# INVESTIGATION OF THE ANTIVIRAL ACTIVITY OF DUCK VIPERIN AGAINST INFLUENZA A VIRUS

by

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**Abstract**

Ducks are the natural reservoir of influenza A virus. Normally, the virus replicates in their intestine and is passed in the feces, although highly pathogenic influenza A virus replicates in the lungs. Influenza infection in ducks is frequently asymptomatic. During infection with highly pathogenic avian influenza H5N1, *viperin* (virus-induced protein endoplasmic reticulum-associated interferon-inducible) was the most up-regulated innate immune gene in the duck lungs but was not upregulated in the duck intestine. Here we determined the effect of duck viperin on influenza A virus during infection. Chicken fibroblast cells stably expressing duck viperin or vector only were infected with low pathogenic influenza A virus A/chicken/CA/431/2000. Viperin decreased the viral titre after 24, 48 and 72 hours of infection. Viperin was suspected to decrease the lipid rafts through interacting with farnesyl diphosphate synthase. Using confocal microscopy and flow cytometry, we showed that duck viperin expression decreased the lipid rafts in the chicken fibroblast cells. The addition of exogenous farnesol, which works downstream of farnesyl diphosphate synthase, reversed the antiviral effect of duck viperin. Finally, we identified the critical regions and residues important for function of duck viperin. The central domain contains radical S-Adenosyl methionine motifs, mutation of which leads to loss of viperin antiviral effect. Viperin, which is induced by highly pathogenic influenza A virus, has an antiviral function.

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**Abbreviations**

| | |
|---|---|
| Ado | 5 -deoxyadenosyl radical |
| BSA | Bovine serum albumin |
| CHIKV | Chikungunya virus |
| *cig-33* | Cytomegalovirus-induced gene 33 |
| *cig-5* | Cytomegalovirus-induced gene 5 |
| CTB | Cholera toxin B |
| CTB-FITC | Cholera toxin B -fluorescein isothiocyanate conjugated |
| DENV | Dengue virus |
| DF-1 | Chicken fibroblast cell line |
| DMSO | Dimethyl sulfoxide |
| ER | Endoplasmic reticulum |
| FBS | Fetal bovine serum |
| FITC | Fluorescein isothiocyanate |
| FPPS | Farnesyl diphosphate Synthases |
| FRAP | Fluorescence recovery after photobleaching |
| HA | Hemagglutinin |
| HCMV | Human cytomegalovirus |
| HCV | Hepatitis C virus |
| HeLa cells | Human cervical cancer cells |
| HIV | Human immunodeficeincy virus |
| HPAI | Highly pathogenic avian influenza |
| IAV | Influenza A virus |

| | |
|---|---|
| IFN | Interferon |
| IRF3 | Interferon regulatory factor 3 |
| IRF7 | Interferon regulatory factor 7 |
| LCMV | Lymphocytic choriomeningitis virus infection |
| LPAI | Low pathogenic avian influenza |
| LPS | Lipopolysaccharide |
| M1 | Matrix protein M1 |
| M2 | Matrix protein M2 |
| MDA5 | Melanoma Differentiation-Associate Gene 5 |
| MDCK | Madin-Darby Canine Kidney Epithelial Cells |
| MDMs | Monocyte-derived macrophages |
| MEM | Minimal Essential Media |
| MEF | Mouse Embryo Fibroblast |
| MOI | Multiplicity of infection |
| NA | Neuraminidase |
| NEP | Nuclear export protein |
| NP | Nucleocapsid protein |
| NS1 | Non-structural protein 1 |
| NS2 | Non-structural protein 2 |
| OASL | 2'-5'-oligoadenylate synthetase-like |
| ORF | Open reading frame |
| PA | Polymerase acidic protein |
| PAMPs | Pathogen-associated molecular patterns |

| | |
|---|---|
| PB-2 | Polymerase basic protein 2 |
| PB1 | Polymerase basic protein 1 |
| PB1-F2 | Polymerase basic protein 1-Frame 2 |
| pDCs | Plasmacytoid dendritic cells |
| PFUs | Plaque forming units |
| poly I:C | Polyinosinic: polycytidylic acids |
| PRRs | Pattern recognition receptors |
| PRRSV | Porcine reproductive and respiratory syndrome virus |
| PSF | Streptomycin, Penicillin, Amphotericin B |
| RIG-I | Retinoic acid inducible helicase |
| RNP | Ribonucleoprotein |
| RSAD2 | Radical S-adenosyl methionine domain containing-2 |
| SAM | S-Adenosyl methionine |
| SiRNA | Small interfering RNA |
| TAP1 | Transporter associated with antigen processing subunit 1 |
| TLR | Toll-like receptors |
| TPCK-trypsin | L- (tosylamido-2-phenyl) ethyl chloromethyl ketone–treated trypsin |
| TX-100 | Triton X-100 |
| VAP-A | Vesicle-associated membrane protein-associated protein subtype A |
| VHSV | Viral hemorrhagic septicemia virus |
| *vig-1* | VHSV-induced gene number 1 |
| Viperin | Virus inhibitory protein, endoplasmic reticulum-associated, interferon-inducible |

# Chapter 1. Introduction

## 1.1 Influenza A virus

Influenza A virus (IAV) infection is one of the most common causes of mortality and morbidity worldwide. It is estimated that over 50,000 deaths occur every year due to seasonal influenza infection (Thompson et al., 2003). In addition to the seasonal strains, pandemic strains occasionally emerge. These strains can travel around the world and spread rapidly causing pandemic disease, such as the one caused by the 1918 pandemic strain. The 1918 IAV pandemic strain was responsible for the death of more than 50 million people worldwide (Mills et al. 2004; Ansart et al. 2009; reviewed in Johnson & Mueller 2002).

Influenza A virus is a member of *Orthomyxoviridae* family. It is a pleomorphic virus, which can be either spherical or filamentous, having a size of 80–120 nm in spherical form and 300 nm in filamentous form. It is an enveloped virus that consists of eight negative sense RNA segments. These RNA segments encode 11 different structural and non-structural proteins. These proteins are polymerase basic protein 1 (PB1), polymerase basic protein 1-frame 2 (PB1-F2), polymerase basic protein 2 (PB2), polymerase acidic protein (PA), hemagglutinin (HA), neuraminidase (NA), nucleocapsid protein (NP), matrix protein (M1) and M2, non- structural protein 1 (NS1) and non-structural protein 2 (NS2) (Chen et al. 2001; Lamb et al. 1980; reviewed in Rossman & Lamb 2011). The surface of the virus contains 2 spike glycoproteins HA and NA. IAVs are classified based on their HA and NA proteins. There are eighteen HA and eleven NA

subtypes of which 16 HA circulate in waterfowl and two (H17 and H18) have been isolated from bats (reviewed in Yoon et al. 2014; Alexander et al. 1986; Tong et al. 2013).

