...LYLPL.TVRP---------

Figure 3.1: Structural comparison of chicken IFITM1 protein with various orthologs. (A) Phylogenetic study of IFITM1 across diverse orthologs. Species were categorised by their orders and designated by various hues. The evolutionary tree was constructed using MEGA 6.06. The species name and protein accession number are provided. The scale bar at the bottom denotes the mistake rate. (B) A pairwise identity percentage plot of the IFITM1 protein among multiple orthologs was conducted using the SDT tool. The percentage identity was depicted on the right-hand scale of the graph. (C) Domain architectures of IFITM1 in humans and chickens. Domain structures are denoted as follows: NTD, N-terminal domain (red); IMD, intramembrane domain (orange); CIL, conserved intracellular loop (blue); TMD, transmembrane domain (green); CTD, C-terminal domain (purple). The numbers indicate the number of amino acids within each domain. (D) Amino acid sequence alignment of the whole IFITM1 protein in humans and chickens. The alignment was produced via the ClustalW algorithm within the BioEdit software (Tom Hall, version 7.2).

A heatmap was generated using the transcriptomic datasets in response to influenza infections to determine host cellular genes that are either upregulated or downregulated (**Fig. 3.2a**). Chicken IFITM1 is annotated in the heatmap as well as in the volcano plot, showing a significant expression of chIFITM1 in chicken DF-1 cells.

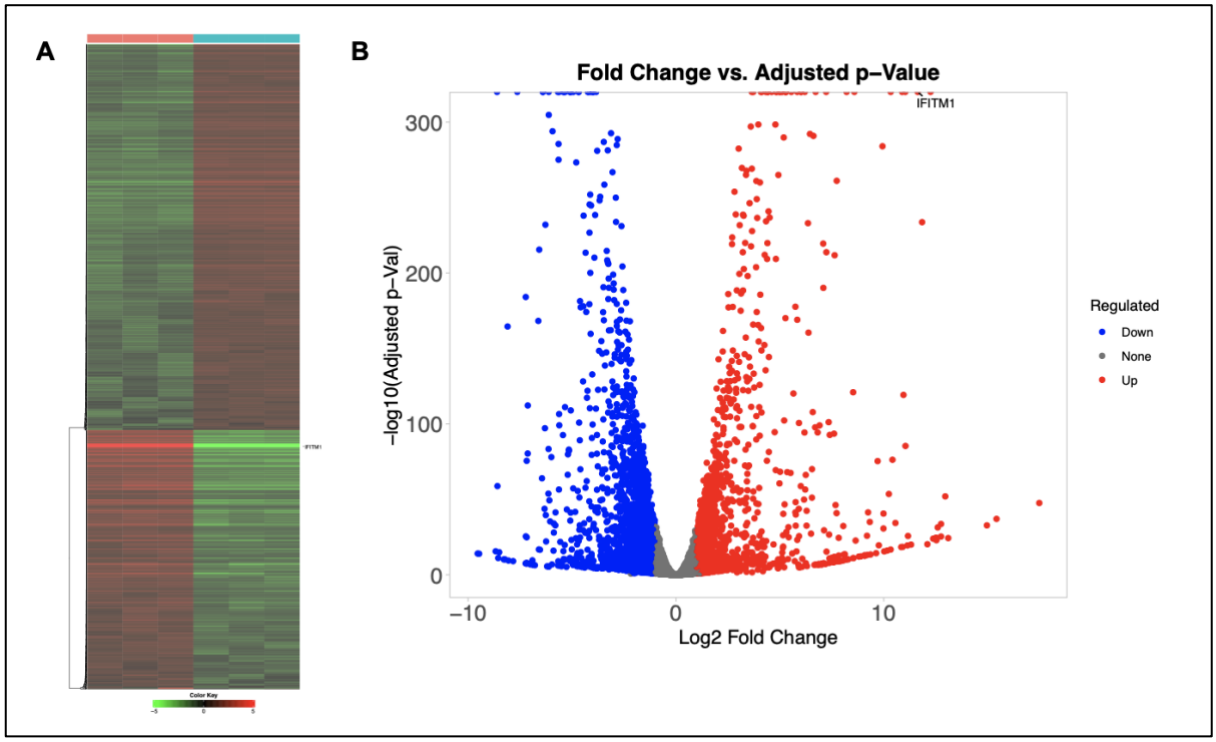


Figure 3.2: Transcriptional regulation of chIFITM1. (A) Heatmap showing the most up- or down-regulated genes where chIFITM1 is highlighted. (B) Volcano plot of up- and down-regulated genes in chicken embryo fibroblasts (DF-1 cells). chIFITM1 is highlighted in the volcano plot.

Different cell lines were infected with the H9N2 influenza virus to determine the relative fold change differences due to the induction of chIFITM1 (**Fig. 3.3**). DF-1 and CEF are a type of embryonic chicken fibroblast cell, whereas LMH are cells that exhibit epithelial morphology that were isolated from the liver of a chicken with hepatocellular carcinoma. Compared with the mock (negative control group), chIFITM1 was significantly expressed in all three avian cells lines. The overexpression of chIFITM1 in avian cell lines exhibited inhibitory effects in cells infected with the H9N2 influenza virus. These data indicate that chIFITM1 is transcriptionally activated in response to influenza infection in all tested cell lines; however, the fold induction of chIFITM1 in LHM was non-significant.

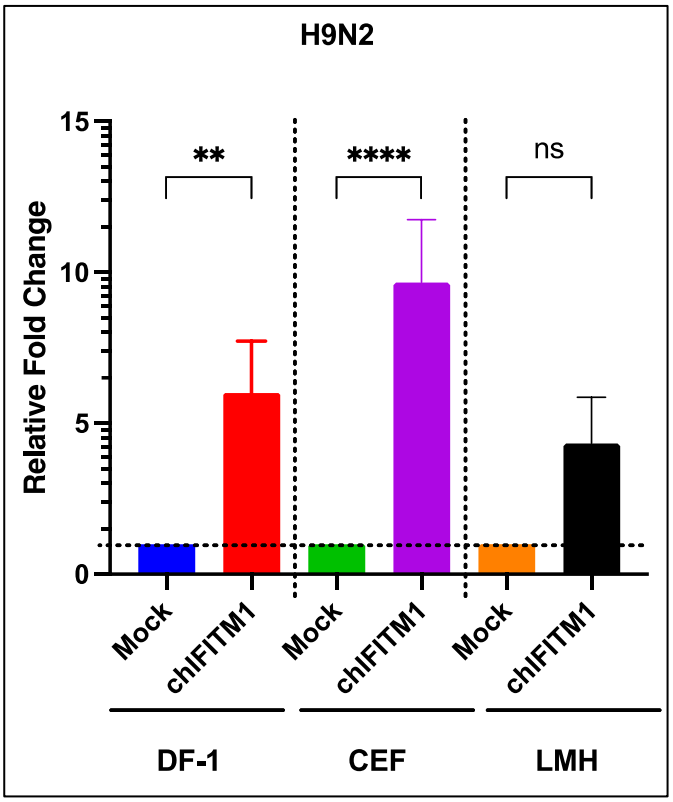


Figure 3.3: Induction of chIFITM1 by the influenza H9N2 in diverse avian cell lines. Chicken cells were infected at a multiplicity of infection (MOI) of 1.0; RNA was collected and quantified for chIFITM1. The induction of chIFITM1 was presented as a fold change relative to unstimulated (mock) cells.

chIFITM1 localises in the cytoplasm of chicken fibroblast cells.

chIFITM1 was expressed in chicken fibroblast (DF-1) cells, and the localisation was assessed by immunofluorescence using an antibody against the C-terminal flag-tag. The analysis indicated that chIFITM1 was localised in the cytoplasm and in the plasma membrane (**Fig. 3.4a**). LAMP1 (a marker for late endosomes and lysosomes) was also expressed in addition to chIFITM1 to mark the localisations in subcellular compartments, and analysis indicated that chIFITM1 co-localised in the lysosomes (**Fig. 3.4b**).

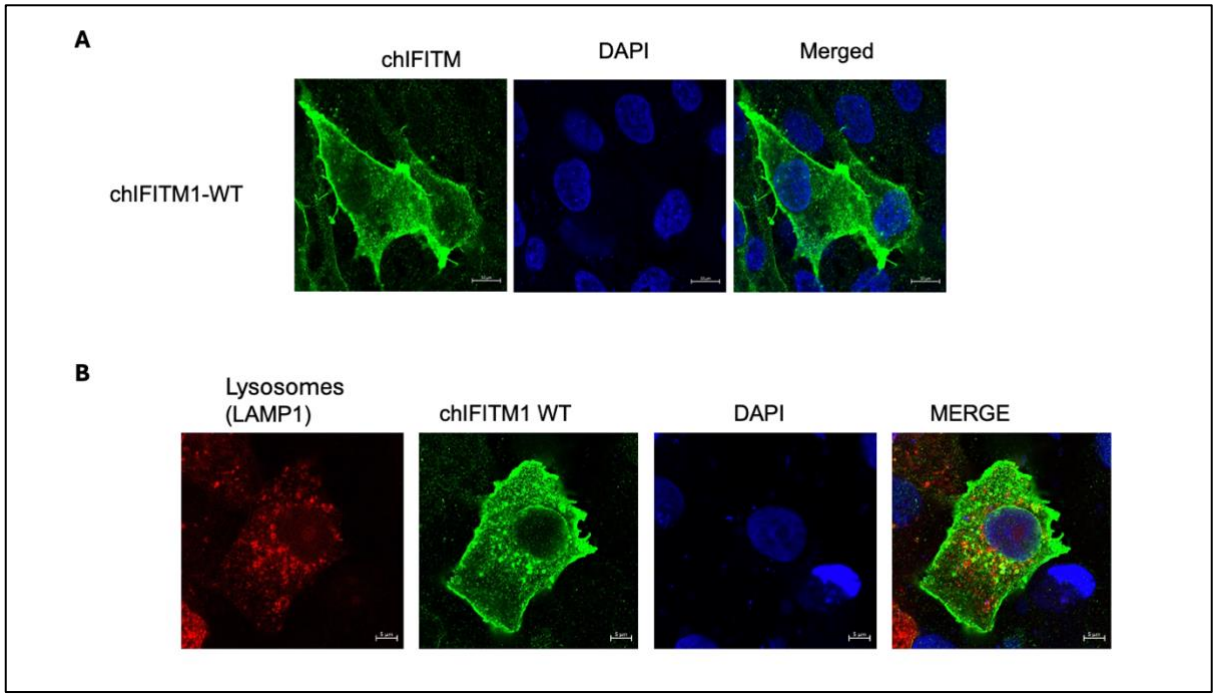


Figure 3.4: Cellular localisation of chicken IFITM1. (A) chIFITM1 localises in the plasma membrane under the normal physiological state of the cells. (B) ChIFITM1 localises in the late lysosomes.

To understand the structural attribution of cellular distribution in the plasma membrane, prediction of transmembrane helices was predicted using TMHMM tools. A total of two

transmembrane domains (WSL.SYSI. and IFN.VFL.) were detected (**Fig. 3.5a**). Based on these predictions, five domains were colour-coded for structural and functional mapping of critical residues for the functionality of chIFITM1 (**Fig. 3.5b).**

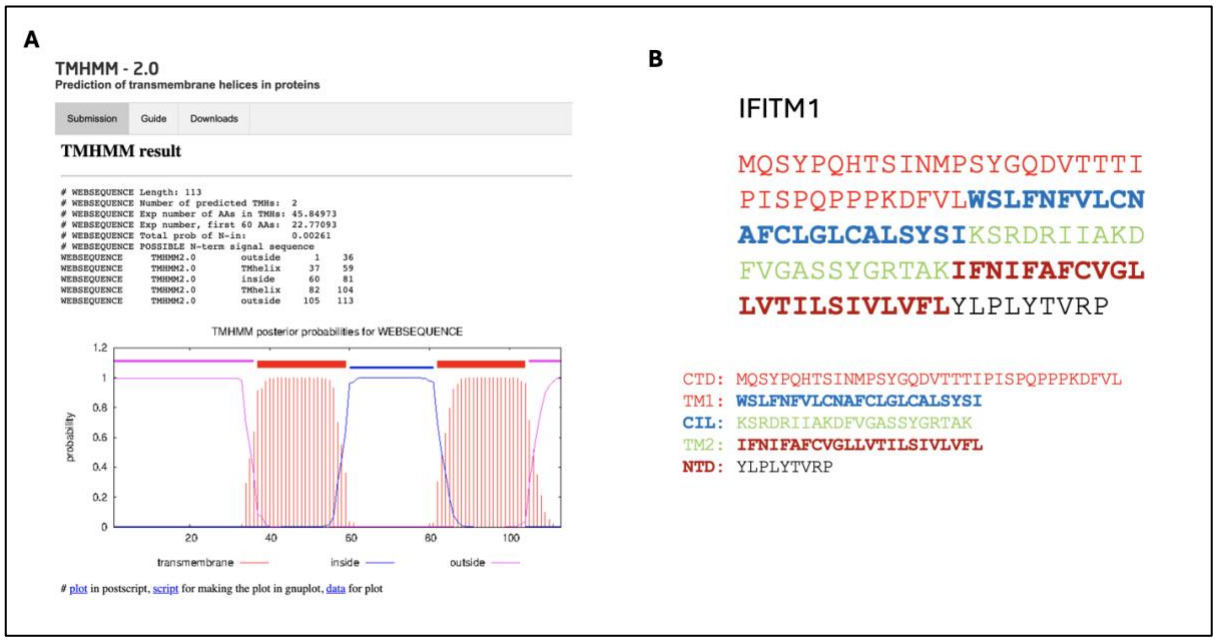


Figure 3.5: Prediction of functional-guided transmembrane domains of chIFITM1

To identify the amino acids crucial for IFITM1 localisation and viral restriction, we created a series of mutant constructs to modify or abolish its amphipathicity. We choose to employ alanine replacements for the residues on the hydrophilic face of the helix (**Fig. 3.6**). This is attributable to alanine's limited hydrophobicity in these places, which should have a negligible effect on the secondary structure. The Iterative Threading Assembly Refinement (I-

TASSER) bioinformatics approach was employed to estimate the structure of chIFITM1, and a confidence score was calculated.

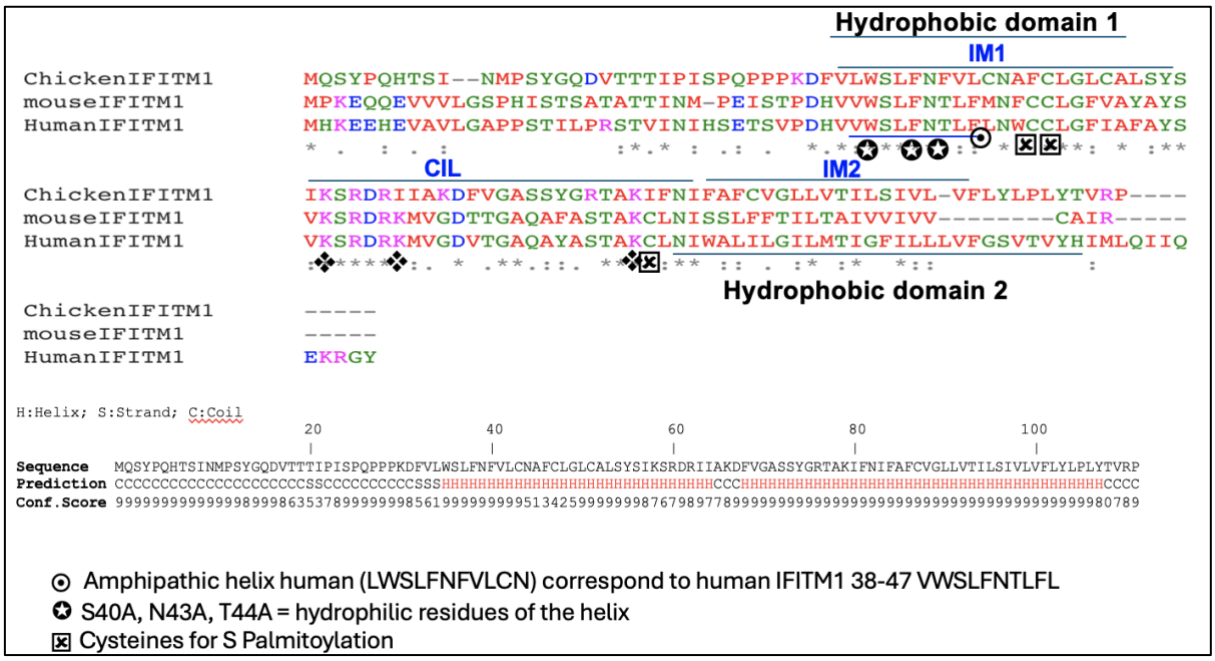


Figure 3.6: Structural annotation of chicken IFITM1 and prediction of several domains, including hydrophobic domains 1 and 2 and hydrophilic residues as well as palmitoylation sites.

Based on the structural annotation outlined in **figure 3.6**, this was further developed to understand the secondary structure of chIFITM1 (**Fig. 3.7**). The chIFITM1 helix, coils, or strands were predicted (**Fig. 3.7.a),** and a 3D model designed from this using the predicted domains was annotated (**Fig. 3.7.b).**

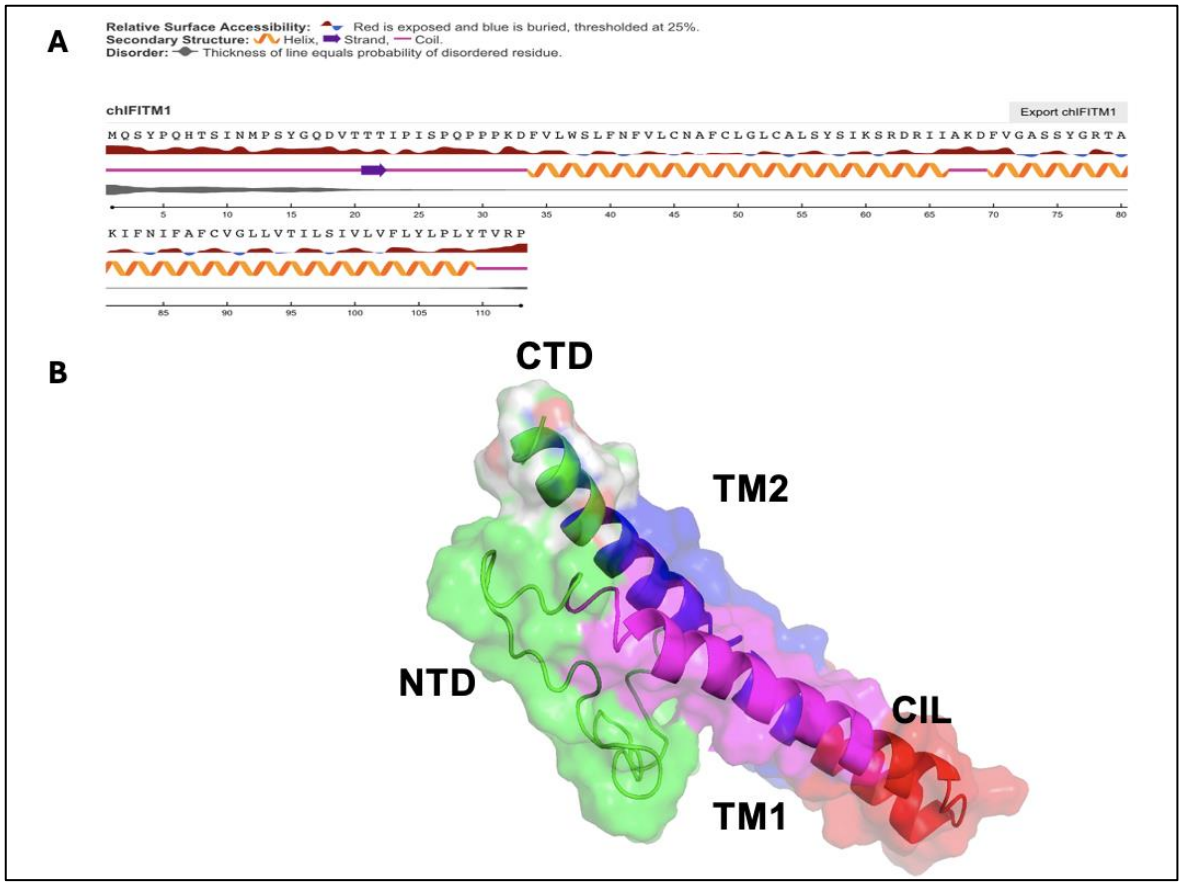


Figure 3.7: Structural prediction and annotation of chicken IFITM1. A) Protein sequence including relative surface accessibility (exposed/buried), secondary structure (helix/strand/coil), and disorder (thickness of line = probability of disordered residue). B) 3D model of the chIFITM1 structure, showing different domains.