HA protein mediates the viral entry into the cells; it recognizes and attaches the virus to the sialic acid (also known as *N-acetylneuraminic acid*) receptors as well as allowing membrane fusion activity. Avian influenza strains preferentially bind to sialic acid residues with α 2,3 linkages, whereas human strains preferentially bind α 2,6 linkages (Connor et al. 1994). Upon attachment to the cells, the virion is internalized either by clathrin-dependent (Matlin et al., 1981) or clathrin-independent (Sieczkarski and Whittaker, 2002) endocytosis. The low pH in the endosomes triggers HA-mediated fusion of the viral–endosomal membranes and activates the M2 ion channel. M2 is a multi-functional, proton-selective ion channel, which has roles both in virus entry as well as in assembly and budding. It mediates proton conduction into the virion core, causing dissociation of the ribonucleoprotein (RNP) core from the M1 protein (Wharton et al., 1994). The RNP core contains the RNA polymerase complex proteins, PB1, PB2 and PA, and the NP that mediates binding and packaging of the viral genome. Dissociation of the RNP allows for its subsequent import into the nucleus and the start of viral replication (reviewed in Pinto & Lamb 2006). NS1, NS2 and PB1-F2 proteins are then expressed and are not incorporated into the mature virion. NS1 inhibits the immune function through inhibiting NF-κB pathway as well as inhibiting interferon-mediated antiviral responses of the host (García-Sastre et al., 1998; Wang et al., 2000). NS2 (also known as nuclear export protein (NEP)) mediates the transport of viral RNPs from the cell nucleus during

viral replication (O'Neill, Talon, & Palese, 1998). PB1-F2 is expressed from the second open reading frame of the PB1 gene. It triggers the proapoptotic function and increases IAV pathogenicity in mice (Chen et al., 2001).

Influenza viruses bud from the apical plasma membrane and from specific membrane microdomains, which are called lipid rafts. The lipid rafts are plasma membrane subdomains that contain high concentrations of cholesterol and glycosphingolipids (Leser & Lamb, 2005; Nayak et al., 2004; and Scheiffele et al., 1999). The lipid rafts are important for the budding of different viruses such as HIV-1, Ebola virus and influenza viruses (reviewed in Suomalainen 2002). With the help of M1 protein, IAV assembly takes place at the plasma membrane where M1 organizes the interaction between the glycoproteins HA and NA and the RNP (Gomez-Puertas, et al. 2000). In influenza viruses, mutation of the M1 transmembrane domain leads to the attenuation of viral replication (Chen et al. 2005). During budding, HA and NA start clustering on the lipid rafts domain to start budding out from the cell. M1 protein binds to the cytoplasmic tails of HA and NA and acts as a docking site for viral RNPs. Polymerization of M1 leads to elongation of the budding virion. M2 protein interacts with M1, which leads to the release of the virus particles (reviewed in Rossman & Lamb 2011).

Cholesterol is an important component of the lipid rafts and essential for its stability (Brown & London, 1998). Cholesterol depleting agents are used to disrupt the lipid rafts, which allow the raft-associated proteins to be soluble in non-ionic detergent

such as Triton X-100 (TX-100) at low temperature. After extraction with an excess of cold TX-100, the resulting detergent-insoluble glycolipid-rich complexes can be floated in sucrose gradients and thereby be separated from other detergent-insoluble material like actin and tubulin ( Brown et al., 1992). Simons and coworkers demonstrated that in influenza virus, HA is associated with the lipid rafts due to its poor extraction by non-ionic detergent such as Triton X-100 at 4°C (Scheiffele, Roth, & Simons, 1997). During IAV infection, HA was more readily extracted from viperin expressing cells by Triton X-100, which suggests a decrease in lipid rafts production during viperin expression ( Wang et al., 2007).

**1.2 Ducks are the natural host of influenza A viruses**

IAVs can infect a wide range of animals, including humans, pigs, ducks and horses. However, ducks and wild waterfowl are the natural reservoir of IAVs. The reservoir and the virus have co-evolved. The host has evolved some defence mechanisms in order to limit the damaging effect of viral infection, while the virus evolved to limit the damage of the host immune response. IAVs replicate in wild ducks. They invade the cells lining the intestinal tract where they replicate, causing no signs of the disease. The virus is then excreted from the intestine with the faeces (reviewed Webster et al. 1992; Webster et al. 1978). This process leads to the transmission of the virus from the contaminated ponds to the dabbling and diving ducks, which allows the spread of the virus (Munster et al., 2007).

IAVs can cross the species barrier from their reservoir (ducks and other waterfowls) to mammalian and other avian species. Infection of other species may lead to their mortality and morbidity, while infections of the natural reservoir species are typically asymptomatic.

IAV infecting poultry can be divided into two distinct groups on the basis of their ability to cause disease in chickens; either low pathogenic avian influenza (LPAI) or high pathogenic avian influenza (HPAI). HPAI strains are the virulent viruses that cause 'fowl plague', in which mortality may reach 100%. The H5 and H7 subtypes have been shown to cause HPAI in susceptible species, but not all H5 and H7 viruses are virulent. All other viruses are known to be LPAI; they cause a much milder, primarily respiratory disease. However, LPAI infection may be exacerbated by other infections or environmental conditions resulting in much more serious disease (reviewed in Alexander 2000; Alexander 2007). IAV HA is an essential determinant of pathogenicity. HA is produced as a precursor HA0, which needs a post-translational cleavage by the host proteases to be functional and cause infection (Rott, 1992). LPAI HA0 precursor has a single arginine at the cleavage site and another one at position 4. These viruses use host proteases such as trypsin- like enzymes to cleave HA0. Thus they are restricted to the site where these enzymes are available in the host, for example the respiratory and intestinal tracts. On the other hand, unlike the LPAI, the HPAI viruses possess multiple basic amino acids like arginine and lysine at their HA0 cleavage sites, which make them liable to cleavage by a ubiquitous protease(s). HPAI replicate throughout the bird, damaging vital organs and tissues which results in disease and death (Rott et al. 1995; reviewed in Alexander 2000).

### 1.3 Innate immune response to influenza A viruses in ducks

IAV has to overcome a number of barriers maintained by the host to successfully establish an infection. One of these barriers is the innate immune system, which acts as the first line of defense against IAV. The rapid detection and subsequent activation of immune effectors is essential in any successful innate immune response. A particularly important part of the antiviral innate immune response is the production of type-I interferon (IFN). The activated type I interferon (IFN-α/β) system, which is among the cytokines, represents the most powerful innate defense mechanism against IAV replication and spread (Wu et al. 2011).

In order to produce IFN, the virus must cross the cell membrane. Once the virus finds its way through the cell membrane, the innate immune response starts by sensing pathogen-associated molecular patterns (PAMPs), such as viral nucleic acids, by pattern recognition receptors (PRRs). There are two classes of PRR that play a role in sensing the IAV infection in ducks; the retinoic acid inducible helicase (RIG-I) (Barber et al., 2010), and the Toll-like receptors (TLR)(reviewed in Takeda & Akira 2004). Once they engage with their receptors, they initiate pathways that lead to the production of type-I IFN. As well, TLR7 senses the viral ssRNA in endosomes (Lund et al., 2004; MacDonald et al. 2008).

RIG-I and Melanoma Differentiation-Associate Gene 5 (MDA5) are the cytosolic RLR receptors that can detect the IAV RNA. RIG-I binds to smaller 5′ triphosphorylated single stranded RNA (Hornung et al., 2010; Pichlmair et al., 2006), whereas MDA5 binds

to longer part of double stranded RNA (Kato et al., 2008). During IAV infection, RIG-I contributes more to the production of type-I IFN compared to MDA5(Kato et al., 2006). Ducks possess both a functional RIG-I (Barber et al., 2010) and MDA5 (Wei et al., 2014) whereas chickens sense IAV infection through MDA5 only as they lack RIG-I (Liniger at al., 2012; Barber et al., 2010).

When White Pekin ducks were infected with LPAI A/mallard/BC/500/05 (BC500) and HPAI A/Vietnam/1203/04 (VN103), the transcriptional profile ISGs in lung tissue showed a remarkably rapid induction of the interferon response, which led to upregulation of key genes at 1 dpi with VN1203. In comparison to the low pathogenic strain BC500 that induced much lower expression of these same antiviral genes in lung or intestine tissue, despite higher replication of this virus in ducks. VN1203 replicates in lung, while BC500 replicates in the intestinal tissues and this difference in replication site might be causing the differences in response to VN1203 and BC500. Otherwise viral replication may be more widespread in VN1203-infected lung as the M gene transcript could be readily detected in VN1203 infected lung, while it was at the limit of detection in BC500 infected intestine tissue. There was an upregulation of ISGs in lung tissue, and only slight upregulation of these genes in intestinal tissue. The increase of the ISGs include, *dIFITM1 ISG12* and *IFIT* (Vanderven et al., 2012).

## 1.4 Virus inhibitory protein, endoplasmic reticulum-associated, interferon-inducible (Viperin)

Viperin was first discovered during the characterization of new fish genes that were expressed in response to viral infection or induction. Benmansour and coworkers infected rainbow trout by viral hemorrhagic septicemia virus (VHSV), a type of rhabdoviruses that is responsible for a viral disease causing massive losses in European trout farms. One of the genes induced during infection was identified as VHSV-induced gene number 1 (*vig-1*). This gene was induced by fish interferon and is encoded by 348 amino acids. The *vig-1* gene was homologus to a cytomegalovirus-induced gene (*cig-5*) that was discovered earlier (Boudinot et al. 1999 ; Zhu et al. 1998) . Zhu and coworkers identified *cig 5* to be a gene stimulated by human cytomegalovirus (HCMV) infection, but they did not know its exact function at that time (Zhu et al. 1998). While examining the IFN response of the primary fibroblast cells infected with HCMV, Cresswell and Chin found a unique IFN response protein almost identical to Cig 5, which they named viperin.

### 1.4.1 Viperin structure

Viperin encompasses three domains; the N-terminal domain, the central domain, and the C-terminal domain with a molecular mass of 42 kDa. The duck viperin gene encodes 363 amino acids with 1092 nucleotides (Figure 1) (Zhong et al. 2015). The open reading frame (ORF) of the human viperin transcript is 1083 nucleotides, encoding a 361 amino acid peptide (Chin & Cresswell, 2001). The fish viperin transcript is 1062 nucleotides, encoding a protein with 354 amino acids (Sun & Nie, 2004). The mouse viperin transcript is 1196 nucleotides, encoding a protein with 362 amino acids

(Boudinot et al., 2000) and the chicken viperin transcript is 1065 nucleotides, encoding 354 amino acids (Goossens et al. 2015).

The N-terminal residues of viperin vary between species and are not conserved (Helbig et al. 2011; Hinson & Cresswell 2009). The human viperin N-terminal consists of an amphipathic α-helix and a leucine zipper. The α-helix domain acts as a membrane-binding domain as it anchors the protein to the ER membrane (Hinson & Cresswell, 2009; Hristova et al., 1999). The leucine zipper domain appears as a periodic repetition of leucine at every seventh amino acid, which facilitates the dimerization of different polypeptides (Landschulz et al.1988). This domain might play an important role in protein-protein interaction (Helbig et al., 2011).

The central domain consists of four S-Adenosyl methionine (SAM) motifs, which is why viperin is also known as radical S-adenosyl methionine domain containing-2 (RSAD2). The first one of these SAM motifs in viperin contains the canonical CxxxCxxC (Duschene & Broderick, 2010; Shaveta, et al. 2010; Upadhyay et al., 2014). The SAM domains are known for their ability to bind to the Fe-S cluster and catalyze the slow cleavage of SAM to form a highly reactive radical Ado• (5 -deoxyadenosyl radical). This Ado• is highly active and is able to abstract a hydrogen atom from a substrate. This step is considered a common important step in a wide variety of chemical transformations including anaerobic oxidation, sulfur formation, isomerization, sulfur insertion, ring formation and protein radical formation. SAM domains also play a key role in DNA precursor, vitamin and cofactor formation and biodegradation pathways

(Sofia et al. 2001; Broderick et al. 2014). Song and colleagues carried out spectroscopic studies on purified N-terminally truncated viperin heterologously expressed in *Escherichia coli,* and showed that viperin has an active SAM domain and can bind to the iron-sulfur cluster (Shaveta et al., 2010). On the other hand, Duschene and Broderick demonstrated SAM in viperin can produce the active Ado•, the classical product of SAM through a combination of HPLC and mass spectrometry analysis (Duschene & Broderick, 2010). The first SAM motif in viperin that contains the CxxxCxxC motif is important for the formation of a [4Fe-4S] cluster, while the other three motifs appear to function in the binding of SAM (Duschene & Broderick, 2012) .

The C-terminal domain of viperin which is highly conserved among species, is important for viperin dimerization (Hinson & Cresswell, 2009). Its exact role however is still unclear. Guo and coworkers demonstrated that an aromatic amino acid residue at the C-terminal domain of viperin is important for proper antiviral function against hepatitis C virus (HCV) (Jiang et al., 2008). This suggests that the C-terminal domain might be involved in protein-protein interactions and/or substrate recognition required for mediating an enzyme activity (Jiang et al. 2010; reviewed in Seo et al. 2011; Helbig et al. 2011). During HIV infection of monocyte-derived macrophages (MDMs) in vitro, viperin activity was inhibited by a short C-terminal domain truncation but not with a longer truncation. This difference suggests that the short truncation may destabilize viperin and alter its tertiary structure (Nasr et al., 2012).

### 1.4.2 Viperin Regulation

Normally, viperin normal expression levels are low; however, its expression is strongly induced by IFN from a variety of cell types, through double-stranded (ds) DNA, polyinosinic: polycytidylic acids (poly I:C) which is an RNA analog, lipopolysaccharide (LPS), and infection with a range of viruses (Nasr et al. 2012; reviewed in Helbig & Beard 2014; Chin & Cresswell 2001; reviewed in Mattijssen & Pruijn 2012). Viperin upregulation evidence has been reported in different studies in which both the host cell and virus varied. In these studies both IFN-dependent and IFN independent pathways were implicated. The discovery of both *cig-5* and *vig-1,* during the infection of HCMV in the human fibroblast and the VHSV in the rainbow trout fish has shown that viperin is capable of being directly induced by a virus as well as indirectly through an IFN-α-dependent pathway (Boudinot et al., 1999; Chin & Cresswell, 2001). While studying the IFN pathway of human macrophages during the HCMV infection, Chin and Cresswell identified an IFN-γ-responsive gene, viperin (Chin & Cresswell, 2001). Viperin was also produced independently from IFN, expression of mouse viperin was found to be still induced in the presence of anti-IFN antibodies or cycloheximide, which prevent IFN activity (Boudinot et al., 2000). The IFN-independent viperin induction by VSV appears to use IRFs activated by the mitochondrial anti viral signaling protein (MAVS), in this case localized to peroxisomes rather than mitochondria. Upon viral infection, peroxisomal MAVS induces rapid and transient IFN-independent viperin expression, whereas mitochondrial MAVS activates IFN-mediated viperin expression with delayed kinetics (Dixit et al., 2010).

Viperin activity is not just active against viruses but also active against bacteria. Tilapia fish viperin is expressed and regulated by bacterial infection and has antibacterial properties. As well, electrotransfering of a viperin-expressing plasmid into zebrafish muscles was found to reduce bacterial counts (Lee et al., 2013). Viperin was also expressed during atherosclerosis and induced in vascular cells by inflammatory stimuli (Olofsson et al., 2005).