Mutating chIFITM1 at specific residues results in a change in protein localisation within DF-1 cells, as well as an increase in plaque-forming units and viral titer levels.

All mutants underwent structural depictions to understand the location and domain of the mutation. The following mutations occurred all within the conserved intracellular loop (CIL) (**Fig. 3.8a)** by replacing the native amino acids with a stretch of alanines. **Fig. 3.8b** compares the cellular localisation of the chIFITM1-wt and chIFITM1 mutants. DAPI has stained the nucleus blue and the green represents the proteins. In each of the figures, chIFITM1-wt is localised to the cellular membrane as expected, whereas each of the mutants is localised in the cytoplasm of the cell. To determine the antiviral effect of chIFITM1-wt and chIFITM1

mutant, DF-1 cells were transiently transfected, followed by infection with influenza A virus. Quantification of virus particles in the supernatant indicated that chIFITM1 mutant (58-63) is as antiviral as chIFITM1-wt (**Fig 3.8c**). Additionally, the cell-to-cell fusion assay showed a comparable fusion by both mutant and wt IFITM1 (**Fig. 3.8d).**

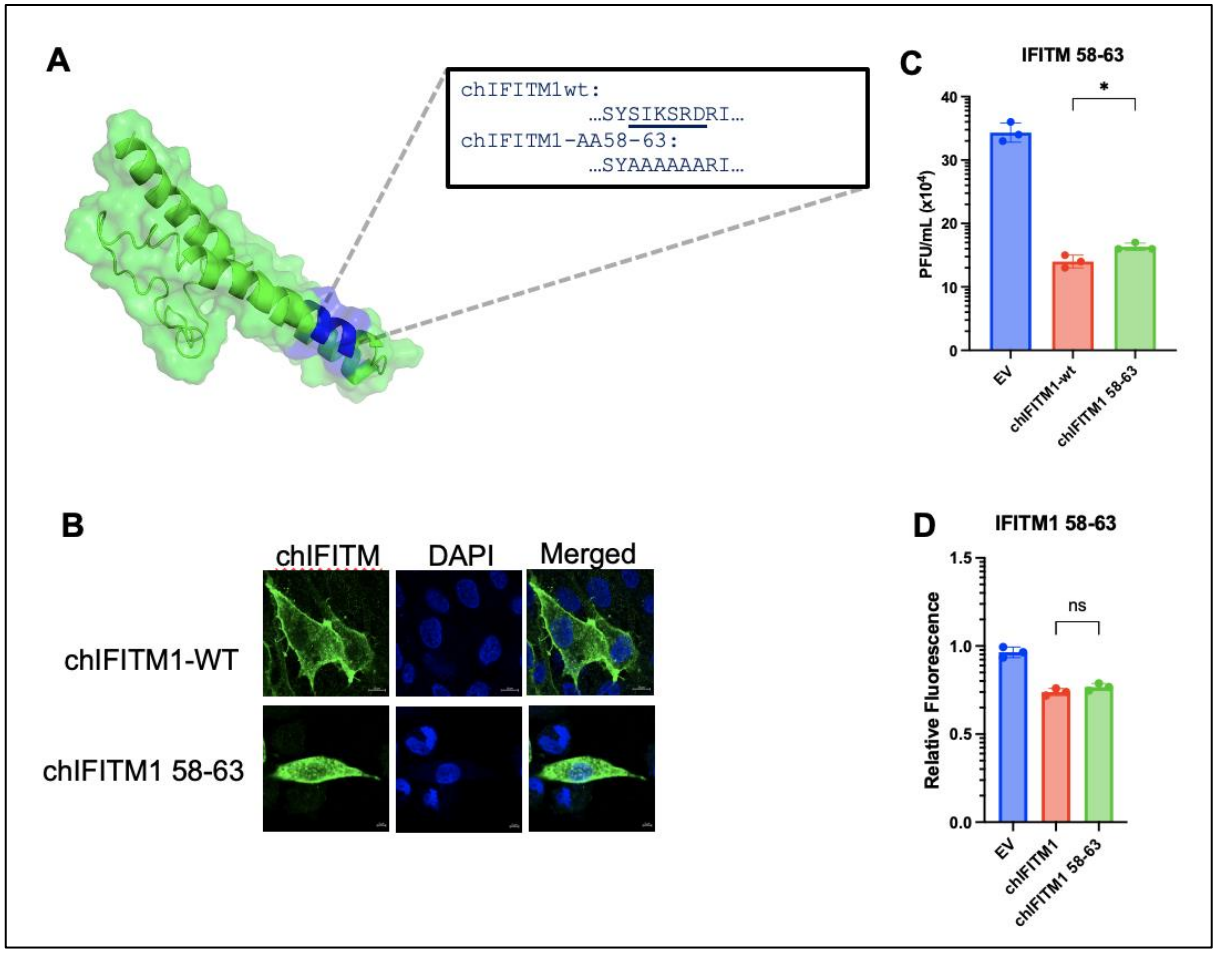


Figure 3.8: Functional characterisation of chIFITM1-AA58-63. A) Structural 3D model of chIFITM1-AA58-63, detailing the change in amino acids that were replaced with alanine's. B) Localisation of mutant protein expression was compared to that of the wild type chIFITM1. HA-tagged proteins are shown in green. C) Plaque-forming unit per mL (x100,000) comparison of infected influenza virus DF-1 cells, of an empty vector, chIFITMwt, and chIFITM1-AA-58-63. D) Cell-cell fusion mediated by chIFITMwt and chIFITM1-AA-58-63 in DF-1 cells compared to an empty vector was quantified using relative fluorescence assays.

Next, a similar set of mutations was introduced as outlined in the 3D model of chIFIMT1 (**Fig. 3.9a**). The chIFITM1 64-69 mutant localised to punctate foci in the cell cytoplasm

compared to the plasma membrane location of chIFITM1-wt. While both mutant and wt-chIFITM1 exerted significant antiviral impact on influenza virus replication, chIFITM 64-69 was significantly lower compared to chIFITM1-wt (**Fig. 3.9c**). A similar trend was also observed for cell fusion (**Fig. 3.9d**), indicating the roles of ammino acids from 64-69 in the functionality of chIFITM1.

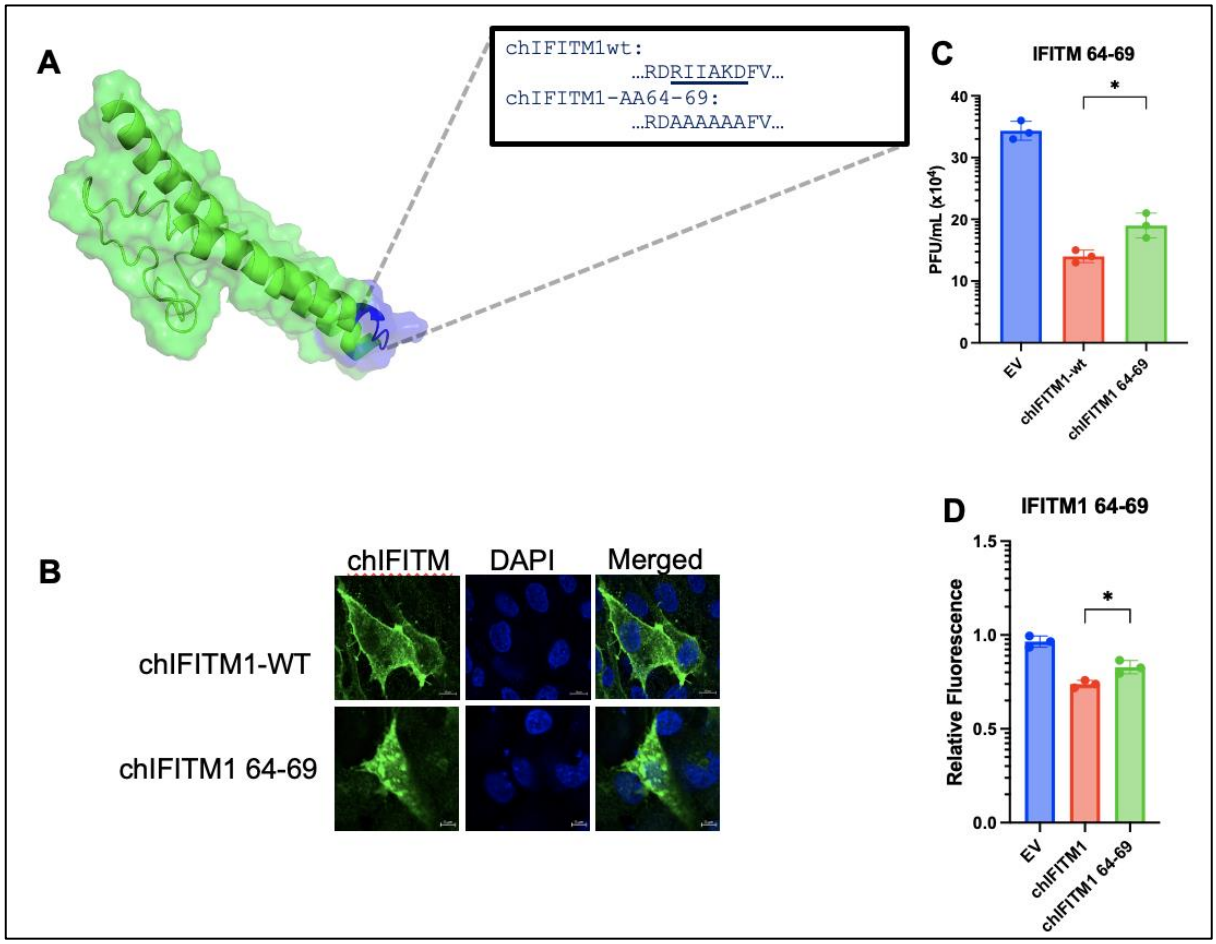


Figure 3.9: Functional characterisation of chIFITM1-AA64-69. A) Structural 3D model of chIFITM1-AA64-69, detailing the change in amino acids that are not alanine's. B) Localisation of mutant protein expression was compared to that of the wild type chIFITM1. HA-tagged proteins are shown in green. C) Plaque-forming unit per mL (x100,000) comparison of infected influenza virus DF-1 cells, of an empty vector, chIFITMwt, and chIFITM1-AA-64-69. D) Cell fusion in DF-1 cells mediated by an empty vector, chIFITMwt, and chIFITM1-AA-64-69.

An additional 6 amino acids (FVGASS) were replaced with 6 As (**Fig. 3.10a),** and the cellular distribution indicated a localisation of the mutant in the plasma membrane; however,

a distinct cellular distribution was also observed (**Fig. 3.10b).** Both mutant and wt-chIFITM1 exerted significant antiviral impact on influenza virus replication; chIFITM 70-75 was significantly lower compared to chIFITM1-wt (**Fig. 3.10c**). A similar trend was also observed for cell fusion (**Fig. 3.10d**), indicating the roles of amino acids from 70 to 75 in the functionality of chIFITM1.

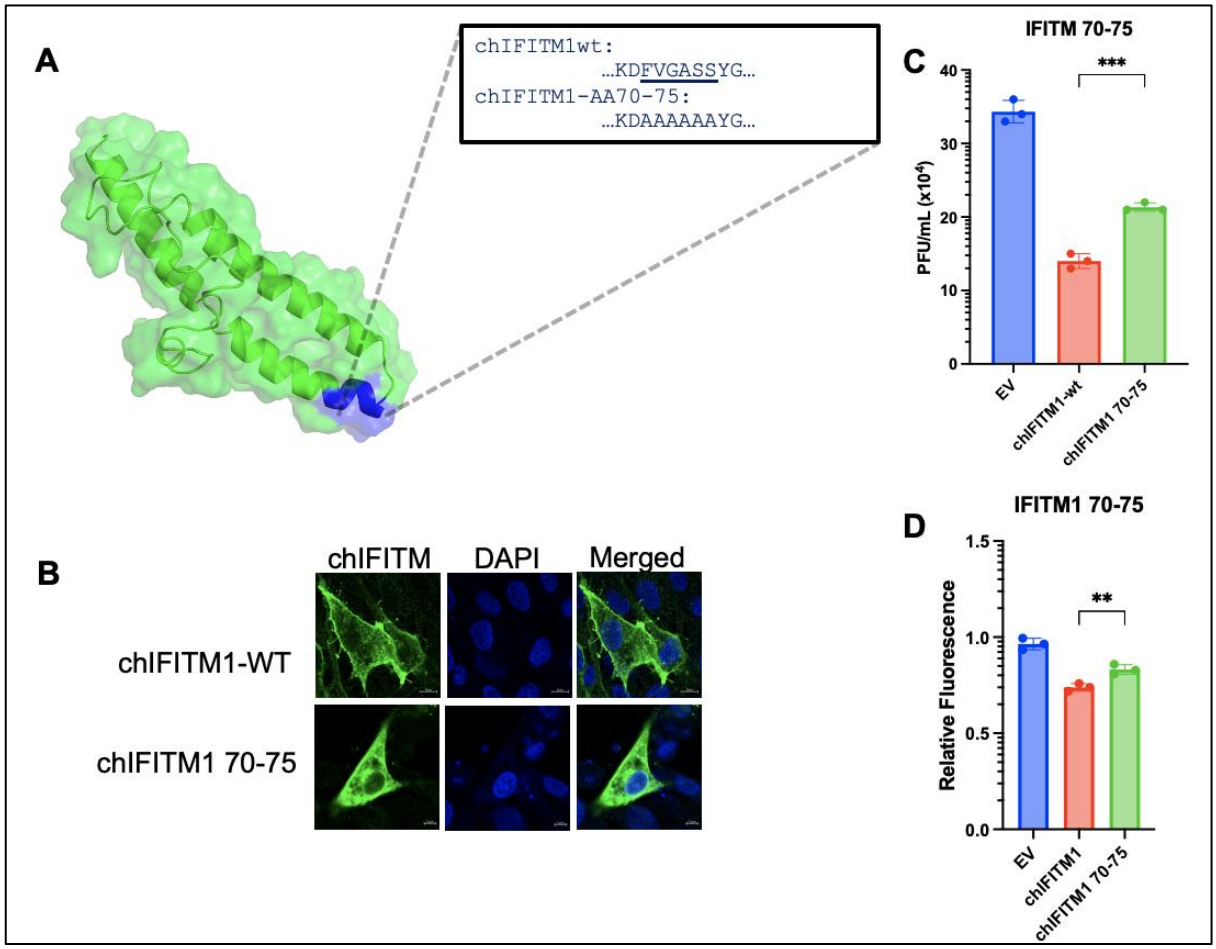


Figure 3.10: Functional characterisation of chIFITM1-AA70-75. A) Structural 3D model of chIFITM1-AA70-75, detailing the change in amino acids that are not alanine's. B) Localisation of mutant protein expression was compared to that of the wild type chIFITM1. HA-tagged proteins are shown in green. C) Plaque-forming unit per mL (x100,000) comparison of infected influenza virus DF-1 cells, of an empty vector, chIFITMwt, and chIFITM1-AA-70-75. D) Cell fusion of DF-1 cells mediated by an empty vector, chIFITMwt and chIFITM1-AA-70-75.

Similarly, 6 additional amino acids (YGRTAK) were replaced with 6 As (**Fig. 3.11a**). The analysis of the cellular distribution indicated a localisation of mutants exclusively in the

cytoplasm around the nucleus (**Fig. 3.11b).** Both mutant and wt-chIFITM1 exerted significant antiviral impact on influenza virus replication; chIFITM 76-81 was significantly lower compared to chIFITM1-wt (**Fig. 3.11c**). A similar trend was also observed for cell fusion (**Fig. 3.11d**), indicating the roles of amino acids from 76–81 in the functionality of chIFITM1 in regulating virus replication, cell fusion, and expression dynamics in the cells.

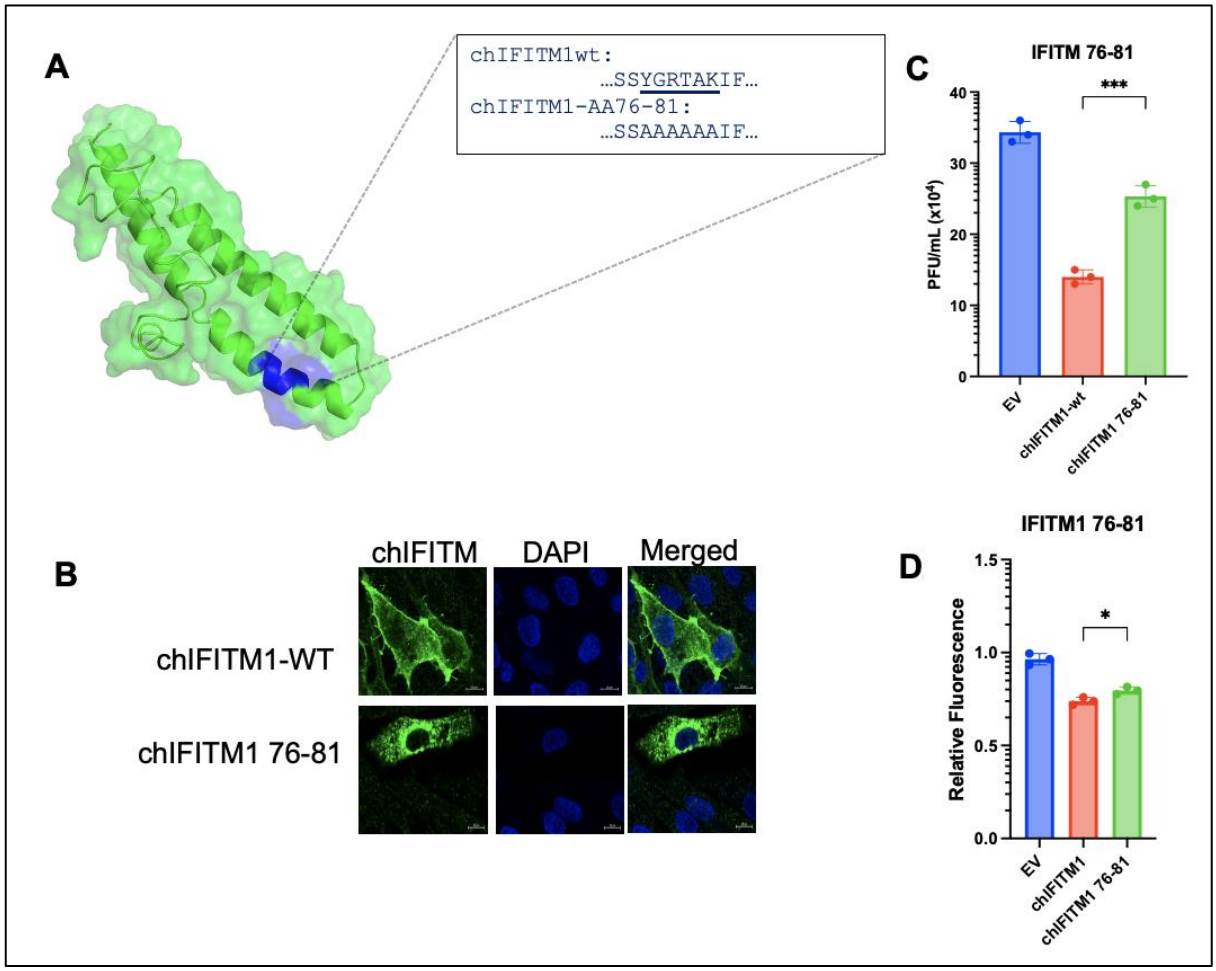


Figure 3.11: Functional characterisation of chIFITM1_AA76-81. A) Structural 3D-model of chIFITM1-AA76-81, detailing the change in amino acids that are no alanine's. B) Localisation of mutant protein expression was compared to that of the wild type chIFITM1. HA-tagged proteins are shown in green. C) Plaque-forming unit per mL (x100,000) comparison of infected influenza virus DF-1 cells, of an empty vector, chIFITMwt, and chIFITM1-AA-76-81. D) Cell fusion of DF-1 cells, elicited by an empty vector, chIFITMwt and chIFITM1-AA-76-81.