In ducks, viperin was induced in the spleen, kidneys, liver, brain, and blood during Newcastle disease virus infection. Viperin was also upregulated with poly (I:C) (Zhong et al. 2015). During IAV infection in mouse embryo fibroblast (MEF) and MEF with RIG-I knock down. Viperin was not detected in the RIG-$I^{-/-}$, it was only detected in the presence of RIG-I (Loo et al., 2008).

### 1.4.3 Celluar localization of viperin

Viperin uses the N-terminal domain to anchor onto the endoplasmic reticulum (ER) membrane (Wang et al. 2007; Hinson & Cresswell 2009; reviewed in Seo et al. 2011). Cresswell and his colleagues were able to identify the precise topology and the ER localization signal of viperin. In order to investigate whether viperin is localized to the luminal or cytosolic face of the ER, they used anti-tapasin and anti-calnexin antibodies. Tapasin is localized in the lumenal side of the ER membrane, while calnexin is localized at the cytosolic part of the ER. With the help of these antibodies along with an anti-viperin monoclonal antibody, streptolysin O was used for permeabilization of the cells' plasma membrane, allowing the aforementioned antibodies to enter the cells. Using confocal microscopy under these conditions, the co-localization of calnexin and viperin

was observed, while the ER lumenal epitope of tapasin was detected only after the permeabilization with saponin, which caused permeabilization of the ER. This finding confirms that viperin is localized in the cytosolic side of the ER membrane. Further investigation showed that viperin co-localized with a human transporter associated with antigen processing subunit 1 (TAP1) protein. Their next step was to identify which of the three domains of viperin are responsible for viperin localization to the cytoplasmic face of the ER. They found that deleting the α-helix of viperin N-terminal domain localized viperin to the cytosol, however when this domain was added again, viperin was re-localized to the ER membrane (Hinson & Cresswell, 2009). ER is where the synthesis of bulk lipids and cholesterol takes place in conjunction with the Golgi. Then the lipids and cholesterol must be transported to other organelles such as endosomes, mitochondria and plasma membranes. This process is important for the viral entry, assembly and budding, as well as for virus replication in some viruses like influenza virus and HCV (Raychaudhuri & Prinz 2010; reviewed in Helbig & Beard 2014).

While examining the co-localization of viperin, Cresswell and colleagues observed that the cells expressing viperin have a crystalloid ER, distorting the smooth ER into a lattice like structure. This crystalloid ER structure was only formed in the presence of the wild type viperin not the amphipathic α-helix part only, or the viperin lacking the N-terminal domain viperin. They also found that viperin dimerization or multimerization can be done independently from the N-terminal domain that contains the amphipathic α-helix. They showed that the viperin C-terminal domain is responsible for protein dimerization, which caused crystalloid ER formation (Hinson & Cresswell, 2009).

Viperin is also localized to the lipid droplet of neutrophils during some viral infections such as lymphocytic choriomeningitis virus infection (LCMV) and HCV (Hinson et al., 2010). In other viral infections, such as a HCMV infection, viperin expression was found to be induced and is relocated to the mitochondria (Chin & Cresswell, 2001).

### 1.4.4 Antiviral roles of viperin

Viperin has a wide range of antiviral activities. It has been reported as one of the IFN genes that are highly upregulated in response to viral infections. Viperin has an antiviral effect against medically significant arbovirus, as such as dengue virus. Dengue virus (DENV) is the cause of dengue fever, which is a mosquito-borne viral disease that is widespread in tropical regions. To date, no specific treatment to DENV has been identified (Bhatt et al., 2013). During DENV infection *viperin* was found to be the most upregulated gene in the type I IFN response pathway (Fink et al., 2007). DENV is recognized by the TLR3, RIG-I and MDA5 pathways to induce the IFN response, viperin then inhibits early post-entry DENV-2 RNA replication. The anti-DENV activity was found to reside in the C-terminal end of viperin (Helbig et al., 2013).

Viperin also has demonstrated antiviral activity against HCV (Helbig et al. 2005). During HCV infection viperin is localized in the lipid droplets, which are an essential component of the HCV life cycle. HCV dispatches two of its viral proteins, core and NS5A, onto the surface of intracellular lipid droplets where the virus assembly takes place. Viperin interacts with HCV NS5A and core proteins at the lipid droplets interface while interacting with the proviral host factor, vesicle-associated membrane protein-

associated protein subtype A (VAP-A). This host factor interacts with NS5A (and NS5B) and is required for the efficient replication of HCV genomic RNA. The anti-HCV activity of viperin depends on the C-terminal region of viperin and the amphipathic helix of the N-terminal. The C-terminal domain is essential for viperin to interact with HCV NS5A and exert its anti-HCV action, while the amphipathic helix of the N-terminal domain is important for localizing viperin to the ER and lipid droplets (Helbig et al., 2011).The viperin central domain contains SAM motifs, which plays an important role in viperin anti-HCV activity (Jiang et al., 2008).

Chikungunya (CHIKV) is a viral disease transmitted to humans by infected mosquitoes. It causes fever and severe joint pain. During CHIKV infection, Teng et al. found that viperin was highly induced in the monocytes, as the major target CHIKV is the blood. *In vivo*, viperin deficient mice, Rsad2$^{-/-}$mice, suffered from higher viremia levels and more pronounced joint inflammation. The N-terminal amphipathic α-helical domain of viperin is important for its antiviral activity against CHIKV (Teng et al., 2012)

In monocyte-derived macrophages (MDMs) cells, viperin was the most up-regulated IFN-stimulated gene during HIV infection, and it significantly inhibited HIV-1 production (Nasr et al., 2012). Viperin disrupted the lipid rafts, which are important for the entry and egress of the HIV. SiRNA knockout of *viperin* leads to increased HIV-1 replication. SAM motifs at the central domain are important for viperin anti-HIV activity. Nevertheless, the addition of exogenous farnesol reversed viperin inhibition of the HIV (Nasr et al., 2012).

Viperin produced in other animals has antiviral activity across species. For example, oyster viperin has an antiviral activity against DENV (Green et al., 2015). As well, rainbow trout viperin has an antiviral activity against viral hemorrhagic septicemia virus, which is a trout pathogen (Boudinot et al., 1999). Furthermore, monkey viperin was found to restrict Porcine reproductive and respiratory syndrome virus (PRRSV), a pathogen that is responsible for huge economic losses to the global swine industry. Monkey viperin acts by inhibiting PRRSV genome replication and translation (Fang et al., 2016).

### 1.4.5 Viperin and influenza A viruses

Viperin expression has been shown to inhibit influenza A virus. Cresswell and colleagues demonstrated the viperin anti-influenza effect. They constructed cell lines expressing mouse viperin under tetracycline control. They found that mouse viperin expression inhibited the release of influenza virus from the cells through perturbing the lipid rafts where influenza virus is released from the cells. Mouse viperin interacted directly with Farnesyl Pyrophosphate Synthase (FPPS) and decreased its activity. FPPS is a key enzyme in the synthesis of Farnesyl Pyrophosphate (FPP), which is a precursor of cholesterol, farnesylated and geranylated proteins (Figure 2) (Bloch 1965; reviewed in Szkopińska & Płochocka 2005). Over expression of FPPS, through transfecting the cells with a plasmid that encodes FPPS reversed the anti-influenza effect of viperin. The role of FPPS was also confirmed using siRNA of FPPS. The FPPS siRNA decreased the FPPS levels and decreased the influenza virus titres as in viperin expressing cells (Wang et al., 2007). Wang et al., also tried to determine whether viperin activity is mediated by a

decrease in protein isoprenylation, they treated the cell with an inhibitor of HMG reductase, lovastatin, which acts upstream of FPPS in the biosynthetic pathway. This treatment led to decreased isoprenylation of some proteins. The isoprenylation of these proteins was not detected during viperin expression in the cells (Wang et al., 2007).