The IFITM1 amphipathic helix is required for inhibition of virus protein-mediated membrane fusion.

The protein sequences of chicken, mouse, and human IFITM1 were analysed to determine the amphipathic helix (**Fig. 3.12**). The three key domains for chIFITM1 were compared, and the amino acid differences were identified. Specifically, an amphipathic helix was identified in chIFITM1 (LWSLFNFVLCN) which corresponded to the huIFITM1 (VWSLFNTLFL) along with two hydrophobic domains in IM1 and IM2, respectively.

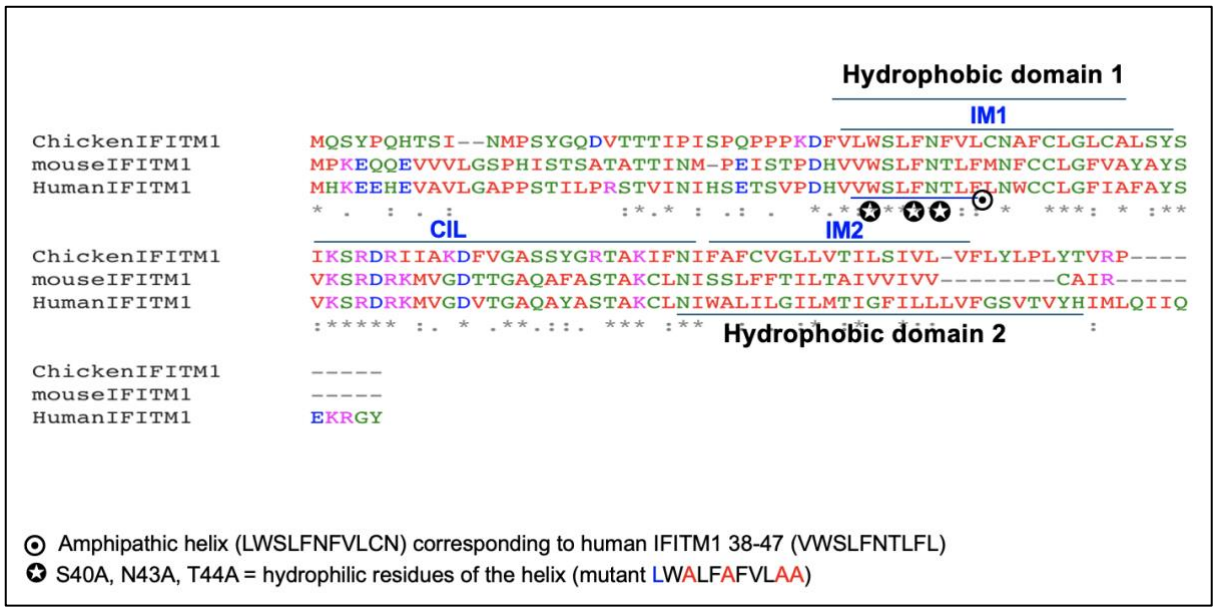


Figure 3.12: Prediction and annotation of amphipathic helix within the chicken IFITM1

The amphipathic helix of chIFITM1 was analysed using HELIQUEST software to determine what changing some of these amino acids to alanines could potentially result in the structure of chIFITM1 (**Fig. 3.13**).

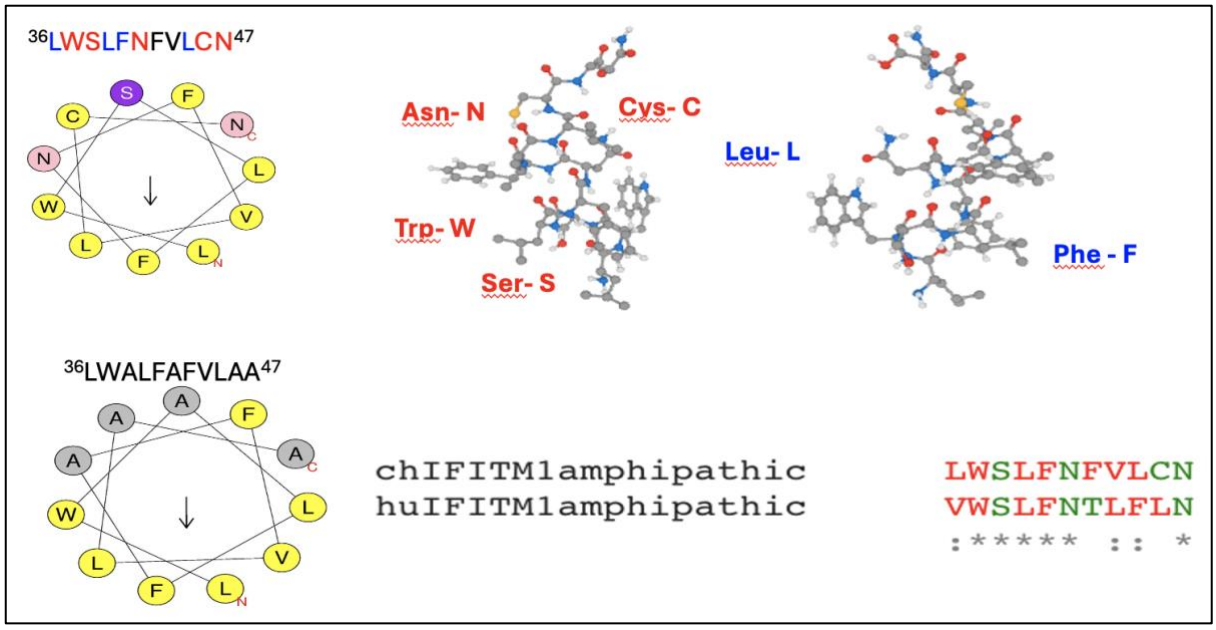


Figure 3.13: Structural attribution of the amphipathic helix within the chicken IFITM1. This image was created using HELIQUEST software. The mutant created to evaluate the amphiphilicity of this helix is also displayed. Hydrophobic residues are represented in grey or yellow, whereas hydrophilic residues are represented in red or blue.

Based on structural prediction and annotation (**Figs. 3.12 and 3.13**), efforts were made to functionally characterise this important motif. For this purpose, two mutants of chIFITM1 were generated within the amphipathic region of chIFITM1 where either the AH domain was deleted or it was mutated with AA (**Fig. 3.14a**). A comparison of the cellular localisation of the chIFITM1-wt and chIFITM1 deleted and mutant indicated that hIFITM1-wt is localised to the cellular membrane as expected, whereas each of the mutants was now localised in the cell cytoplasm. ChIFITM1 with AH deletion made condensed punctate in the cytoplasm, whereas ChIFITM1 with mutated AH domain showed homogenous distribution with somewhat perinuclear localisation of the proteins. Antiviral assays indicate that chIFITM1 mutants (deleted and modified variants) showed a reduced inhibition of influenza virus replication compared to chIFITM1-wt or empty vector (**Fig. 3.14c**). Correspondingly, the cell-to-cell fusion was also reduced in both mutants; however, it was only chIFITM1 with mutated AH that showed non-significant differences (**Fig. 3.14d**). These finding conclude the essential roles of

AH domain in the cellular distribution of chIFITM1, which in turn impacted on the antiviral and cell-cell fusion functionalities of chIFITM1

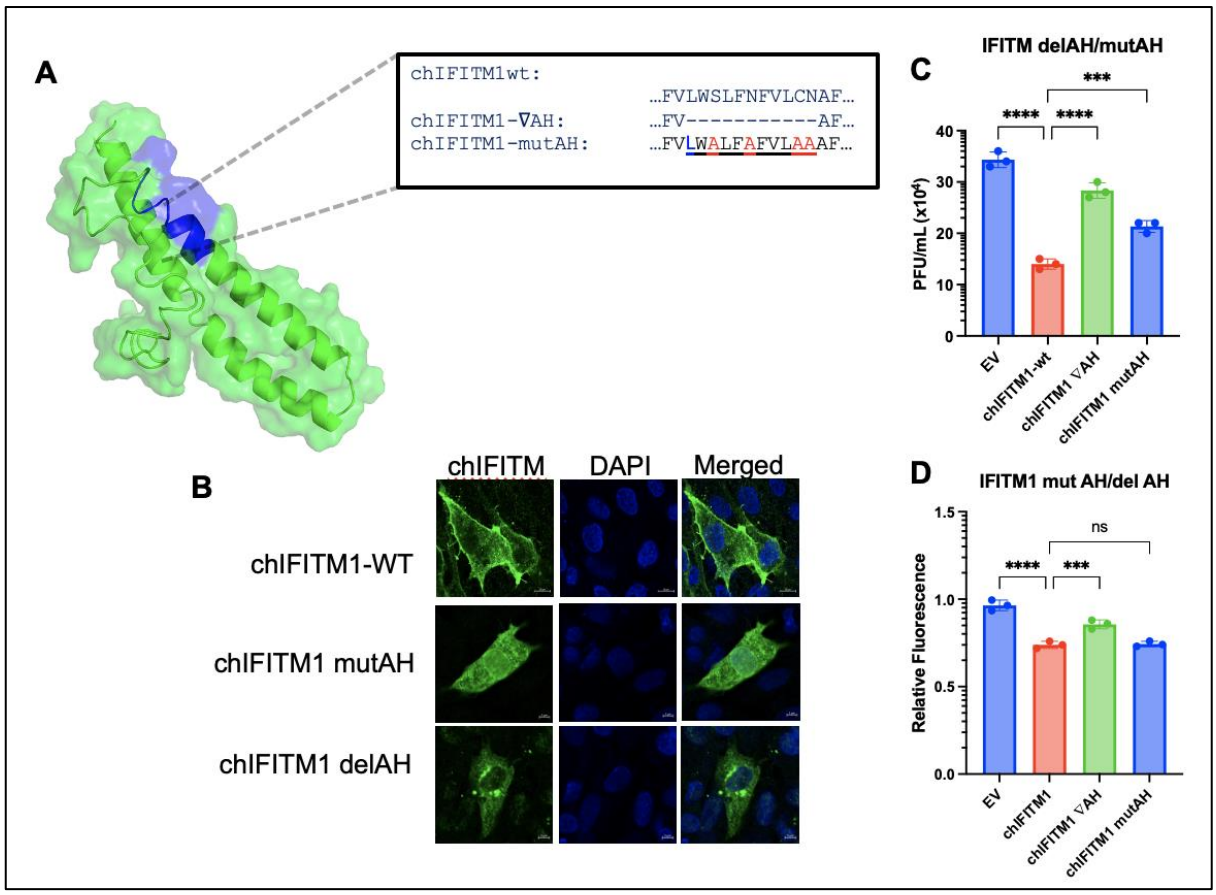


Figure 3.14: Functional characterisation of chIFITM1-∇AH-mutAH. A) Structural 3D model of chIFITM1-AA∇AH-mutAH, detailing the change in amino acids that are not alanine's. B) Localisation of mutant protein expression was compared to that of the wild-type chIFITM1. HA-tagged proteins are shown in green. C) Plaque-forming unit per mL (x100,000) comparison of infected influenza virus DF-1 cells, of an empty vector, chIFITMwt, and chIFITM1-AA∇AH-mutAH. D). Viral titer comparison of infected influenza virus DF-1 cells, of an empty vector, chIFITMwt, and chIFITM1-AA∇AH-mutAH.

3.3 Discussion

In this chapter, we aimed to perform a comparison of mammals, fish, and avian species for their genetic relatedness. Both phylogenetic and similarity (SDT) comparisons clearly reflected that birds display a unique and distinct cluster where *Gallus gallus* IFITM1 (chIFITM1) appeared distinctive compared to all compared bird species. Interestingly, while chIFITM1 was

phylogenetically unique, the domain mapping showed marked similarities in comparison to human IFITM1. However, a range of sequence differences were noticed across the length of chIFIMT1 compared to human IFITM1 in almost all predicted domains. These initial and direct comparisons showed unique evolutionary projects across avian species, especially in chickens.

Like human IFITM1, a marked induction of chIFIMT1 was reported based on the whole genome transcriptomic analysis in response to influenza infection in chicken embryo fibroblasts (DF-1 cells). This indicates that transcriptional activation of chIFITM1 is responsive to viral infection, especially to influenza viruses studied here. Not only that, the chIFITM1 was activated in primary cells (e.g., transcriptomics), but it was also upregulated in established cell lines (DF-1, CEF, and LMH). However, the induction of chIFITM1 in LMH remained nonsignificant. Collectively, this analysis indicates that chIFITM1 is an ISG and is a viral-induced and regulated gene.

We have demonstrated that chIFITM1 is vital for establishing an antiviral state in chicken DF-1 cells against influenza A virus. Importantly, we have for the first time performed sequential mutations of the CIL domain of chIFITM1 and revealed that this domain is essential in determining cellular localisation and antiviral activity. Transiently transduced DF-1 cells were found to express chIFITM1-wt within the plasma membrane, highlighting their roles in establishing the antiviral function by blocking the entry of the influenza viruses. Additionally, we have evidenced that different cellular locations of chIFITM1 can occur through mutation of the CIL domain. Almost all mutations in the form of 6 AAs led to the localisation of chIFITM1 predominantly in the cytoplasm in contrast to the plasma membrane, as was observed in chIFITM1-wt.

Structural annotation followed by cellular location analysis clearly showed localisation of chIFITM1 on the plasma membrane. We attempted extensively to map the structural attributes of chIFITM1 and characterised amphipathic helix in chIFITM1 compared to human IFITM1 recharacterised helix. Additionally, mapping the hydrophilic residues in the helix identified

S40A, N43A, and T44A as key residues. Since palmitoylation is reported as a functionally important character of IFITM1, we identified that out of three well documented cysteines in human and mouse IFITM1, chIFITM1 contained only one conserved cysteine residue.

Taking an extensive and yet systematic approach of sequential 6-residue mutational mapping, we have identified that not only is the cellular distribution impacted, but the function is impaired in terms of antiviral and cell-to-cell fusion assays. This is intriguing to observe that a mutation of only 6 residues altered the phenotype of chIFITM1 in all three assays indicating the sensitivity of chIFITM1 for external mutations. To further characterise the attributes of the amphipathic helix in the functional annotation of chIFITM1, we attempted to not only delete the predicted amphipathic helix but also replace the critical residues with alanine. As anticipated, both deletions and mutations altered the cellular localisation and impacted the function of chicken IFITM1.

Further investigations into how IFITM1 affects avian susceptibility to influenza virus infection are required and if susceptibility is different for other viruses, as not all viruses, especially enveloped viruses, may be restricted by IFITM1. As other viruses may enter the cell by a different route, for example, endocytosis-dependent mechanisms, then they may bypass IFITM1 at the cellular membrane. Subsequently, future research must concentrate on the protein's interaction with the influenza A virus to potentially inhibit its cellular entry. A deeper comprehension of this ISG could enhance the development of innovative antiviral therapies and address avian influenza pandemics globally.

3.4 Conclusion

Deploying a range of structural and functional approaches, functionally important motifs within chIFITM1 were determined that underline the cellular distribution, cell-cell fusion, and subsequent antiviral functions of chIFITM1 against influenza viruses.

Chapter 4

Structural and Mechanistic Regulation of Antiviral Activities of IFITM3 Against Influenza A Virus

4.1 Introduction

Influenza A virus (IAV) is amongst the most prevalent virus infections in avian, and mammalian populations, including humans. IAV belongs to a family called *Orthomyxoviridae* and causes infection by transmission via droplets and results in several well-known symptoms (high fever, cough, body ache, and runny nose). IAV is an enveloped virus, with its envelope derived from the infected host cell by a process known as 'budding off.'. In this step, the newly generated virus particles acquire an outer coat or envelope. Host innate immune system responses are the first line of defence against infection. IAV stimulates these responses through the induction of interferon, resulting in the production of interferon-stimulated genes. During IAV cell entry, viral and cellular signals are sent sequentially through two separate cellular compartments: the endosomes and the cytosol. The innate immune system is conserved and made up of microbial components called pattern recognition receptors (PRRs) to detect pathogen-associated molecular patterns (PAMPs). The viral RNA is the main PAMP and is targeted by the innate immune system to detect the presence of IAV.

The interferon (IFN) system is a subprocess that continues from the innate immune system. Three kinds of IFN exist, and upon activation, they elicit various molecular alterations, encompassing cell proliferation and inflammation. Upon interaction with the cell surface, they generate interferon-stimulated response elements and gamma-activated sequence promoter elements that facilitate the expression of antiviral genes. Type I IFNs are key for targeting IAV infection and are responsible for producing interferon-stimulated genes (ISGs). Currently, IAVs have developed a means to counteract the type I interferon response due to the NS1 protein of the virus being able to inhibit IFN-β production within infected epithelial cells. Interferon-induced transmembrane protein 3 (IFITM3) is a broadly expressed interferon-stimulated gene (ISG) recognised for its role in limiting the *in vivo* replication of dangerous viruses.

IFITM3 was initially discovered by two genome-wide RNAi screens and yeast two-hybrid assays as a host restriction factor for influenza A virus in human research. Previous analysis of IFITM3 has provided evidence that IFITM3 plays an important role in the defence against IAV. Overall chIFITM3 only shares a 42% amino acid identity with its human homologue and therefore provides a great model for investigating the potential antiviral effects in the chicken model. IFITM3 is thought to inhibit the fusing of the IAV virus with target cells during the hemifusion and/or fusion pore generation stages by diminishing membrane fluidity or enhancing the spontaneous positive curvature of the outer membrane. IFITM3 has been shown to co-localise with late endosomes and lysosomal host proteins, which places it at the start of the entry pathway of viruses susceptible to IFITM3. IFITM3 in humans and mice has been extensively characterised, but the homologue in chickens is lacking dramatically.

IFITM3 contains two intramembrane domains (IM1 and IM2), which are separated by a conserved intracellular loop (CIL). The final domains are the c-terminal and n-terminal domains, which display heterogenicity across the paralogs, suggesting that they may directly contribute to the antiviral specificities. There is a significant lack of information regarding these domains and their impact on IAV, and therefore this is a great avenue for prospective research. Recent studies have demonstrated that the N-terminal domain possesses a motif that serves as an endocytic signal crucial for the endocytosis and localisation of IFITM3 to endocytic vesicles and lysosomes. The amphipathic helix plays a critical role in antiviral function by affecting physical properties. The intracellular loop is essential for viral inhibition, but as stated above, the pathway is unknown. The transmembrane domain contains two phenylalanine residues required for IFITM/IFITM interactions for inhibition of viral entry. Finally, the c-terminal domain determines the subcellular localisation of the protein.