In another study done by Chow and colleagues, human viperin *in vitro* has demonstrated antiviral activity against IAV. Surprisingly, using viperin-knockout mice, they showed that innate expression of viperin *in vivo* does not decrease IAV titres in mice lungs. They demonstrated that viperin deficiency in mice does not significantly alter pulmonary damage induced by IAV infection or affect production of neutralizing antibody against IAV (Tan et al., 2012).

### 1.4.6 Viperin expression in ducks during influenza A viruses infection

During IAV infection in humans, the IFN induced MxA protein confers a high degree of resistance to the virus (Pavlovic et al., 1990), however MX is non-functional in ducks (Bazzigher et al., 1993). On the other hand, when exposing the mallard ducks to the HPAI H5N1 (A/duck/Hubei/49/05), one of the most upregulated innate immune genes in the duck lungs was *viperin*. Table 1 shows the top five upregulated transcripts in the lungs of H5N1 infected ducks (unpublished work done by Man Rao). These results were confirmed by qPCR, when White Pekin duck was infected with A/Vietnam/1203/04 (H5N1) (VN1203), as viperin was upregulated 15666 fold in the lungs, and no upregulation of viperin could be seen in the intestine of the infected ducks (Figure 3). It is unclear whether duck viperin has an anti-viral effect to influenza virus.

## 1.5 Hypothesis, experimental aims and results summary

### 1.5.1 Hypothesis

We hypothesize that duck viperin has an anti-viral activity against influenza virus like mouse and human viperin by perturbing the lipid rafts.

### 1.5.2 Experimental objectives and Results summary

As mentioned before viperin is an important innate immunity protein, which is evolutionarily conserved from fish to primates. It has widely been recognized as an innate immunity defense protein against DNA and RNA viruses as well as other pathogens. To date, no studies have been done on duck viperin during IAV infection, which is the most upregulated gene in the natural host of IAV with no or little signs of disease.

*Objective 1. Developing a cell line expressing the duck viperin.*

In order to develop an avian cell that is able to express duck viperin with an advantage of stable and homogeneous expression, duck viperin with the C-terminal V5 tag was previously cloned into the mammalian expression vector pcDNA3.1/Hygro+. Using Lipofectamine 2000, I was then able to stably transfect an empty vector or duck viperin into DF-1 cells (chicken fibroblast cell line). In order to get a monoclonal stable cell line, I plated candidates on 96 well plates for isolation and separation of single clones. To confirm the transfection, the Western blot technique was used to confirm the expression of the tagged viperin in DF-1 cells and a band size at 42 kDa was detected. HeLa cell (human cervical cancer cells) were transiently transfected with duck viperin

with the C-terminal V5 tag. The expression of duck viperin was confirmed using Western blotting.

*Objective 2. Investigating the antiviral effect of the duck viperin against IAV.*

To evaluate the antiviral effect of duck viperin against IAV, DF-1 cells stably expressing viperin or transfected with an empty vector were infected with the low pathogenic avian influenza (LPAI) A/chicken/California/431/00 (H6N2). Virus titres were determined after 24, 48 and 72 hours of infection using plaque assay. The percentage of infected cells was determined after 48 hours of infection using the Operetta High Content Imaging System (Perkin Elmer).

To evaluate the antiviral effect of duck viperin in HeLa cells, cells were transiently transfected with duck viperin and infected with mouse adapted human strain A/Puerto Rico/8/1934 (H1N1) (PR8).x Virus titres were determined using plaque assay after 48 hours of infection.

The virus titres in plaque assay decreased in the presence of cells expressing duck viperin, and the percentage of infected cells decreased in the presence of viperin.

*Objective 3. Investigating the effect of viperin on the cell lipid rafts and the effect of the addition of exogenous farnesol to the cells.*

Viperin interacts with FPPS and decreases its enzyme activity, which perturbs the formation of lipid rafts. Lipid rafts are important for the virus release from the cells (Leser &

Lamb, 2005; Scheiffele et al., 1999) and by disturbing the lipid rafts, the amount of virus released from the cells should be decreased significantly (Xiuyan Wang et al., 2007). In order to detect whether there is a change in lipid rafts, DF-1 cells stably and transiently expressing viperin were used, lipid rafts were stained with cholera toxin B (CTB) fluorescein isothiocyanate (FITC) conjugated, where CTB binds to gangliosides in the lipid rafts (Fujinaga et al., 2003). Gangliosides are composed of glycosphingolipids attached to one or more sialic acids. A decrease in the number of cells with lipid rafts was observed in the DF-1 cells expressing viperin compared to the DF-1 cells transfected with an empty vector. To further confirm these results, the percentages of the cells expressing lipid rafts in both DF-1 expressing either duck viperin or an empty vector were determined by flow cytometry using CTB-FITC.

Farnesyl Pyrophosphate Synthase (FPPS) is a key enzyme in the synthesis of FPP, during mevalonate pathway. FPPS helps in the condensation of dimethyallyl pyrophosphate and isopentenyl diphosphate to form farnesyl diphosphate. Viperin is known to decrease the activity of FPPS. During IAV infection, viperin prevented prenylation of cell membrane proteins by interacting with the enzyme FPPS and decreasing its activity; thus inhibiting influenza budding from the plasma membrane. This inhibition mechanism was reversed by overexpressing FPPS (Wang et al., 2007). During HIV infection of Monocyte Derived Macrophages (MDMs) cells, viperin exerts its inhibitory effects on the virus via interacting with FPPS. The addition of exogenous farnesol, which can converted directly to FPP, prevented lipid raft disruption and permitted viral budding which significantly enhances HIV production in the cells (Nasr et al., 2012). In order to investigate whether duck viperin

antiviral effect will be reversed in the presence of exogenous farnesol, farnesol was added to two different clones of DF-1 cells stably expressing viperin. The changes in the virus titres were determined using plaque assay. A significant increase in virus titres was observed in the presence of farnesol compared to its absence in cells expressing duck viperin. Conversely, no significant difference could be seen in virus titres between cells expressing empty vector in the presence or absence of exogenous farnesol.

*Objective 4. Identifying the critical regions and residues important for the duck viperin function.*

Viperin consists of three distinct domains; an N-terminal domain, a C-terminal domain and a central domain. To determine which one of these three domains is essential to the viperin antiviral effect, an N-terminal truncation mutant, a short C-terminal truncation and a long C-terminal truncation mutants were constructed, as well as mutants in the first and the last SAM motifs in the central domain. These mutants were used to identify the critical regions and residues important for duck viperin function. Mutations in SAM motifs as well as N-terminal and C-terminal short truncations were found to decrease viperin antiviral activity, whereas long C-terminal truncation did not affect viperin antiviral activity. The critical regions and residues that are important for viperin anti-influenza activity have not been identified in viperin fromany of the vertebrates before.

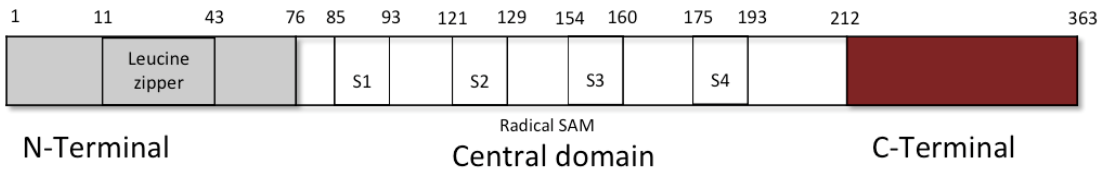



**Figure 1: Schematic representation of the structure of duck viperin**

The schematic structure shows the positions of the putative leucine zipper motif in the N-terminal domain, the central domain with the four SAM motifs and C-terminal domain of duck viperin.