Therefore, the role that IFITM3 plays in respect to blocking viral entry will be determined through investigations with chicken fibroblast cells and chIFITM3. This study will

map the functional and structural insight into the antiviral potential of IAV using a range of techniques.

4.2 Results

Identification of the chicken IFITM locus.

The locus architecture of chIFITM1 and huIFITM1 was initially compared (**Fig. 4.1**). The IFITM gene cluster in chickens is located on chromosome 5, bordered by the genes ATHL1 and B4GALNT4. The area is recognised as syntenic with the IFITM gene cluster located on human chromosome 11. All chIFITMs, similar to huIFITMs, consist of two exons, and the position of the intron-exon boundary is conserved across all chIFITM genes. The chicken genome possesses a complete IFITM region with four putative IFITM genes.

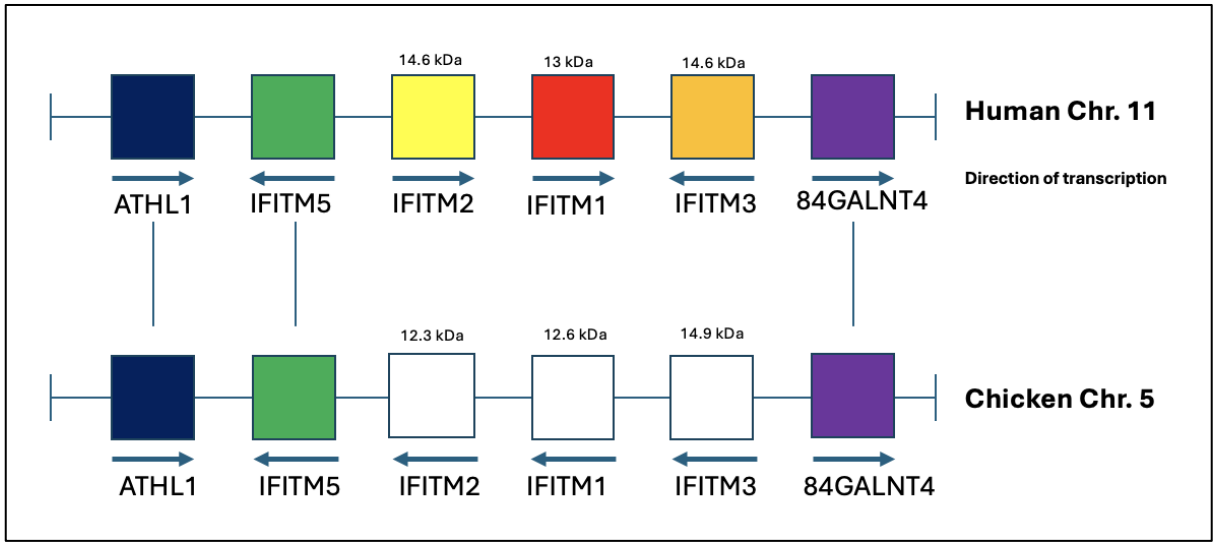


Figure 4.1: The locus architecture of chIFITM and huIFITM. The IFITM gene cluster for chickens is on Gallus gallus chromosome 5.

An in-depth comparison between chIFITM3 and other orthologs.

Previous studies into chIFITM3 have started to analyse the sequence, but we sought to compare it to other orthologs. Firstly, a phylogenetic analysis was carried out, detailing a

variety of mammals, fish, reptiles, and birds (**Fig. 4.2a**), with chickens' closest relation being the Senegal Parrot. The data for these species was then further analysed to develop a complex identity percent plot, detailing the pairwise identity (up to 100%) for each of these (**Fig. 4.2b**). Due to the nature of IFITM3 and its antiviral properties, it is important to always compare it to its human ortholog. Therefore, the domains of chIFITM3 were compared to huIFITM3 (**Fig. 4.2c**) along with their amino acid lengths for each domain. Human IFITM3 is 132 amino acids long due to its N-terminal domain being 18 amino acids longer in length than chIFITM3. All the other domains are the same length of amino acids for both orthologs of IFITM3. The amino acid sequence alignment was then analysed and compared between chIFITM3 and huIFITM3 (**Fig. 4.2d**). This shows the gaps where huIFITM3 has longer domains and where amino acids are present in huIFITM3 and not chIFITM3.

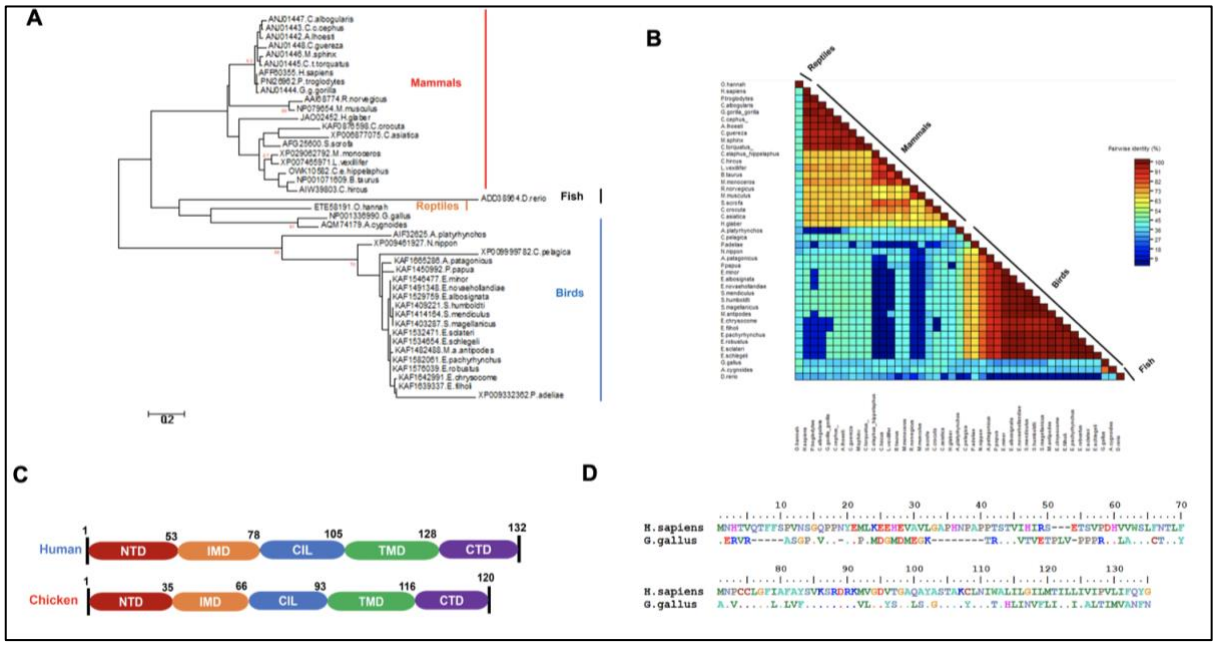


Figure 4.2: In silico characterisation of chicken IFITM3. (A) Phylogenetic investigation of IFITM3 across multiple orthologs categorised species by their orders and designated them with distinct colours. The evolutionary tree was constructed using MEGA 6.06. The species name and the protein accession number are included. The scale bar at the bottom denotes the mistake rate. (B) A pairwise identity percentage plot of the IFITM3 protein among distinct orthologs was conducted utilising the SDT software. The percentage identity was depicted on the right-hand scale of the graph. (C) Domain's architectures of IFITM3 of humans and chickens. Domain structures are represented as NTD, N-terminal domain (red); IMD, intramembrane domain (orange); CIL, conserved intracellular loop (blue);

TMD, transmembrane domain (green); and CTD, C-terminal domain (purple). (D) Amino acid sequence alignment of the entire IFITM3 protein of humans and chickens. The alignment was generated using clustal W algorithm of BioEdit program (Tom Hall, version 7.2)

A heat map was designed using the chicken DF-1 cells to determine all the proteins within this cell line that were upregulated or downregulated (**Fig. 4.3a**). Chicken IFITM3 is annotated on this figure as well as being annotated on the volcano plot (**Fig. 4.3b**), which also shows a plot of the up-regulated and down-regulated genes in chicken DF-1 cells.

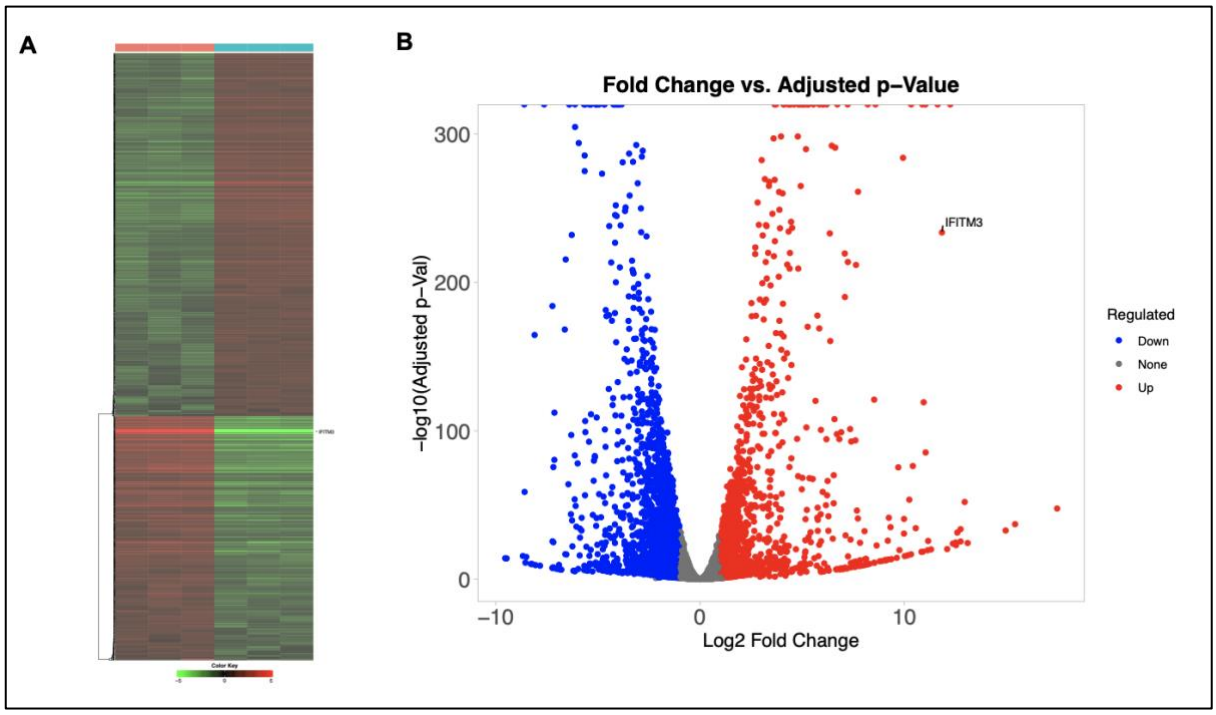


Figure 4.3: Transcriptional regulation of chIFITM3. (A) Heatmap showing the most up or down-regulated genes where chIFITM3 is highlighted. (B) Volcano plot of up- and down-regulated genes in chicken embryo fibroblasts (DF-1 cells). chIFITM3 is highlighted in the volcano plot.

Different cell lines were infected with the H9N2 influenza virus to determine the relative fold change differences due to the induction of chIFITM3 (**Fig. 4.4**). DF-1 and CEF are a type of embryonic chicken fibroblast cells, whereas LMH are cells that exhibit epithelial morphology that were isolated from the liver of a chicken hepatocellular carcinoma. Compared with the mock (negative control group), chIFITM3 was significantly expressed in all three avian cell

lines. The overexpression of chIFITM3 in avian cell lines exhibits inhibitory effects on cells infected with H9N2 influenza virus.

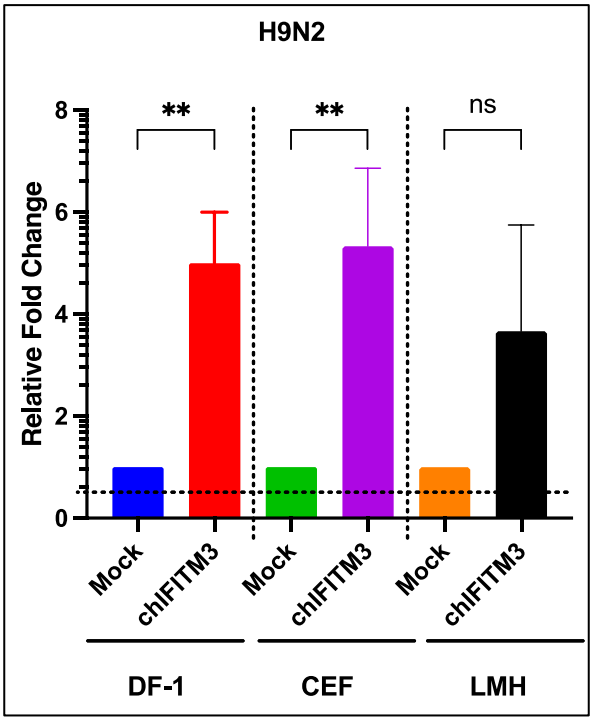


Figure 4.4: Induction of chIFITM3 by the influenza H9N2 in diverse avian cell lines.

chIFITM3 localises in the cytoplasm of chicken fibroblast cells.

chIFITM3 was expressed in chicken fibroblast (DF-1) cells and the localisation was established by immunofluorescence using an antibody against the c-terminal HA-tag (**Fig. 4.5**). LAMP 1 (a marker for lysosomes), RAB 5 (a marker for early endosomes), and RAB 7 (a marker for late endosomes) were also expressed in addition to chIFITM3 (**Fig. 4.6**) to understand where else chIFITM3 localises within the cells.

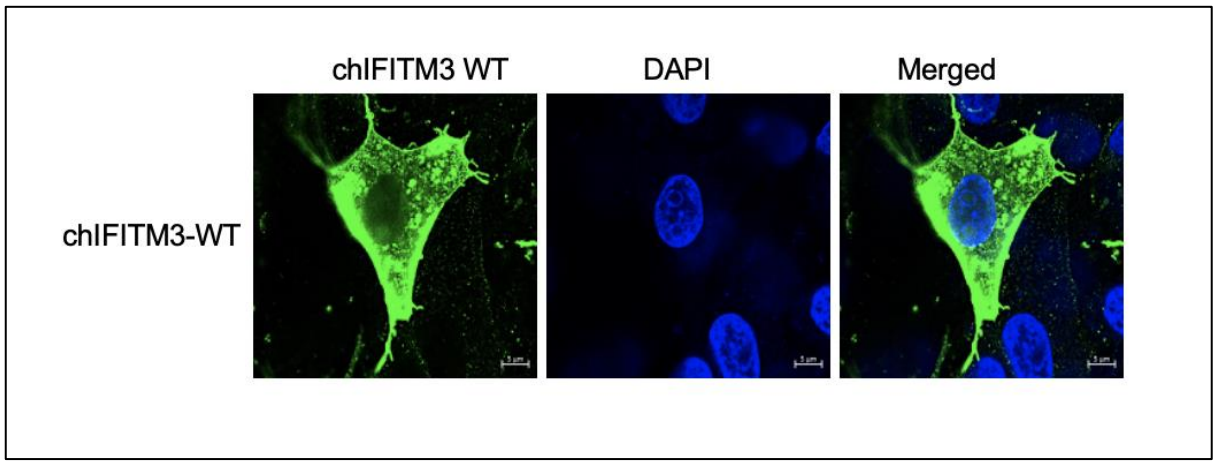


Figure 4.5: Cellular localisation of chicken IFITM3. chIFITM3 localises in the plasma membrane under the normal physiological state of the cells.

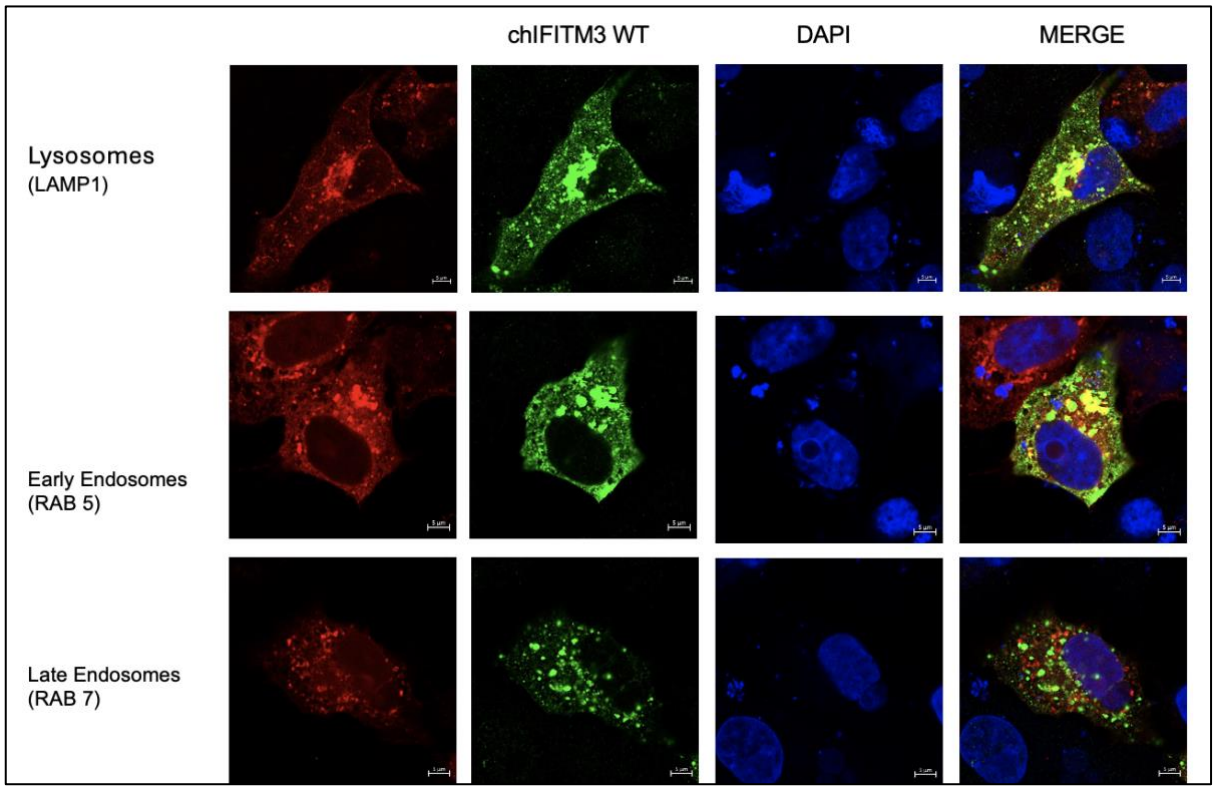


Figure 4.6: Cellular localisation of chicken IFIT3. chIFITM3 localises in late and early endosomes and lysosomes.