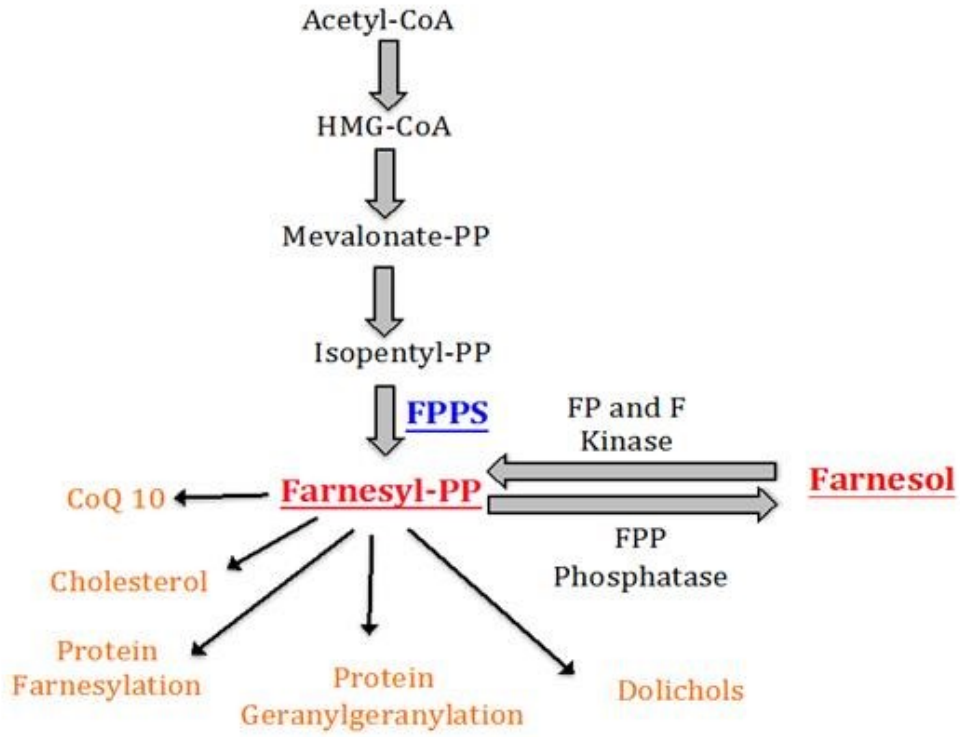



**Figure 2. FPPS pathway and formation of cholesterol, farnesylated and geranylated proteins**

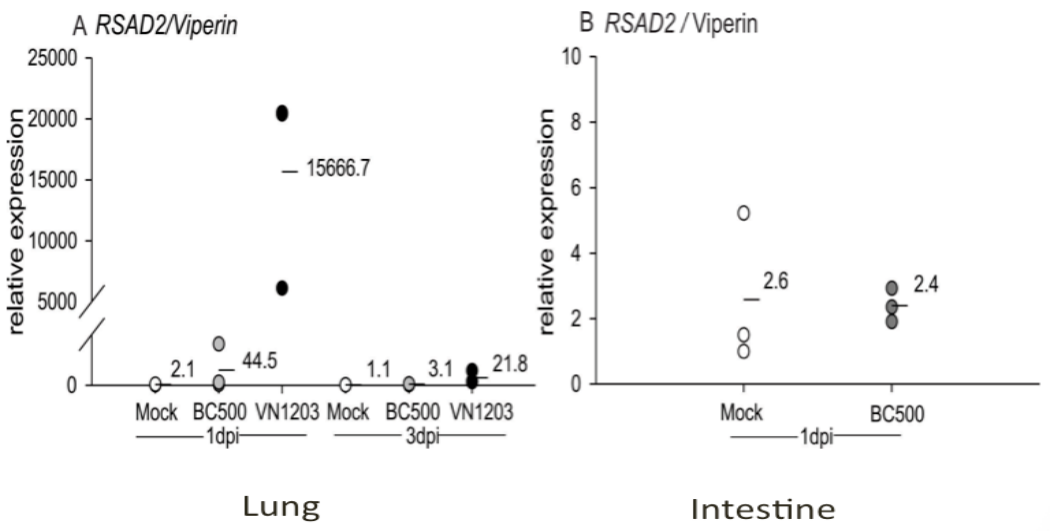



**Figure 3. Viperin expression levels by qPCR.**

White Pekin ducks were infected with A/Vietnam/1203/04 (H5N1) (VN1203) and A/mallard/BC/500/05 (H5N2) (BC500). The relative expression of viperin was determined using qPCR at 1-day post infection (dpi) and 3 dpi in the lungs and 1 dpi in the intestine.

**Table 1. The top 5 most upregulated transcripts in lung of H5N1 infected ducks.**
Transcriptome analyses of the top 5-upregulated genes after infecting the mallard duck with the HPAI (A/duck/Hubei/49/05) H5N1 virus.

| Gene | Fold upregulation 1dpi | Fold upregulation 2dpi |
|---|---|---|
| *Viperin* | 342.322 | 675.296 |
| *IFIT5* | 210.667 | 288.608 |
| *CCL4* | 128.442 | 243.037 |
| *PKR* | 173.977 | 223.074 |
| *OASL* | 114.799 | 174.119 |

## Chapter 2. Materials and Methods

### 2.1 Sequencing and alignment of duck viperin and duck FPPS with other viperin

Viperin-V5 tagged at the C-terminal domain was previously cloned into pcDNA3.1/Hygro+ and transformed into *Escherichia coli* DH5α (Invitrogen). *E. coli* DH5α was grown at 37 °C in an Innova 4300 incubator shaker (New Brunswick Scientific laboratories) for 16 hours. I then isolated the plasmid from *E. coli* using the Qiagen Spin Miniprep Kit (Qiagen Inc., Oakville, ON) following the manufacturers' instructions. The extracted plasmid was sequenced in the forward and reverse direction using BigDye Terminator v3.1 (Applied Biosystems) using vector specific primers T7-pgem and BGHR primers (Table 2). Sequences were analyzed using ContigExpress (Invitrogen). Nucleotide sequence alignment was done by SnapGene software (from GSL Biotech; available at snapgene.com). Amino acid sequence alignment and the percent identity of duck viperin, chicken viperin, human and mouse viperin (Table 3) were determined using Clustal Omega. The phylogenetic tree was created using Clustal Omega (Li et al., 2015; McWilliam et al., 2013; Sievers et al., 2011). Accession numbers of vertebrate viperin proteins are provided in Table 3.

Alignment of amino acids sequences of FPPS, also known as Farnesyl Diphosphate Synthase (FPPS) of the duck, chicken and human and percentage identity were determined using Clustal Omega. Phylogenetic trees were created using Clustal Omega (Li et al., 2015; McWilliam et al., 2013; Sievers et al., 2011). Accession numbers of vertebrate FPPS are provided in table 4.