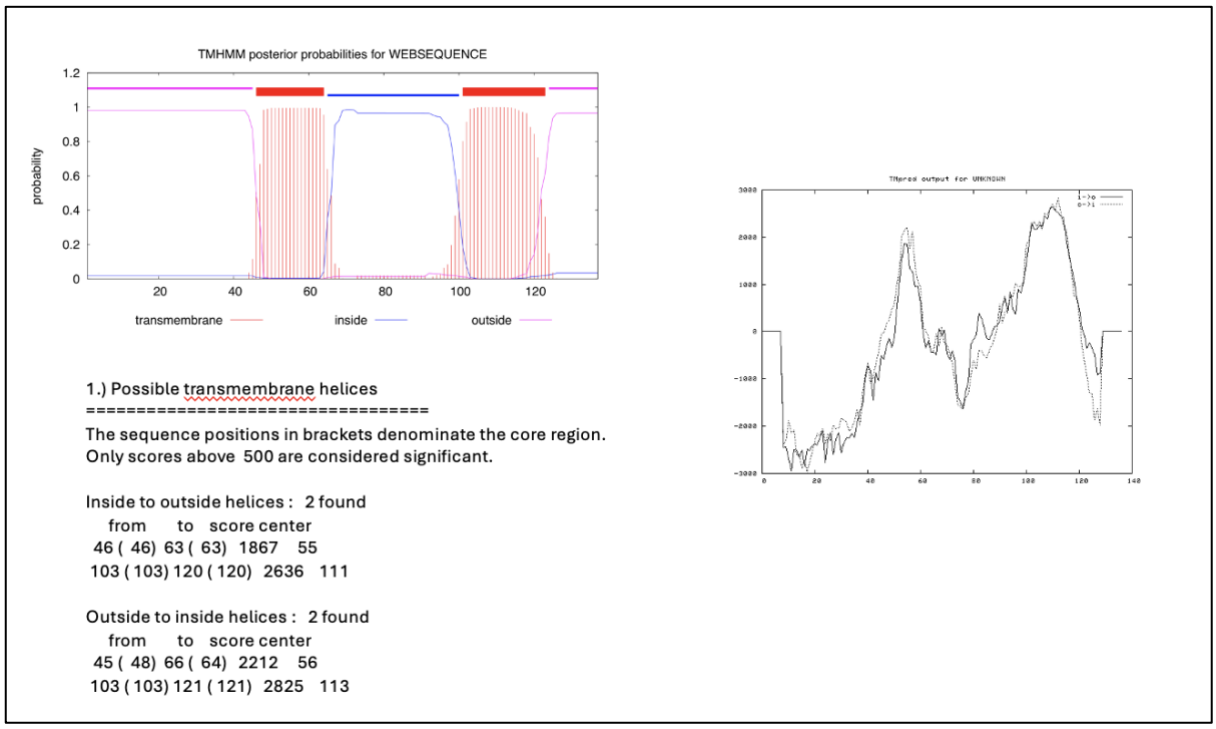


Figure 4.7: Prediction of functional-guided transmembrane domains of chIFITM3

To identify the amino acids critical for IFITM3 localisation and viral limitation, we created a series of mutant constructs to modify or remove amino acids in segments of six throughout the gene. We choose to employ alanine replacements for the residues on the hydrophilic face of the helix (**Fig. 4.8**). This is attributable to alanine's limited hydrophobicity in these places, which should have a negligible effect on the secondary structure. The Iterative Threading Assembly Refinement (I-TASSER) bioinformatics approach was employed to estimate the structure of chIFITM3, and a confidence score was established.

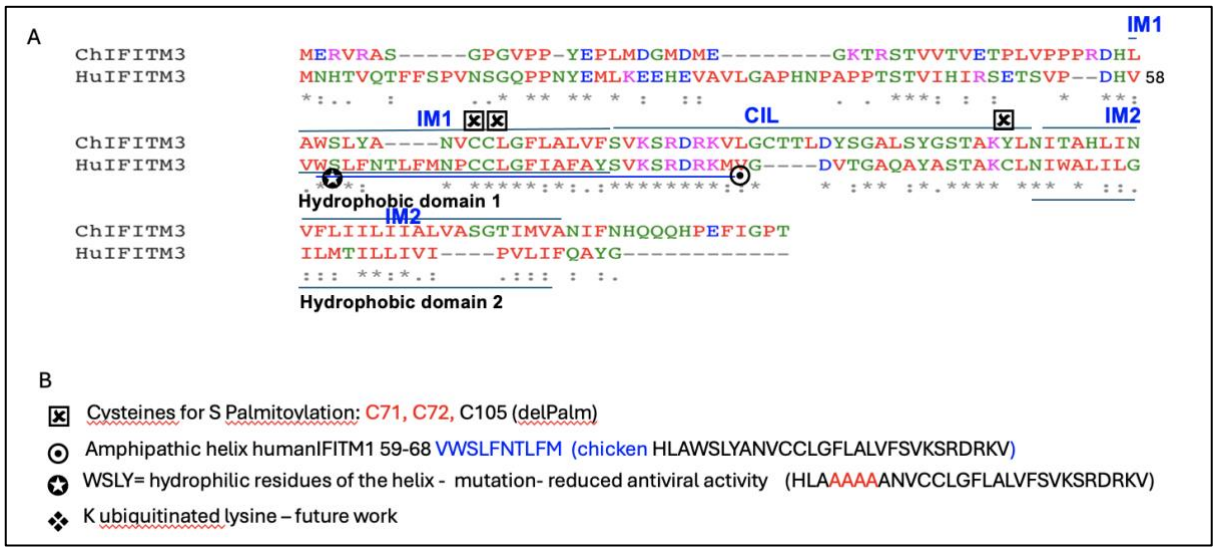


Figure 4.8: Structural annotation of chicken IFITM3

Based on the structural annotation in figure 4.8, this was further developed to understand the secondary structure of chIFITM3 (**Fig. 4.9**). The parts of the sequence that are helix, coils, or strands were determined (**Fig. 4.9a),** and a 3D model was designed from this using the predicted domains (**Fig. 4.9b**). These structural annotations highlight previously characterised corresponding domains in chIFITM3 compared to huIFITM3.

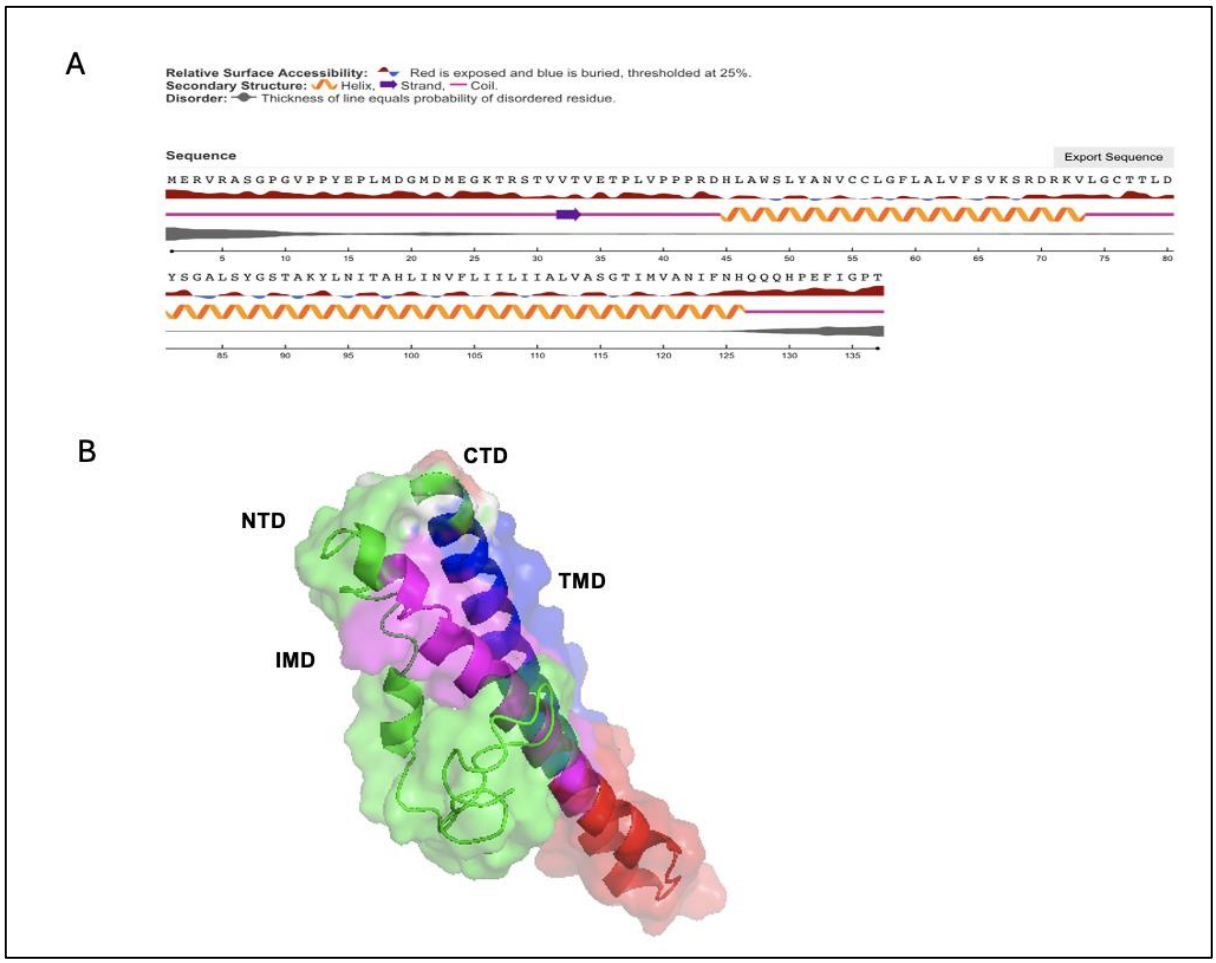


Figure 4.9: Structural prediction and annotation of chicken IFITM3

The determination of secondary structures determined critical domains and residues that highlight structural similarities of chIFITM3 with corresponding mammalian IFITM3 (**Fig. 4.10**). While IFITM3 helices in chicken were mapped corresponding to human, only 4 residues were found to be identical. These differences in the short protein of IFITM3 may be attributed to the functional differences between chicken and human IFITM3.

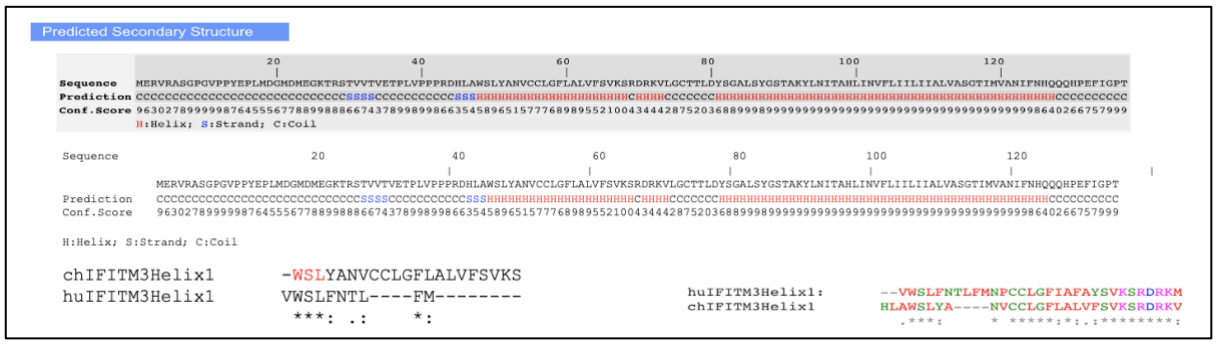


Figure 4.10: Structural annotation of chicken IFITM3 and prediction of helices in chIFITM3

Mutating in the NTD of chIFITM3 impacted the cellular localisation and antiviral potential of chIFITM3.

To map the functional attribute to NTD of chIFITM3, a span of 6 alanine mutations was introduced in the entire length of chIFITM3 NTD (**Fig. 4.11a**). Cellular distribution of all mutants (n=7) showed a punctate and condensed expression almost exclusively in the cytoplasm in contrast to chFITM3-wt and chIFITM3 1-6 which show peri-plasma membrane expression. This indicates that 6 mutations at any given position within the NTD drastically alter the expression kinetics of chIFITM3 (**Fig. 4.11b**). While the expression in the cell was affected, only 4 mutants showed a non-significant inhibition of influenza viruses (**Fig. 4.11c**).

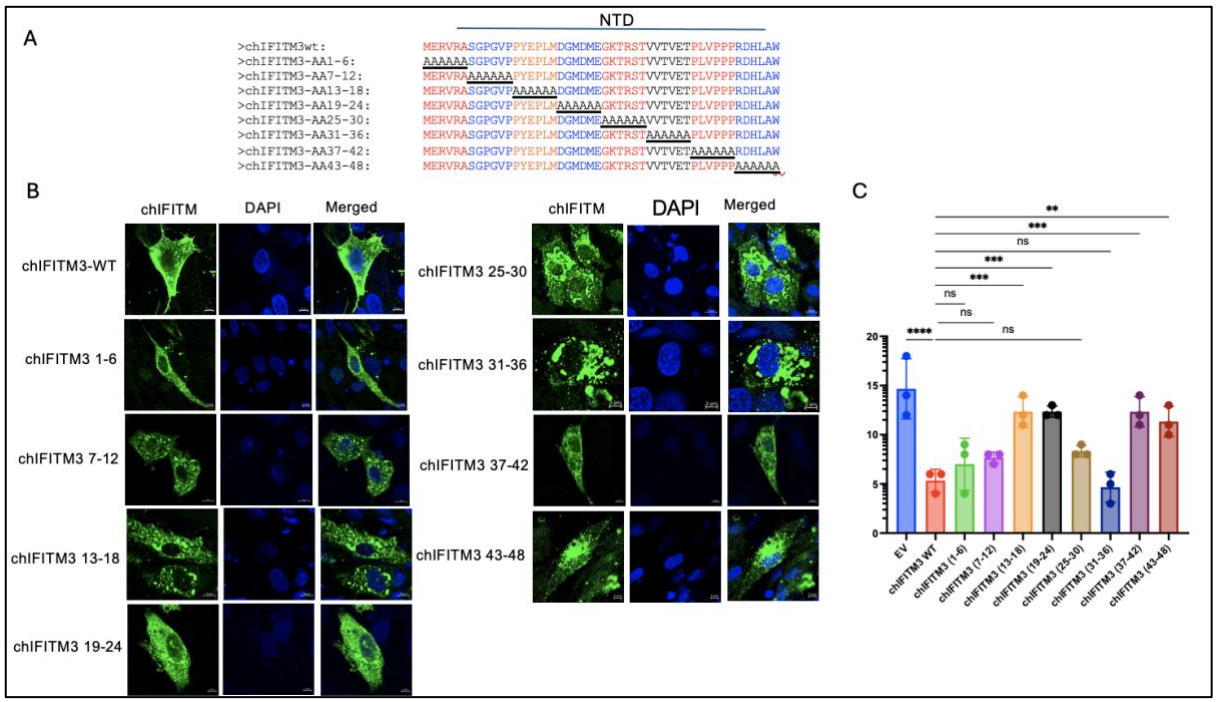


Figure 4.11: Functional characterisation of NTD domains of chIFITM3

Next, we deployed similar approaches to mutate TM1 and CIL (**Fig. 4.12a**). All these 6A mutations altered the expression of chIFITM3 in cellular compartments (**Fig. 4.12b**). The analysis of antiviral potential identified that all these mutations not only re-directed the chIFITM3 in the cells but have also ablated the ability of chIFITM3 in inhibiting influenza viruses except chIFITM3 91-96 (**Fig. 4.12c**).

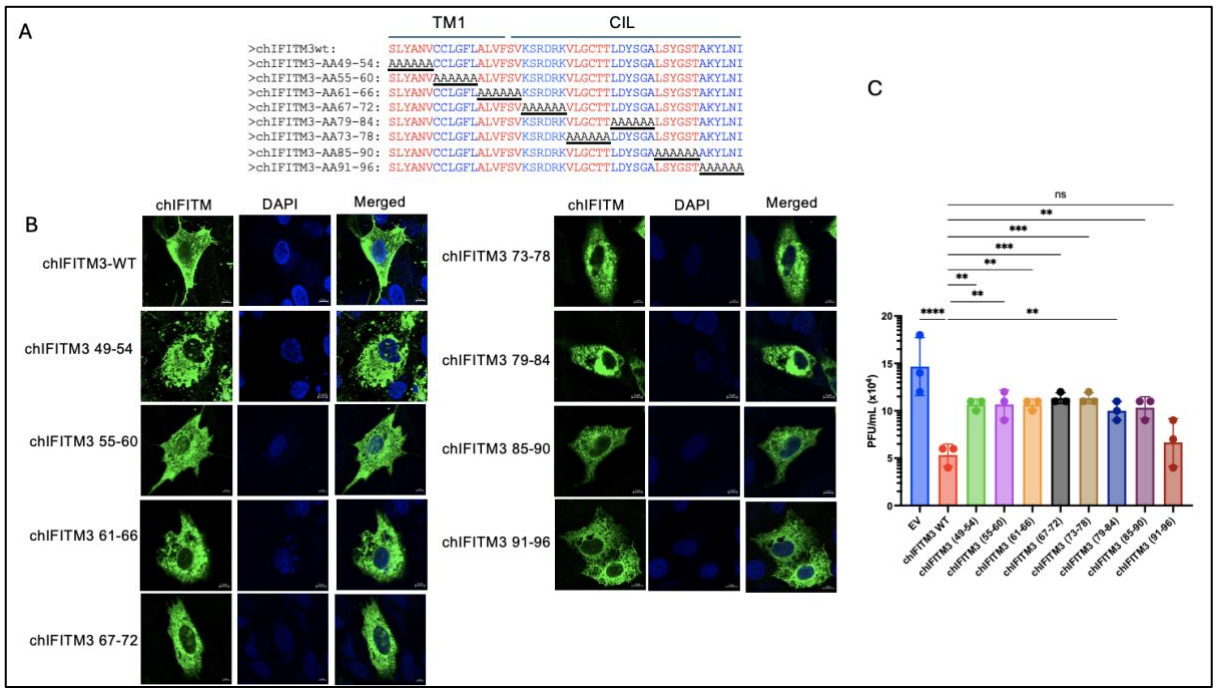


Figure 4.12: Functional characterisation of TM1 and CIL domains of chIFITM3

Mutational mapping of TM2 and CTD (**Fig. 4.13a**) showed that cellular distribution was altered in all mutants of chIFITM3 (**Fig. 4.13b**). Interestingly, all mutants of chIFITM3 retained the antiviral activities except the chIFITM3 97-102 mutant (**Fig. 4.13c**).

Taken together, mapping residues involved in cellular distribution showed sensitivity of chIFITM3 for mutational changes, whereas the antiviral activities are tolerated within the NTD, TM2, and CTD.

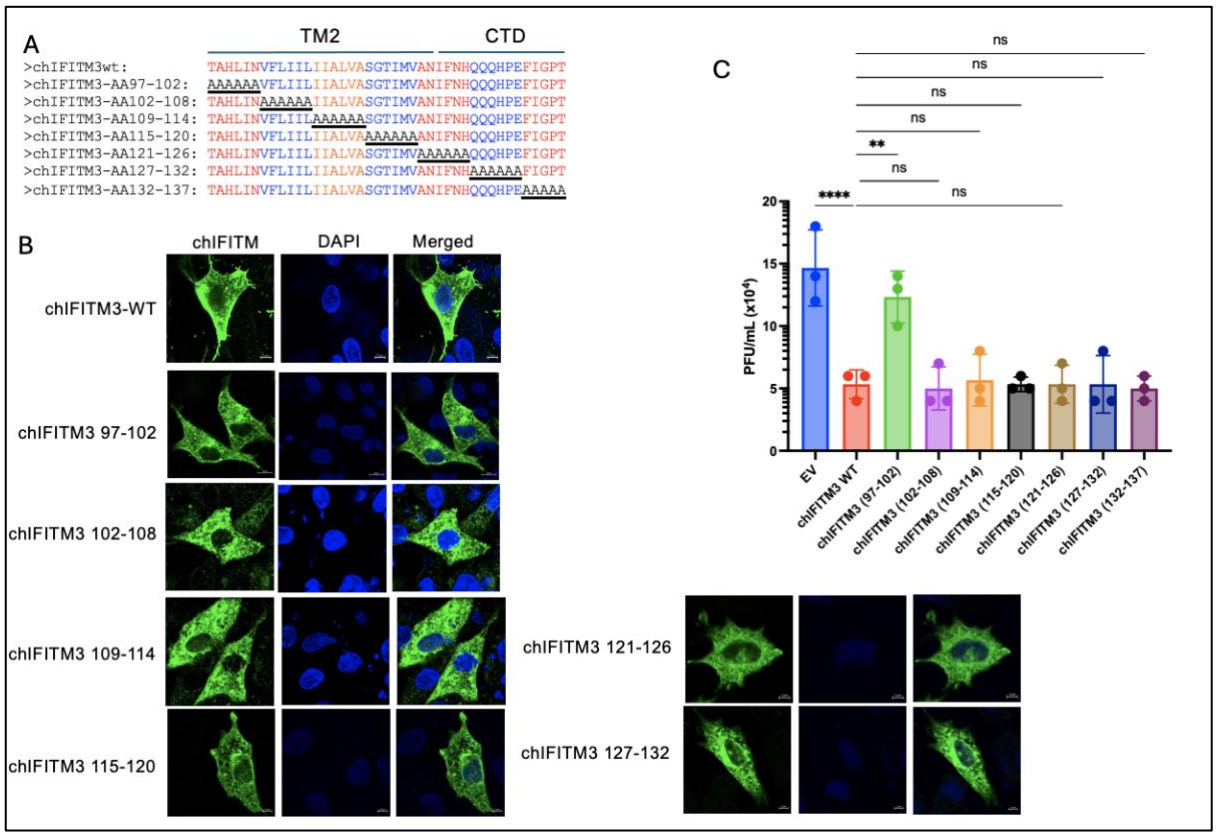


Figure 4.13: Functional characterisation of TM2 and CTD domains of chIFITM3

Furthermore, investigations were made into the chIFITM3 helix for hydrophobic and hydrophilic nature (**Figs. 4.14a and b**, respectively). The interface of the mutations (WSL into AAA) was identified to be critical for the structure of the chIFITM3 (**Fig. 4.14c and d**).