**2.2 Cell culture, transfections and generation of stable cell lines expressing V5 tagged viperin**

DF-1 cells, a spontaneously immortalized chicken fibroblast cell line, were cultured in Dulbecco's modified Eagles Medium (DMEM) (Life Technologies) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich) at 39°C and 5% $CO_2$. For transient transfections, cells were seeded overnight in 6-well plates ($8x10^4$ cells) and 24 hours later transfected with 1.5 μg of plasmid DNA/well using 3.75 μL of Lipofectamine 2000 (Invitrogen). Infections were performed 24 hours after transfection. For stable transfections DF-1 cells stably expressing viperin were generated by seeding cells overnight in 6-well plates ($8x10^5$ cells) and 24 hours later were transfected with 1.25 μg of linearized plasmid DNA/well using 3.75 μL of Lipofectamine 2000 (Invitrogen). Plasmid DNA was linearized by digestion with BglII (New England Biolabs). Forty-eight hours after transfection, cells were put under selection using hygromycin (500 μg/mL) and surviving cells were expanded. After approximately 10 days under selection, the concentration of hygromycin was lowered to a maintenance dose (250 μg/mL). Individual clones were isolated by limiting dilution, and expression of duck viperin in individual clones screened by Western blot.

**2.3 Western blots**

Whole cell lysates of DF-1 cells were collected using Lysis Buffer (50 mM Tris-HCl (pH 7.2), 150 mM NaCl, 1% (v/v) Triton X-100) with cOmplete Mini, EDTA-free proteinase inhibitor (Roche Diagnostics). Cell lysates were boiled in 1x Laemmli buffer for 10 minutes before separation by SDS-PAGE electrophoresis, and were transfered to a

nitrocellulose membrane. Membranes were blocked using 5% (w/v) skim milk powder in 1X PBS for one hour. Western blotting was performed using a primary mouse anti-V5 antibody at 1:5000 (Life Technologies) and subsequent blotting with a secondary goat anti-mouse-HRP at 1:5000 (BioRad). Proteins were visualized by chemiluminescence using the ECL kit (GE-Healthcare).

**2.4 Cell culture and virus infections**

A/chicken/California/431/2000 (H6N2), and A/Puerto Rico/8/1934 (H1N1) were propagated in 10 day old embryonated chicken eggs. The titre of all stocks was determined by plaque assay on MDCK cells. Twenty-four hours later after transfection or plating of stably expressing DF-1 cells, cells were challenged with H6N2. HeLa cells were challenged with H1N1 at the multiplicity of infection (MOI) of 0.05. For influenza infections, DF-1 cells were cultured in DMEM supplemented with 0.3% BSA and sodium bicarbonate only, while, HeLa cells were cultured in DMEM supplemented with 0.3% BSA, sodium bicarbonate and L- (tosylamido-2-phenyl) ethyl chloromethyl ketone–treated trypsin (TPCK-trypsin) (Worthington Biochemical) (0.1 µg/mL), DF-1 cells were infected with influenza A virus for 60 minutes. During infection the plates were rocked every 15 min before changing media, and cells washed once with 1X PBS to remove unbound virus. Fresh media was added and cells were incubated for different times before fixing or plaque assay (24, 48 and 72 hours).

**2.6 Plaque assay**

Madin-Darby Canine Kidney (MDCK) epithelial cells were cultured in 1x Minimal Essential Media (MEM) supplemented with 10% fetal bovine serum (FBS) (Sigma-Aldrich), sodium bicarbonate 7.5% (Life Technologies Inc.), PSF (Streptomycin, Penicillin, Amphotericin B) (Sigma-Aldrich) to prevent any bacterial or fungal contamination, MEM vitamin growth supplement for cell culture medium (Sigma-Aldrich), and L-glutamine (Gibco). Infection media for MDCK cells contains the same ingredients as growth media, except 10 % FBS is replaced with 0.3% BSA and 1 µg/mL of TPCK (Worthington Biochemical) added. Supernatants from infected DF-1 cells were collected 24 or 48 or 72 hours post infection, and serially diluted in infection media. Monolayers of MDCK cells were infected with serially diluted supernatants. After one hour with rocking every 15 minutes, supernatants were removed and cells were washed twice with 1X PBS. Cells were then overlaid with infection media containing 0.9% agar. After 72-96 hours, cells were stained with 0.1% crystal violet solution and the number of plaques were counted to calculate the number of plaque forming units per milliliters (PFUs/ml).

**2.7 Fluorescent microscopy analysis of viral infection**

Forty-eight hours after infection with influenza A virus, cells were fixed in 1% paraformaldehyde, then treated with 0.1% Triton X-100 for 10 minutes. Cells were washed three times with 1X PBS and blocked for 1 hour with 4% BSA. The cells were then stained with anti-nucleoprotein-FITC (Argene) for 1 hour, followed by staining with Hoechst 33342 (Life Technologies). The former stains the IAV nucleoprotein and the

latter stains the nucleus. Images were taken with the Operetta High Content Imaging System (Perkin Elmer) to determine the percentage of infected cells.

**2.8 Fluorescent microscopy and lipid rafts detection**

DF-1 cells stably expressing viperin or an empty vector were seeded (8×105 cells/well) onto sterile glass coverslips in 6 well plates. Twenty-four hours post-transfection, seeded cells were stained with Cholera Toxin B subunit FITC conjugate (CTB-FITC) (Sigma). CTB is used as a marker for lipid rafts, which are membrane microdomains enriched in cholesterol and sphingolipids. CTB binds to GM1-gangliosides in the lipid rafts. FITC (green) has an excitation wavelength peak at 490 nm and emission wavelength peak at 520 nm. Seeded cells were fixed overnight in 1% paraformaldehyde at 4°C, treated with 0.05% Triton X-100 for 10 minutes, then stained with rabbit anti-V5 conjugated to Dylight650 (Abcam) (red) to stain V5 tagged viperin with excitation wavelength peak at 654 nm and emission wavelength peak at 673 nm, and then stained with Hoechst 33342 (Molecular Probes$^{TM}$) nucleic acid stain which is a cell-permeant nuclear counterstain that emits blue fluorescence when bound to dsDNA, its excitation wavelength peak at 35 nm and emission wavelength peak at 461. The cells were imaged using the Leica TCS SP5 confocal microscope. Image analysis was done using ImageJ program (Schindelin et al., 2012).

DF-1 cells were seeded ($8\times10^5$ cells/well) onto sterile glass coverslips in 6 well plates. Twenty-four hours later, cells were transiently transfected with 1.5 µg of V5-tagged viperin or pcDNA3.1/Hygro+ per well using 3.75µL of Lipofectamine 2000

(Invitrogen). Twenty- four hours after transfection, cells were stained with CTB-FITC (Sigma-Aldrich, Canada), cells were then fixed overnight in 1% paraformaldehyde at 4°C, treated with 0.1% Triton X-100 for 10 minutes, then stained with a primary mouse anti-V5 primary monoclonal antibody (Invitrogen), followed by secondary detection with a goat anti-mouse antibody conjugated to Alexa Fluor® 594 (Novex™). The nuclei of cells were stained with Hoechst 33342 (Molecular Probes™). Cells were imaged using The Zeiss AxioObserver confocal microscope (Zeiss LSM 710). Image analysis was done using the program Zen 2011 lite edition (Carl Zeiss Microscopy GmbH, Germany).

**2.9 Addition of farnesol**

DF-1 cells expressing either viperin or an empty vector were grown in media supplemented with farnesol (Sigma-Aldrich, Canada) at concentrations of 10 μM for 10 days before infection. Liquid farnesol from stock was diluted in dimethyl sulfoxide (DMSO) to get 500 mM then diluted 1:1000 in DMEM (Life Technologies) supplemented with 10% FBS to get 500 μM. Then finally diluted in cell media to reach the final concentration of 10 μM.