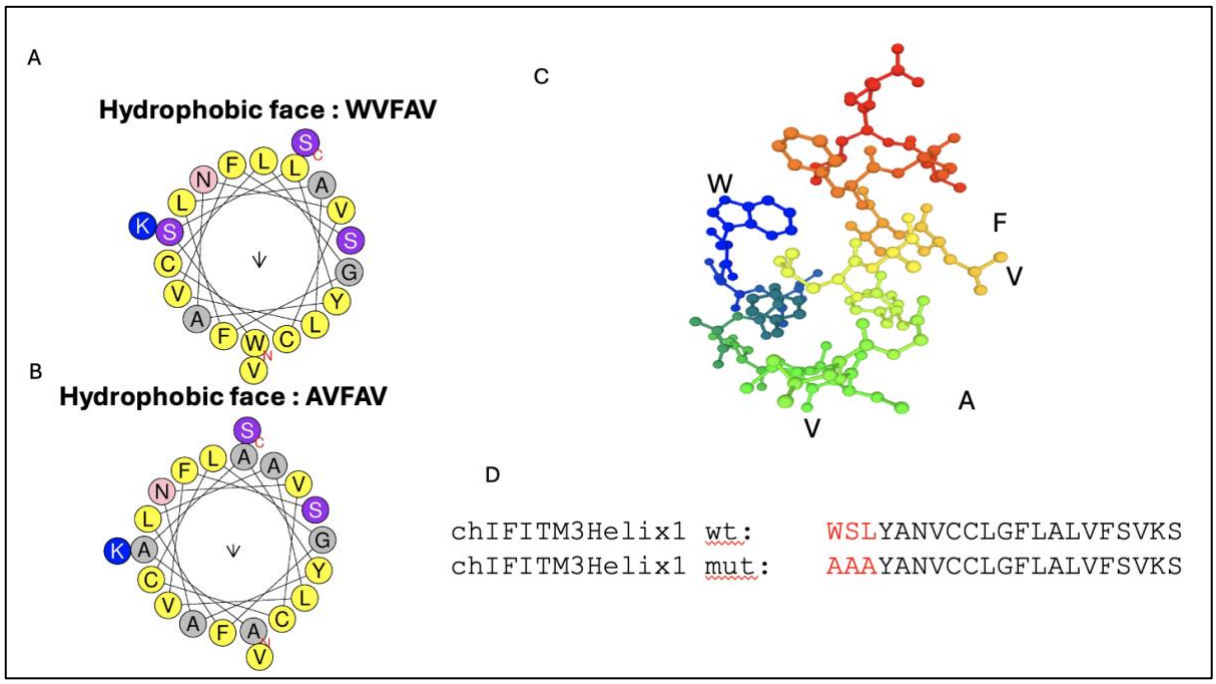


Figure 4.14: Prediction of hydrophobic faces and helices of chIFITM3

Based on the finding of the hydrophobic faces and helices (**Fig. 4.14**), we attempted to mutate the predicted AH region (mutAH) or delete the AH domain from the chIFITM3 (delAH) (**Fig. 4.15a**). We have observed that either mutation or deletion of the AH domain from chIFITM3 directed the expression of chIFITM3 from the plasma membrane to the cytoplasm in the form of punctate (**Fig. 4.15b**). Assessment of antiviral potential indicated that while both chIFITM3 mutants (mutAH and delAH) showed antiviral activities, they have markedly reduced antiviral potential compared to chIFITM3-wt against influenza A viruses (**Fig. 4.15c**).

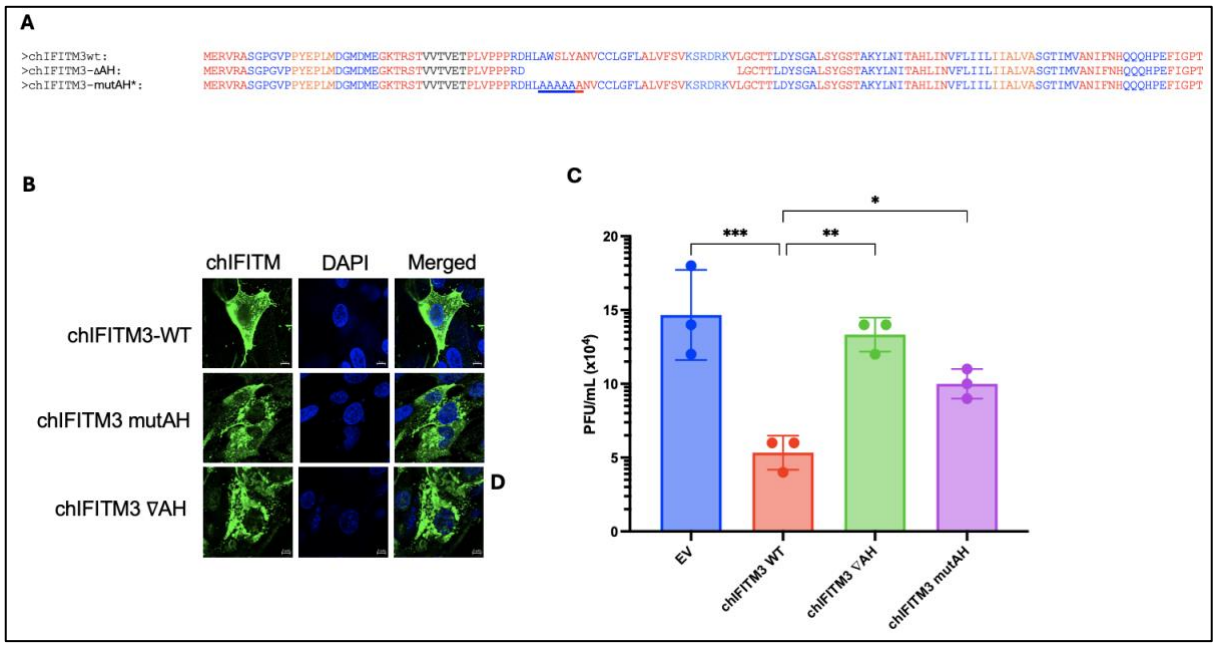


Figure 4.15: Functional characterisation of chIFITM3_∇AH-mutAH

A comparison of the chicken and human IFITM3 indicated high conservation across different domains. Importantly, two critical palmitoylation in cysteines were conserved within the IM1, whereas the third palmitoylation within the CIL was different between chicken and human IFITM3 (**Fig. 4.16**).

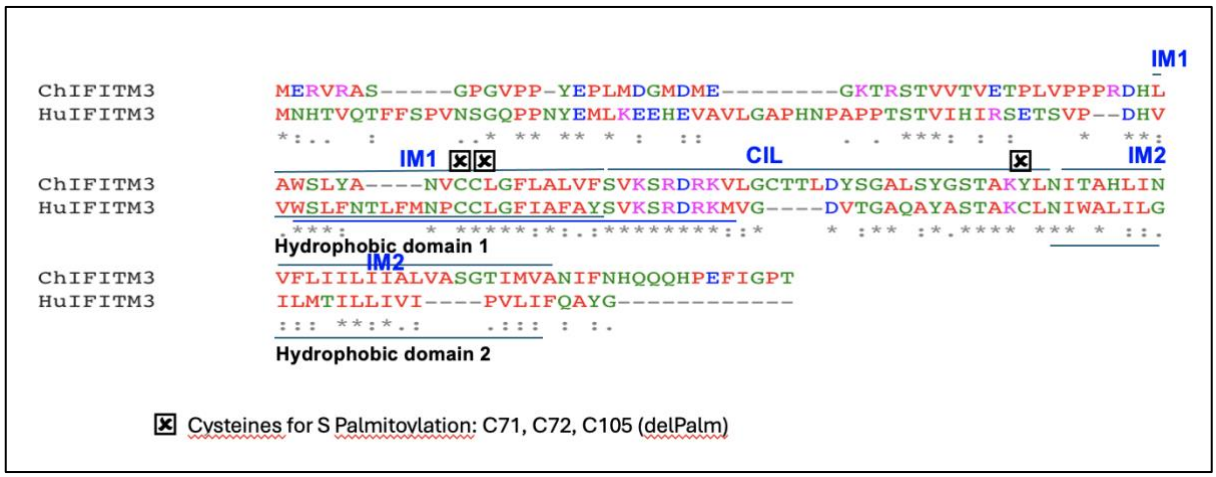


Figure 4.16: Prediction and annotation of chicken IFITM3

Based on the prediction of conservation shown in **Fig. 4.16**, we next aimed to generate a Palm deletion version of the chIFITM3 (**Fig. 4.17a**). We observed that deletion of two cysteines dramatically altered the cellular distribution of chIFITM3 in chicken fibroblasts (DF-

1 cells) (**Fig. 4.17b**). Intriguingly, ablation of palmitoylation significantly abrogated the ability of the chIFITM3 in inhibiting influenza virus compared to chIFITM3-wt (**Fig. 4.17c**).

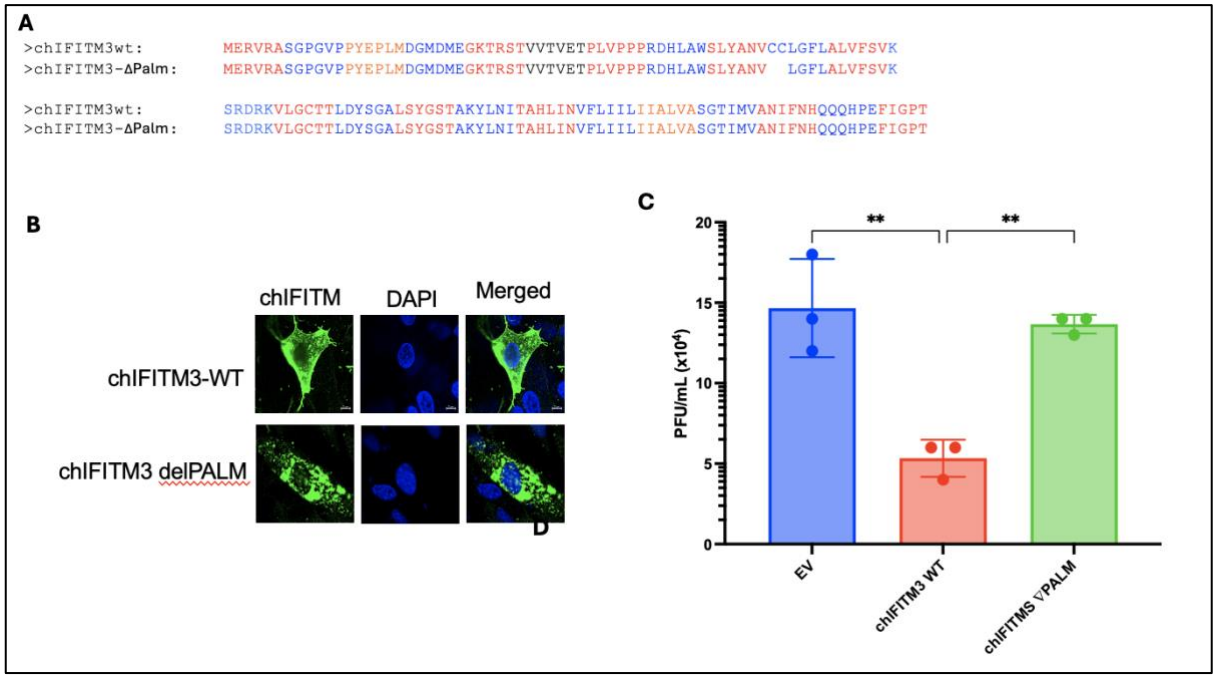


Figure 4.17: Functional characterisation of chIFITM3_∇Palm

4.3 Discussion

The findings of this study shed light on the structural and functional properties of chicken IFITM3 (chIFITM3), as well as its role in inhibiting influenza A virus (IAV). By systematically analysing chIFITM3's localisation, structural domains, and mutational consequences, we have uncovered critical aspects of its antiviral mechanisms. These findings not only expand our understanding of IFITM3 in avian species but also highlight the protein's evolutionary conservation and divergence across species, providing new insights into its antiviral function. This study's key finding is that chIFITM3 is predominantly localise in the cytoplasm, particularly in late and early endosomes and lysosomes, as demonstrated by its co-localisation with markers such as LAMP1, RAB5, and RAB7 (**Fig 4.5 and 4.6**). This localisation pattern is consistent with previous studies on human IFITM3 (huIFITM3), which

also localised in endosomal compartments and is known to inhibit viral entry during hemifusion or fusion pore formation (Bailey et al., 2014; Desai et al., 2014). However, unlike huIFITM3, which can localise to the plasma membrane under certain conditions, chIFITM3 has a more prominent cytoplasmic distribution. This suggests that there may be species-specific differences in the regulation of IFITM3 localisation, which could affect its antiviral activity.

Our results revealed that mutations in chIFITM3's N-terminal domain (NTD), transmembrane domains (TM1 and TM2), and conserved intracellular loop (CIL) have a significant impact on its antiviral activity (**Fig 4.11, 4.12, and 4.13**). For example, mutations in the NTD affect the cellular localisation of chIFITM3, resulting in a punctate and condensed expression pattern in the cytoplasm, which coincided with a lack of antiviral efficacy in some mutants. These results suggest that the NTD is important for the localisation and function of chIFITM3. Interestingly, while plasma membrane localisation was not required for antiviral activity, mutations in the TM1 and CIL domains eliminated chIFITM3's ability to block IAV infection, indicating the importance of these domains in the chIFITM3 antiviral action. These results are consistent with previous studies on huIFITM3, which has revealed that the TM1 and CIL domains are required for antiviral action (John et al., 2013; Li et al., 2013). However, the unique susceptibility of chIFITM3 to mutations in these domains emphasises the significance of studying IFITM3 in different species to fully understand its antiviral mechanisms.

Furthermore, structural analysis of chIFITM3 revealed several key properties, such as the existence of two intramembrane domains (IM1 and IM2) separated by a conserved intracellular loop (CIL) (**Fig 4.9 and 4.10**). The secondary structure prediction of chIFITM3 revealed a high degree of similarity to huIFITM3, notably in the helical sections, which are crucial for antiviral activity. Despite these structural similarities, just four residues in the helical regions of chIFITM3 and huIFITM3 were found to be identical, implying that the functional differences between the two proteins could be related to these sequence variations. These

findings emphasise the significance of even minor variations in the protein sequence to affect the antiviral specificity and efficacy. We have identified that the amphipathic helix (AH) in chIFITM3 is a key structural element for its antiviral activity (**Fig 4.14**). However, mutations or deletions in the AH domain significantly reduced the chIFITM3's antiviral activity; the protein retained some ability to inhibit IAV infection (**Fig 4.15**). This is consistent with previous findings in huIFITM3, where the AH domain was found to play an important function in modulating membrane fluidity and curvature, thereby preventing viral fusion (Chesarino et al., 2017). The conservation of this domain across different species emphasises its importance in IFITM3's antiviral activity, whereas observed differences in functional impact between species.

On the other hand, post-translational modifications, particularly palmitoylation, have been demonstrated to be crucial to IFITM3 activity. In this study, we identified two conserved cysteine residues in the IM1 domain of chIFITM3 that are essential for its antiviral action (**Fig 4.16**). However, the deletion of these palmitoylation sites significantly altered the cellular distribution of chIFITM3 and eliminated its capacity to inhibit IAV infection (**Fig 4.17**). These findings are consistent with previous studies on huIFITM3, which have shown that palmitoylation is required for its antiviral action (Yount et al., 2010; Jia et al., 2014). Meanwhile, the third palmitoylation site in the CIL domain differed between chIFITM3 and huIFITM3, suggesting that post-translational modifications may regulate IFITM3 activity differently between species. These findings raise intriguing questions regarding how various species evolved distinct mechanisms for regulating the IFITM3 activity, maybe in response to different viral threats.

The results of this study build upon previous research on the antiviral mechanisms of IFITM3, particularly in human and mouse models. However, this study sheds novel insights on the functional and structural properties of chIFITM3, which have not been extensively characterised before. The identification of key domains and residues in chIFITM3 that are essential for its antiviral activity, as well as the demonstration of its efficacy to mutations,

represents a substantial advancement in our understanding of IFITM3 in avian species. This study presents a novel and detailed mutational analysis of chIFITM3, demonstrating that the NTD, TM1, and CIL domains are essential for its antiviral function. While previous research has explored the role of these domains in huIFITM3, this study offers the first comprehensive examination of their significance in chIFITM3. Likewise, the discovery of species-specific differences in IFITM3 regulation, particularly in post-translational modifications, underscores the need to study IFITM3 across different species to fully understand its antiviral mechanisms.

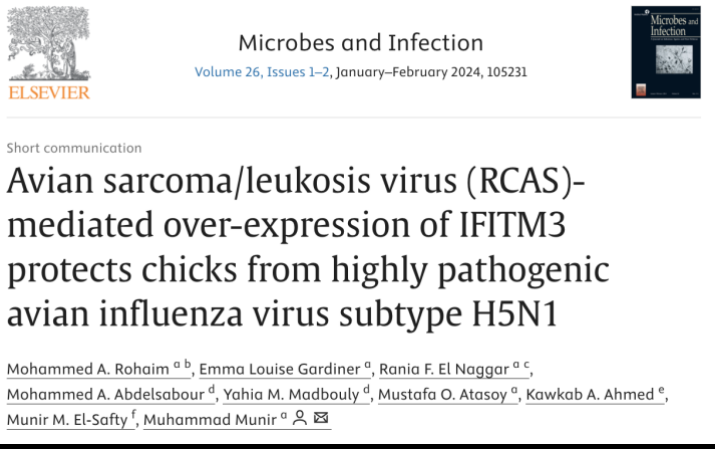
4.4 Conclusion

The findings of this study enhance our knowledge of IFITM3 and lay the groundwork for future comparative studies. Additionally, the findings of this study have significant implications for future research on IFITM3 and its role in antiviral defence. The sensitivity of chIFITM3 to mutations, particularly in the NTD, TM1, and CIL domains, suggests these regions could serve as potential targets for novel antiviral strategies. Stabilising these domains with small molecules or peptides may enhance IFITM3's antiviral activity, offering a new approach to combating influenza virus infections. In addition, the species-specific differences in IFITM3 regulation, particularly in post-translational modifications, emphasise the need for further research on how these variations influence viral susceptibility across species. Understanding these differences could provide valuable insights into the evolution of antiviral defence mechanisms and aid in developing species-specific antiviral therapies. To conclude, this study presents a comprehensive analysis of chIFITM3's structural and functional characteristics in inhibiting IAV infection. The results underscore the critical role of specific domains and residues in chIFITM3's antiviral activity while shedding light on the evolutionary conservation and divergence of IFITM3 across species. These insights are crucial for developing novel antiviral strategies and preventing avian influenza pandemics, guiding future research on IFITM3's role in antiviral defence across different species.

Chapter 5

Avian sarcoma/leukosis virus (RCAS)-mediated over expression of IFITM3 protects chicks from highly pathogenic avian influenza subtype H5N1

This chapter is based on our publication in a peer-reviewed journal:



5.1 Introduction

The host innate immune system responses serve as the primary defence mechanism against infection (Olive, 2012). IAV induces these responses by promoting interferon production, which leads to the expression of interferon-stimulated genes (ISGs) (Diamond and Farzan, 2012). The entry of IAV into cells involves a sequential delivery of viral and cellular signals across two separate cellular compartments: the endosomes and the cytosol (Wrensch et al., 2015). The innate immune system comprises microbial components known as pattern recognition receptors (PRRs) that identify pathogen-associated molecular patterns (PAMPs). Viral RNA serves as a primary PAMP, which the innate immune system targets to recognise the presence of influenza A virus (IAV) (Killip, Fodor and Randall).

Three types of interferons exist, and their activation leads to various molecular alterations, encompassing cell growth and inflammation (Graber and Dhib-Jalbut, 2014). Upon interaction with the cell surface, these induce the production of IFN-stimulated response elements and gamma-activated sequence promoter elements, which facilitate the expression of antiviral genes (White et al., 2008). Type I interferons are essential for addressing influenza A virus infection and play a crucial role in the induction of interferon-stimulated genes (ISGs) (Zhang et al., 2019). Currently, IAVs have evolved mechanisms to counteract the type I interferon response, as the NS1 protein of the virus inhibits IFN-β production in infected epithelial cells (Anastasina et al., 2016).

Interferon-induced transmembrane protein 3 (IFITM3) is a broadly expressed interferon-stimulated gene (ISG) recognised for its role in inhibiting the replication of pathogenic viruses. A host restriction factor for IAV in human investigations was initially identified as human IFITM3 through two genome-wide screens of RNAi and yeast-two hybrid (Winkler et al., 2019). IFITM3 in humans and mice has been extensively characterised, but the homologue in chickens is lacking dramatically. Overall, chIFITM3 only shares a 42% amino acid identity with its human homologue (Brass et al., 2012) and therefore provides a great model for investigating the potential antiviral effects in the chicken model. IFITM3 is thought to inhibit

the fusion of the IAV virus with target cells during the hemifusion and/or fusion pore formation stages by decreasing membrane fluidity or enhancing the spontaneous positive curvature of the outer membrane (Desai et al., 2014).

IFITM3 contains two intramembrane domains (IM1 and IM2), which are separated by a conserved intracellular loop (CIL) (Chesarino et al., 2017). The final domains are the C-terminal and N-terminal domains, which display heterogenicity across the paralogs, suggesting that they may directly contribute to the antiviral specificities (Jai et al., 2012). Recent reports indicate that the N-terminal domain encompasses a motif that serves as an endocytic signal crucial for the endocytosis and localisation of IFITM3 to endocytic vesicles and lysosomes. The amphipathic helix plays a critical role in antiviral function by affecting physical properties. The intracellular loop is essential for viral inhibition, but as stated above, the pathway is unknown. The transmembrane domain contains two phenylalanine residues required for IFITM/IFITM interactions for inhibition of viral entry. Finally, the C-terminal domain determines the subcellular localisation of the protein. There is a significant lack of information regarding these domains and their impact on IAV, and therefore this is a great avenue for prospective research. In-depth research has been done on IFITM3 as a restriction factor that provides widespread resistance to viral infection (Brass et al., 2009; Anafu et al., 2013). IFITM3 employs an endosomal entrance mechanism against both enveloped and non-enveloped viruses (Diamond MS, Farzan, 2012; Perreira et al., 2013). By preventing the virus-endosome fusion, IFITM3 limits their reproduction (Feeley et al., 2011; Huang et al., 2011). The increase of cholesterol in late endosomes and multivesicular bodies caused by IFITM3 may be the underlying mechanism that prevents intraluminal virion-containing vesicles and endosomes from fusing together (Amini-Bavil-Olyaee et al., 2013). To promote cell survival and increase cell resistance to influenza virus infection, IFITM3 is also significantly maintained in resident memory CD8+ T cells (Wakim et al., 2013).

In the current study, the role that IFITM3 plays in respect to blocking viral entry will be determined through investigations with chicken fibroblast cells and chIFITM3. This study will map the functional and structural insight in the antiviral potential of IAV using a range of techniques.

5.2 Results

To assess the *in vivo* antiviral efficacy of chIFITM3 against the highly pathogenic avian influenza virus (HPAIV) subtype H5N1 (clade 2.2.1.2), chicks stably expressing chIFITM3 were produced. Avian retroviruses were utilised through a vector-based expression system for this aim. The complete open reading frame of chIFITM3 was inserted into an RCASBP(A) vector at two distinct restriction sites to effectively produce a capped and polyadenylated chIFITM3 transcript, designated as RCASBP(A)-chIFITM3 (**Fig. 5.1**). RCASBP(A)-WT served as a negative control in the overexpression experiment. The overexpression of chIFITM3 was verified in stably infected RCASBP(A)-chIFITM3 chicken embryo fibroblast (DF-1) cells by an immunofluorescence experiment (**Fig. 5.1b**). The expression of chIFITM3 was primarily localised in the cytoplasm, where the protein appeared in dense punctate formations (**Fig. 5.1b**). Consistent with expectations, DF-1 cells stably infected with RCASBP(A)-chIFITM3 exhibited substantial antiviral efficacy against HPAIV subtype H5N1 in comparison to wild-type DF-1 cells (**Fig 5.1c**). These findings validate that RCASBP(A)-mediated expression of chIFITM3 is functionally stable and embodies the inherent antiviral activity against HPAIV, and these infectious cells can be utilised for the overexpression of the transgene (i.e., IFITM3) in developing chicks.

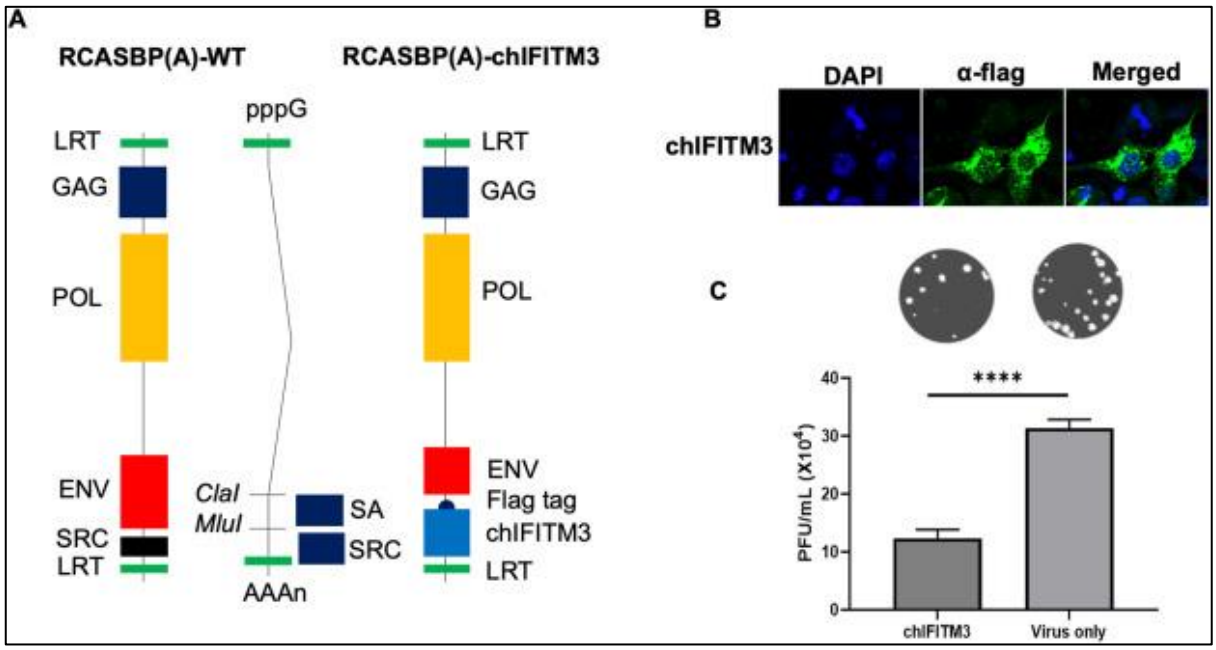


Figure 5.1: Construction and confirmation of RCASBP(A) expression system for chIFITM3. (A) Strategy for generating recombinant RCASBP viruses in which the src gene has been substituted with chIFITM3. (B) Confirmation of steady expression of chIFITM3 mediated by RCASBP(A) utilising an immunofluorescence test. Cells stably infected and expressing chIFITM3 were fixed, permeabilized, and stained with anti-flag antibodies that target the N-terminal flag fused to chIFITM3 (C). Stable cells expressing chIFITM3 or wild-type cells were infected with HPAIV for 24 hours at a multiplicity of infection of 1.0. The cell supernatant was utilised to quantify the released virus via plaque assay. The data signify experiments performed in triplicate. **** denotes a significance level at a p-value of less than 0.0001.

To generate chIFITM3-expressing chicks, 2-day-old embryonated eggs (ED2) were inoculated with recombinant RCAS virus (RCASBP(A)-chIFITM3 or RCASBP(A)-WT) infected DF-1 cells (**Fig 5.2a**). The hatched chicks at ED21 were transferred to isolators in groups until they were exposed to a clinical dosage (104 EID50) of HPAI H5N1 at 15 days post-hatching (PH15). Two independent experiments were conducted to verify that the expression of chIFITM3 did not adversely affect embryonic development and hatchability of RCASBP(A)-chIFITM3 infected eggs in comparison to the dummy group (**Fig. 5.2b**). It was observed that all chicks infected with RCASBP(A)-chIFITM3 or RCASBP(A)-WT exhibited a non-significant reduction in body weight (**Fig. 5.2c**), characterised by a slight decline in weight gain in chIFITM3-overexpressing chicks shortly after hatching. Nevertheless, these chicks

subsequently restored their body weight to match that of the mock-infected group (negative control, inoculated with PBS) by the fifteenth day post-hatch. A quantitative RT-PCR specific to the chIFITM3 transgene was conducted to verify its effective expression in developing chicks. Due to the expression of codon-optimized chIFITM3 via RCASBP(A), the PCR differentiated the transgene from the endogenously produced chIFITM3. This technique revealed a markedly elevated level of chIFITM3 in tracheal RNA from hens infected with RCASBP(A)-chIFITM3 compared to control groups (either transgene-expressing chicks or non-transgene-carrying chicks), demonstrating the effective expression of chIFITM3 (**Fig. 5.2d**). The data indicate that the RCASBP(A) virus induces no major visible changes in chicks, allowing for a reliable challenge with HPIAV to illustrate the antiviral effects of chIFITM3 *in vivo*.

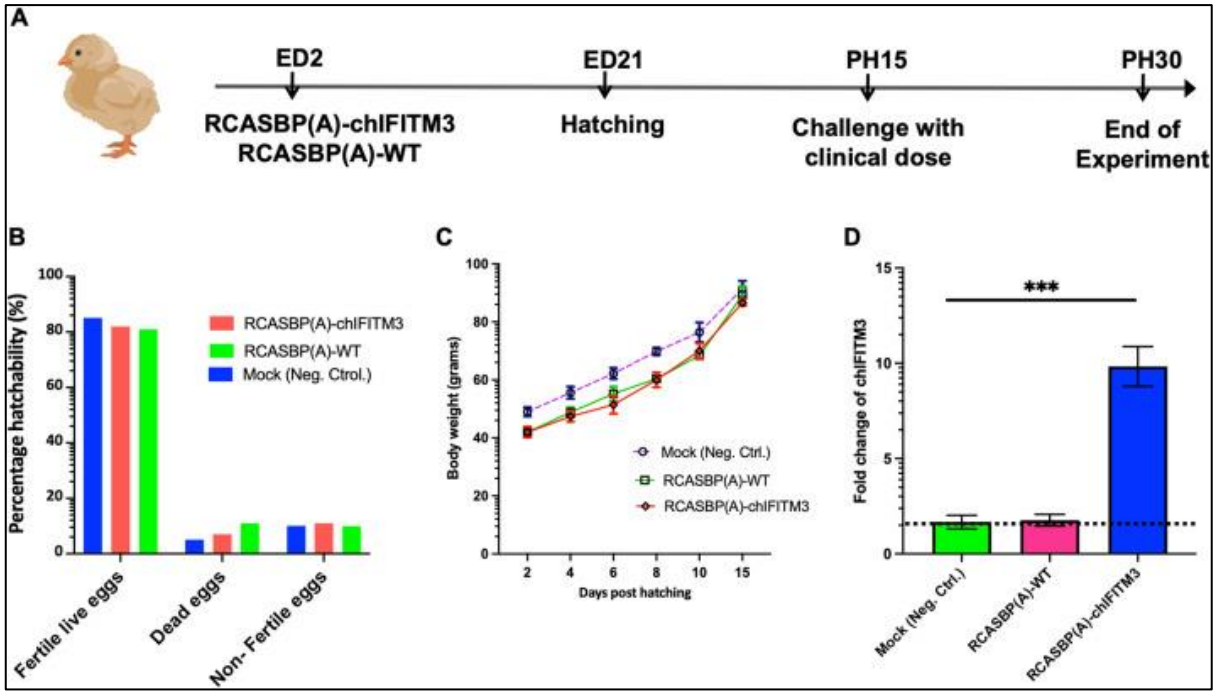


Figure 5.2: Experimental layout for transgene overexpression and impact of chIFITM3 on hatchability and weight gain of hatched chicks. (A) Experimental design for the production of chicks expressing chFITM3. ED denotes embryonation day, while PH signifies post-hatching days. (B) Comparison of hatchability percentages for eggs following chIFITM3 or mock inoculation. (C) Average percentage of body weight gain in chicks after hatching relative to control chicks. (D) Expression of chIFITM3 in chicks infected with RCASBP(A)-chIFITM3 and challenged

with H5N1 was compared to chicks infected with RASBP(A)-WT and those that were mock inoculated (negative control). Asterisks (∗∗∗∗) denote the level of significant differences (p = 0.0001).

A direct correlation exists between the dose of infectious virus and the severity of clinical infections (Kothlow et al., 2010). The clinical outcome of H5N1 infection is influenced by both the genetics of the virus and the host. It was essential to ascertain the inoculum titre of the H5N1 virus capable of inducing clinical disease in chickens. In accordance with prior research [14], a pre-optimised dose of 104 EID50 (referred to as the clinical dose) of the H5N1 strain A/chicken/Egypt_128s_2012 (clade 2.2.1.2) was employed as a challenge virus to evaluate the antiviral efficacy of chIFITM3 in chicks.

ChIFITM3-expressing chicks exhibited complete protection from clinical signs following a challenge with a clinical dose of the H5N1 virus. In contrast, chicks challenged with H5N1 exhibited severe clinical signs starting from the third day post-virus inoculation, whereas chicks in the mock and non-challenged (negative control) groups remained healthy. Furthermore, 60% of chicks expressing chIFITM3 demonstrated protection from clinical challenge, exhibiting no evident clinical disease (**Fig. 5.3a**). The findings indicate that chicks with overexpression of chIFITM3 exhibit disease tolerance, leading to a delay in clinical signs by a minimum of 7 days. The findings indicate that the overexpression of chIFITM3 significantly influenced the outcomes of H5N1 infection, including disease progression and mortality rates.

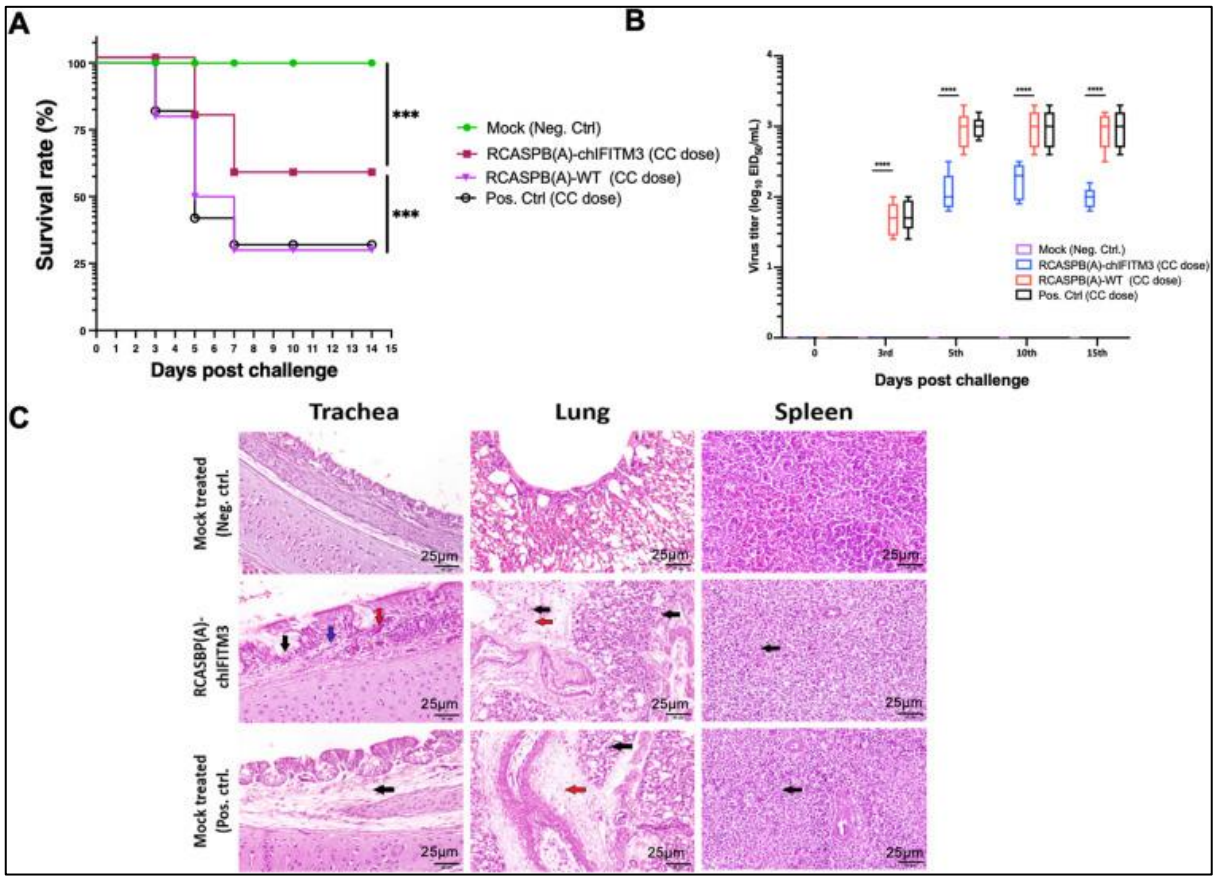


Figure 5.3: Clinical outcome of transgene-expressing chicks compared to wild-type chicks challenged with HPAIV. (A) Percentage survival rates of RCASBP(A)-chIFITM3 and RCASBP(A)-WT infected and challenged chicks with clinical doses of H5N1 were compared to mock inoculated chicks, serving as negative and positive control groups. (B) Evaluation of viral shedding from oropharyngeal swabs of virus-challenged chicks, as well as those infected with RCASBP(A)-chIFITM3 and RCASBP(A)-WT, in comparison to mock-inoculated chicks serving as negative and positive control groups. (C) Photomicrographs depict H&E-stained sections of tracheas, lungs, and spleen obtained from RCASBP(A)-chIFITM3-infected and H5N1-challenged chicks, contrasted with mock-inoculated chicks (negative and positive groups) at post-hatching day 30. Scale bar = 25 µm. Significance is indicated by **** at a p value of less than 0.0001.

Oropharyngeal swabs were obtained from all groups (RCASBP(A)-chIFITM3, RCASBP(A)-WT, and mock-treated) prior to the challenge and every other day following the clinical challenge to assess whether chIFITM3 can facilitate a decrease in viral shedding via oropharyngeal pathways. The results of virus quantification indicated that chicks overexpressing chIFITM3 exhibited a significant reduction in virus shedding following clinical

challenge (**Fig. 5.3b**). This suggests that chIFITM3 plays a crucial role in virus replication, contributing to the decrease in influenza viral shedding.

Trachea, lung, and spleen organs were collected from chIFITM3-overexpressing challenged chicks, as well as from non-challenged and non-inoculated (negative control) chicks at PH30 and subsequently underwent histopathological examination. The mock negative control exhibited no detectable histopathological lesions. The trachea of chIFITM3-expressing chicks exhibited focal necrosis of the lamina epithelialis (black arrow), congestion (red arrow), and infiltration of inflammatory cells (blue arrow) (**Fig. 5.3b**). The trachea of the mock positive control exhibited oedema in the lamina propria/sub-mucosal layer (arrow). Furthermore, the lungs of chIFITM3-expressing and mock-positive control chicks exhibited diminished inflammatory cell infiltration in the air capillaries (black arrow) linked to interlobular oedema (red arrow) when compared to control lungs, suggesting a reduction in pathological lesions within the respiratory tract. The spleen of chIFITM3-expressing and mock-positive control exhibited lymphocytic necrosis and depletion (arrow) in contrast to the negative control. The histopathological findings demonstrate that chIFITM3 overexpression mitigated H5N1-induced pathology in the examined organs, leading to diminished clinical signs in chicks.

5.3 Discussion

The regulation of the innate immune system is governed by cytokines, chemokines, and interferon, which are activated through direct viral infection or intrinsic responses to pathogens (Kothlow et al., 2010). Innate immune responses mediated by viruses and their mechanistic observations differ between avian species and mammals. By mapping cross-species host restriction factors, it may be possible to elucidate the steps involved in the virus-mediated immune response. The host factors of interest specifically include those that influence the zoonotic potential and pathobiology of influenza viruses (Barber et al., 2010). The initial defence mechanism against viral infection involves the activation of the innate immune

response in nearly all vertebrate cells (Munoz-Moreno et al., 2016). A cascade of interferon-stimulated genes (ISGs) is expressed because of the type I interferon (IFN) cytokine group's activity to establish an antiviral state (Blyth et al., 2016). These ISGs encode antiviral proteins that block many viral lifecycle phases, including viral entry, translation, replication, assembly, and release (Chen et al., 2016; Diamond, M.S.; Farzan, 2013). IFITMs are found in a wide variety of animals, including mammals, fish, birds, and amphibians. On chromosome 5, where the chicken IFITM (chIFITM) genes are clustered, four genes have been identified: chIFITM1, chIFITM3, chIFITM5, and chIFITM10 (Smith et al., 2013). A conserved CD225 domain consisting of two intramembrane (IM) regions and a conserved intracellular loop (CIL) defines the topology of the mammalian IFITM proteins, which are very short (approximately 130 amino acids) (Yu et al., 2015; Munoz-Moreno et al., 2016). The structure of the chIFITMs is less well understood since they exhibit considerable genetic divergence from mammals. IFITMs' special capacity to restrict viral entry into host cells emphasises the significance of innate immunity by blocking cell entry, therefore preventing viral replication and disease (Yu and Shi, 2017). Chicken IFITM1 and IFITM3 have been described functionally (Smith et al., 2013), and although most studies provide data at the cellular level, the results provide key concepts for how these proteins provide key antiviral functions and suggest avenues for further investigations.

The interferon-inducible transmembrane proteins (IFITMs) are proteins that are induced by interferon and are expressed widely. They play a crucial role in preventing infections caused by various viruses, including influenza A virus (IAV), West Nile virus (WNV), dengue virus (DENV), severe acute respiratory syndrome coronavirus (SARS-CoV), vesicular stomatitis virus (VSV), and hepatitis C virus (HCV) (Bras et al., 2009; Huang et al., 2011; Diamond and Farzan, 2013; Wilkins et al., 2013; Smith et al., 2014; Smith et al., 2015; Blyth et al., 2016; Chen et al., 2016; Wilkins et al., 2016). Understanding the mechanisms behind the IFITM proteins' antiviral action has been the goal of several investigations (Lu et al., 2011; Li et al., 2013; Desai et al., 2014; Weston et al., 2016; Zhao et al., 2018). IFITMs are found on

endosomal membranes and were proven to inhibit viral particles that have limited virus receptors (Perreira et al., 2013). Viruses' sensitive to IFITM are thought to be obstructed at the cell surface and confined within the endosomal pathway, which ultimately hinders viral fusion by modifying the properties of cellular membranes (Perreira et al., 2013; Desai et al., 2014; Amini-Bavil-Olyaee et al., 2013).

In vivo viral pathogenicity is restricted by IFITM3, according to studies employing mouse infection models. Ifitm3-/- mice had increased vulnerability to respiratory syncytial virus (Everitt et al., 2012) West Nile virus (Gorman et al., 2016), arthritogenic and encephalitic alphaviruses (Poddar et al., 2016), and influenza (Everitt et al., 2012; Bailey et al., 2012). Although previous research suggested a connection between IFITM3 and the control of antiviral immunity, the direct effect of IFITM3 on viral replication has not been clearly separated from any of IFITM3's immune-regulatory roles. Additionally, research on influenza infection has shown that uncontrolled immune cell infection might result in decreased antiviral immune responses in Ifitm3-/- mice (Wakim et al., 2013; Infusini G. et al., 2015). Experimental data indicate that enhanced viral pathogenicity in hosts with weak or impaired IFITM3 activity results from reduced restriction of virus entry and replication (Stacey et al., 2017). This research was conducted to assess the impact of CHITM3 on the pathogenesis of the influenza virus *in vivo*. The checkpoint regulator chIFITM3 is identified as a critical element in influenza-induced immunological dysfunction during *in vivo* infection, as demonstrated through the RCAS retrovirus gene transfer paradigm. chIFITM3 activity serves as a regulator of antiviral immunity, influencing the pathogenic outcome of influenza virus infection, as it does not completely inhibit influenza virus replication.

The RCAS retrovirus gene transfer method provides a straightforward, cost-effective, and less labour-intensive approach for retroviral-mediated transgenic expression. The results indicated a non-significant reduction in body weight in transgenic chicks at hatching. Hatched chicks demonstrated efficient weight recovery, achieving sizes comparable to those of non-transgenic chicks. This study involved the generation of mosaic transgenic chicks that stably

express chIFITM3, aimed at investigating the *in vivo* antiviral function of chIFITM3 against the highly pathogenic avian influenza subtype H5N1. The findings indicate that chIFITM3 offers partial protection (60%) to transgenic chicks against clinical doses of the H5N1 avian influenza virus, which induces clinical disease symptoms. The current study was conducted to investigate the impact of chIFITM3 on predetermined clinical doses, in light of significant changes in the environment and poultry susceptibility to environmental stressors. The findings demonstrated that the clinical dose of H5N1 significantly improved clinical outcomes in transgenic chickens, suggesting that innate immunity can provide protection against HPAI H5N1. Overexpression of chIFITM3 leads to a decrease in clinical disease signs of H5N1 in infected chickens, as well as a reduction in virus-induced pathological lesions and virus shedding.

5.4 Conclusion

Virus pathogenesis differs between avian species and mammals because of cross-species host restriction factors. The primary defence mechanism in nearly all vertebrate cells against viral infection is the activation of the innate immune response. IFITM genes are among the most significantly upregulated ISGs. Specifically, chIFITM1 and chIFITM3 have been genetically and functionally characterised, demonstrating essential antiviral functions and indicating potential directions for further research. The findings indicate that chIFITM3 has the potential to reduce mortality and improve clinical outcomes of infection in chicks, underscoring the significance of innate immunity in the defence against viral infections. This has demonstrated the ability to create virus-resistant transgenic chickens, which can protect food sources and potentially reduce the transmission of zoonotic viruses to humans over the long term. Enhancing the understanding of factors that affect poultry susceptibility to avian influenza viruses will contribute to mitigating risks to both animals including human health.

Chapter 6

General Discussion

6.1 General Discussion

Influenza A virus (IAV) infects many humans annually, and with the use of molecular analysis, we can track key strains that cause infection. The flu vaccine is administered within the UK in the hope that an outbreak will not occur. But due to this vaccine consisting of the four most infectious strains of the previous year, we are always behind. The deaths in the UK alone because of influenza in 2020 exceeded 130,000, displaying just how serious it is that we find a medical treatment that is efficient (*Deaths in the UK from influenza in 2020—Office for National Statistics, 2021)*. These high death rates show the substantial need for research to tackle IAV and determine an effective treatment strategy.

As discussed, ISGs are substantially involved in the immune system, initially upon first IAV infection but also playing a role in secondary infection. Chickens have become a key avian species for providing a bridging model between *Homo sapiens* and mammals. Chickens have developed to become a key model in immunological and microbiological investigations due to the genetic and biological characteristics they possess (Vainio and Imhof, 1995). The first key objective is determining the mechanism of action that chicken ISGs have on the cells post-IAV infection, and this is initially investigated within this report.

Here it is demonstrated that both chIFITM1 and chIFITM3 show a range of antiviral functions that result in the restriction of replication of IAV infection. The above findings suggest that cellular localisation of the protein is a critical role to determine its function as well as key domains within the structure and its topology. Therefore, the mutations within domains that affect cellular localisation and antiviral function provide critical effects to the cells. The topology has previously been discussed, but it is noted that the N-terminal domain residues enter the cytoplasm, and the C-terminal domain of both chIFITM1 and chIFITM3 remain extracellular (Diamond and Farzan, 2012), and therefore they are exposed to cytoplasmic enzymes. It has been confirmed that the N-terminal domain interacts with cytoplasmic enzymes to modulate the antiviral activity of IFITM1 and IFITM3 by regulating the cellular localisation (Jai et al., 2014). The amphipathic helix domain has been widely researched, and particularly within

chIFITM3, the amphipathicity of this domain is required for IFITM3-dependent inhibition of IAV infection (Chesarino et al., 2017). The homologous amphipathic helix in IFITM1 is essential for the suppression of IAV infection; however, the underlying mechanisms remain unidentified. This demonstrates that IFITM proteins have a conserved mechanism of antiviral activity.

The chIFITM1-WT follows recent findings detailing that it localises to the cellular membrane of its cells (**Fig. 3.4**)**,** and as suggested before, it uses this function to inhibit viral infection (Kim, Won, and Jeong, 2021) at the point of entry. When ISGs are associated with the cellular membrane shown through localisation studies, it is predicted that they are linked to early endosomes. This is compared to what we already know about the influence of early endosomes on viral entry, and therefore further experimental analysis will be carried out to compare chIFITM1 with endosome markers. The markers particularly involved with early endosomes are Rab5, EEA1, and Pi(3)K and are linked to the receptor molecule HA to provide viral attachment and entry.

The chIFITM3-WT has been shown to localise to late endosomes within the cytoplasm to prevent viral invasion (**Fig. 4.5**). The protein possesses a brief, conserved sorting signal essential for entry into the endosomal pathway, located within the N-terminal. Research has shown when huIFITM3 undergoes a single nucleotide polymorphism (SNP), the antiviral ability of the protein changes. A single nucleotide polymorphism (SNP) identified as rs12252 is situated at a splicing acceptor location and leads to a shortened N-terminal. This SNP is associated with the severity of H1N1 influenza infection during the 2009 pandemic and underscores the significance of the N-terminal domain in regulating the antiviral properties of IFITM. Our data confirms previous findings of chIFITM3-WT localising to the cytoplasm and showing antiviral properties through the investigation of a plaque assay.

The sequential mutations of chIFITM1 within the CIL domain and mutations of the amphipathic helix domain revealed that all mutants showed localisations to the cellular membrane. Comparing this data to chIFITM1-WT predictions allows for inferences on the antiviral effects of these proteins, indicating that these mutations may have significant

consequences for the cell. These implications will affect the pathways involved with viral activity, and due to localisation within the cell, it is apparent this influence will be upon early endosomes (Deblandre et al., 1995). The exact pathway that this viral inhibition occurs for chIFITM1 is still very much unknown, but through further investigation, particular parts may be unravelled. A suggested pathway for chIFITM1 is that its mechanism of action is to alter the fluidity of the cellular membrane, which results in fusion prevention with influenza A virus envelopes. Therefore, through alteration of the plasma membrane, it can restrict viruses at the initial point of infection (Li et al., 2013). The antiviral assay for all the chFITM1 mutants will be investigated further to determine if the CIL domain is essential for viral activity. There are also mutations within the amphipathic helix domain, and we know through previous research that this domain is of critical influence on viral activity. Research has suggested that mutations within this region not only remove the viral restriction properties but also convert IFITM1 into an enhancer of IAV infection (Shi et al., 2020). Future efforts can be made to determine that when these mutants are transfected into IAV-infected cells, they promote infection above normal levels or just lack the restriction properties.

The sequential mutation of chIFITM3 within all domains of the sequence has revealed how much of a necessity all the domains within this protein are. The data above shows a range of mutations within each of the domains and subsequently determines that when each mutation occurred, the viral rate of infection increased dramatically. Thus, resulting in critical implications for the cell line and showing that when these mutations occur, chIFITM3 cannot function correctly to salvage the cell from influenza A virus infection. The mutations of chIFITM3 within the N-terminal domain can be linked to research that suggests the N-terminal domain's key function is to contain a motif that is an endocytic signal essential for endocytosis and localisation of IFITM3. As discussed in the introduction, the viral entry pathway that is currently known either uses early, middle, or late endosomes. Therefore, the N-terminal is crucial for determining which pathway the protein follows and what endosomes this influences with respect to viral entry. These assumptions for N-terminal domain mutations of chIFITM3

need to be further investigated to conclude how viral entry is affected if the cell localises and influences early endosomes. There are many unanswered questions as to whether viral entry is fully inhibited or not. Within the CD255, we showed several mutations from amino acids 67-102. These mutations lessened IFITM3's restriction of influenza A virus and showed significant viral infection levels. Confocal images revealed that mutations within this domain localise to the cytoplasm, suggesting that this protein influences intracellular distribution. These findings correlate to the function of these domains to provide critical pathways for anti-viral function through affecting physical properties as well as viral inhibition (Winkler et al., 2015).

Overall research has shown that when SNPs in huIFITMs occur, the viral activity of IAV increases, causing strains that can potentially become epidemics. This is due to the genetic polymorphisms in disease-associated genes influencing the susceptibility to gene onset. Therefore, it is important to identify what mutants within the chIFITM1 and chIFITM3 sequences have a key influence on IAV infection. Further investigations into how both chIFITM1 and chIFITM3 affect susceptibility to influenza A virus infection are required. Future studies will need to have a primary objective to focus on how these proteins and their mutants interact with the influenza A virus to prevent entry into the cell. By providing an improved understanding of the function of both IFITM1 and IFITM3 in the control of influenza A virus, this could also inform the design of novel antiviral strategies.

Innate immune responses mediated by viruses and mechanistic observations differ between avian species and mammals. These cross-species host restriction factors may influence the processes involved in viral pathogenesis. The initial defence mechanism against viral infection involves the activation of the innate immune response in nearly all vertebrate cells (Munoz-Moreno et al., 2016; Blyth et al., 2015). IFITM genes represent a significantly upregulated category of interferon-stimulated genes (ISGs) and are present across a diverse range of animal taxa, including mammals, fish, birds, and amphibians. The chIFITM1 and chIFITM3 have undergone genetic and functional characterisation (Yu et al., 2015). While

most studies present *in vitro* data exclusively, the findings indicate significant antiviral functions and propose directions for future research.

IFITMs are broadly expressed, interferon-inducible proteins that inhibit infection by various viruses, including influenza A virus (IAV), West Nile virus (WNV), dengue virus (DENV), severe acute respiratory syndrome coronavirus (SARS-CoV), vesicular stomatitis virus (VSV), and hepatitis C virus (HCV) (Diamond et al., 2013). Studies indicate that Ifitm3−/− mice exhibit heightened susceptibility to respiratory syncytial virus (Poddar et al., 2016), West Nile Virus (Bailey et al., 2019), arthritogenic and encephalitic alphaviruses (Infusini et al., 2015), and influenza (Navid et al., 2021). Previous research has indicated a link between IFITM3 and the regulation of antiviral immunity; however, the direct impact of IFITM3 on viral replication remains inadequately distinguished from its immune-regulatory functions. Experimental data indicate that enhanced viral pathogenicity in hosts with weak or impaired IFITM3 activity results from reduced restriction of virus entry and replication (Diamond et al., 2013).

We employed an overexpression approach to assess the impact of chIFITM3 on the pathogenesis of the influenza virus *in vivo*. Various methods for generating transgenic chickens have been utilised to investigate microbial pathogenesis and chicken physiology (Bednarczyk et al., 2018). The RCAS vector system provides a straightforward and effective means to examine the functions of cellular genes (Rohaim et al., 2013). The checkpoint regulator chIFITM3 is identified as a critical element in influenza-induced immunological dysfunction during *in vivo* infection, as demonstrated through the RCAS retrovirus gene transfer system. chIFITM3 acts as a regulator of antiviral immunity, influencing the pathogenic consequences of influenza virus infection, as it does not completely inhibit influenza virus replication. The results indicated a non-significant reduction in body weight among RCAS-mediated transgene-expressing chicks post-hatching. Hatched chicks demonstrated efficient weight recovery and achieved sizes comparable to those of non-transgene-expressing chicks. This study established chIFITM3-overexpressing chicks to investigate the *in vivo* antiviral

function of chIFITM3 against HPAIV subtype H5N1. The findings demonstrate that chIFITM3 offers substantial protection (approximately 60%) to transgene-expressing chicks against the clinical dose of the H5N1 avian influenza virus responsible for clinical disease. Given the variable conditions affecting poultry susceptibility to infections (Qadir et al., 2022; Shahzad et al., 2022), further investigation was conducted to assess the impact of chIFITM3 against a specified clinical dose of H5N1. The findings demonstrated that the clinical dose of H5N1 significantly diminished the clinical outcome in transgene-overexpressing chicks, suggesting the capacity of innate immunity to confer protection against HPAIV H5N1. In prior research, we employed a comparable methodology to assess the effects of chIFITM1 on the influenza virus, observing a more significant inhibition of influenza-induced pathology in chickens (Rohaim et al., 2021) than that reported for chIFITM3 in this study. Our findings indicate that the overexpression of chIFITM3 or chIFITM1 diminishes clinical disease in H5N1-infected chicks, as well as reduces virus-induced pathological lesions and virus shedding.

To conclude, findings indicate the importance of the innate immune system in establishing an antiviral state against HPAIV. The presented data provide proof of the capacity to generate virus-resistant chickens, which can protect food and inhibit the long-term spread of zoonotic viruses to humans. Gaining a further understanding of factors that influence the susceptibility of poultry to avian influenza viruses will help reduce the risks to animals including human health.

Chapter 7

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7.1 References

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