**2.10 Flow cytometric analysis of the lipid rafts**

DF-1 cells expressing viperin or an empty vector were seeded ($8\times10^5$ cells/well) for 24 hours in a 6 well plate, then they were treated and stained by 3 different ways in order to detect the lipid rafts. Cells were trypsinized then stained with CTB-FITC (Sigma-Aldrich, Canada) for 1 hour. Cells were stained with CTB-FITC and then collected using cell scraper. Or cells were collected with cell scraper then they were

stained with CTB-FITC. All the collected cells were then fixed overnight at 4°C with 1% paraformaldehyde. The percentages of fluorescent cells were detected and processed using flowJ software.

### 2.11 Central domain targeted mutagenesis.

Viperin central domain contains four SAM motifs. Motif one contains the CXXXCXXC structure that is important for the formation of the iron-sulfur cluster while the other 3 motifs are important to support that structure. Here, the first radical SAM motif was mutated through mutating the amino acids containing sulfur into amino acids without sulfur to prevent the iron-sulfur cluster formation. In the first motif, the CXXXCXXC was mutated into AXXXAXXA, at positions 85, 89 and 92 using the primers SAM1_mutant_REV and SAM1_mutant_FWD. The last motif was mutated using the primers SAM4_ REV and SAM4_FWD (Table 5). All mutants were V5 tagged at the end of the C-terminal domain and all primers were ordered from Integrated DNA Technologies, Inc. (Coralville, Iowa). PCR amplifications were carried out using Phusion High-Fidelity DNA Polymerases (Thermo-Fisher, Burlington, ON). Following the reaction, the products were purified using QIAquick PCR Purification Kit (Qiagen, Inc.), then digested with DpnI. DpnI only cleaves at methylated sites, so it cuts the template plasmid but not the PCR product (Zheng, 2004). Following digestion, the products were successfully transferred to One Shot® TOP10 Chemically Competent *E. coli. E.coli* expressing the required construct grew on selective agar containing carbenicillin. The plasmid was then extracted using a Qiagen Spin Miniprep Kit (Qiagen Inc., Oakville, ON) and sequenced using BigDye Terminator v3.1 (Applied Biosystems) using specific primers (Table 2). DF-1 cells were transiently transfected with either the generated

mutants or an empty vector (control) and relative fold changes in infectivity were determined using plaque assay.

**2.13 N-terminal domain and C-terminal domain truncations mutants**

Proteins truncated at the N-terminal or C-terminal domain of duck viperin were generated by overlap extension PCR. All truncated mutants were V5 tagged at the end of the C-terminal domain. Briefly, the N-terminal region of duck viperin was amplified using specific primers (Table 5) to delete the 33 amino acids following the starting codon methionine. For the C-terminal truncation mutants there was a short truncation and a long truncation; the short truncation where 33 amino acids from the end of the C-terminal domain was deleted, and a long truncation where 100 amino acids from the end of the C-terminal domain were deleted using specific primers (Table 5). All primers were ordered from Integrated DNA Technologies, Inc. (Coralville, Iowa). PCR amplifications were carried out using Phusion High-Fidelity DNA Polymerases (Thermo-Fisher, Burlington, ON). Following the reaction, the products were purified using QIAquick PCR Purification Kit (Qiagen, Inc.), then digested with DpnI. Following digestion, the products were treated with T4 Polynucleotide Kinase (T4Pnk) to circularize. T4Pnk is an enzyme that catalyzes the transfer of a phosphate from ATP to the 5' end of the DNA. Then the products were treated with T4 ligase. T4 ligase facilitated the joining of DNA strands together by catalyzing the formation of a phosphodiester bond using the 5' phosphate that is added in the previous step. The products were then successfully transferred to One Shot® TOP10 Chemically Competent *E. coli*. *E. coli* expressing the required construct grew on selective agar containing carbenicillin. Then the plasmid was

extracted using a Qiagen Spin Miniprep Kit (Qiagen Inc., Oakville, ON) and sequenced using BigDye Terminator v3.1 (Applied Biosystems) using specific primers (Table 2). DF-1 cells were transiently transfected with the generated mutants or an empty vector as a control and relative fold changes in infectivity were determined using plaque assay.

**2.14 Statistical analysis**

Statistical analysis was done using Statplus software.

**Table 2. Primer sequences for sequencing the duck viperin construct and duck viperin mutants.**

| Primer | Primer Sequence (5′➔3′) |
|---|---|
| T7-pgem | TAATACGACTCACTATAGGG |
| BGHR | TAGAAGGCACAGTCGAGG |
| RSAD2 F1 | GGCCAGCTGGTCCAGTTCTGCA |
| RSAD2 R1 | CCAGCGCACAGGGTTGAGAGCT |

**Table 3. Accession numbers for viperin and FPPS from vertebrates**

| Animal | Accession number |
|---|---|
| Duck viperin | NP_001297730.1 |
| Chicken viperin | EU427332.1 |
| Human viperin | NP_542388.2 |
| Duck FPPS (predicted) | XP_005022878 |
| Chicken FPPS | P08836 |
| Human FPPS | AIC48744.1 |
| Mouse FPPS | CAJ18572 |

**Table 4: Primer sequences for generation of duck mutant viperin proteins**

| Position | Primer | Primer Sequence (5′➔3′) |
|---|---|---|
| SAM1 motif | SAM1-FWD | GGCGGTGTGGAAGGCGAAGCCGGCCTTATAGTTGGCCTGCCGGGTGAAG |
| | SAM1-REV | CTTCACCCGGCAGGCCAACTATAAGGCCGGCTTCGCCTTCCACACCGCC |
| SAM4 Motif | SAM4-FWD | GCCCGATTAAAACATTGACCTCCTCATTAAAAGCTAGACATGCAATTGCCAGAATGTCCAAATATTTACCGTACT |
| | SAM4-REV | AGTACGGTAAATATTTGGACATTCTGGCAATTGCATGTCTAGCTTTTAATGAGGAGGTCAATGTTTTAATCGGGC |
| N-terminal domain | Δ17 NTD-F | GCTGGCTAGCATGGCGGCGCTCCGAG |
| | Δ17 NTD-R | CATGCTAGCCAGCTTGGGTC |
| C-terminal domain Short Truncation (Δ33 amino acids) | Δ33/100 CTD-F | GGCAAGCCCATCCCCAAC |
| | Δ33 CTD-R | ACCAACATCCAGGATAGAGTTGGAA |
| C-terminal domain Long Truncation (Δ100 amino acids) | Δ33/100 CTD-F | GGCAAGCCCATCCCCAAC |
| | Δ100 CTD-R | ATCATCACCACTGTTCTCCCC |

## Chapter 3. Results

### 3.1 Sequencing and alignment of duck viperin.

Previously, using the transcriptome analysis our lab found that viperin was the most up-regulated gene in the lungs after infecting a mallard duck with HPAI (A/duck/Hubei/49/05) H5N1(analysis done by Man Rao), and then these results were confirmed with qPCR using with A/Vietnam/1203/04 (H5N1) (VN1203) (unpublished results). Bianca Mutis constructed viperin with a V5 tag at the C-terminal end in pcDNA3.1/Hygro+ (Figure 4).

To compare duck viperin was to viperin of other species, I generated a sequence alignment of duck viperin with viperin from other vertebrate species. The duck viperin amino acid sequence showed 90.11% identity with chicken viperin, 76.35 % with human viperin and 73.35% with mouse viperin (Figure 5a). However, there was a significant divergence between the duck N-terminal domain and chicken N-terminal domain, they shared 67.65 % identity in the amino acid sequence, while duck viperin shared 27.94 %, and 30.43% with human, and mouse respectively (Figure 5).

### 3.2 Isolation of clones expressing duck viperin.

DF-1 cells were stably transfected with V5 tagged viperin. Cells were kept under the antibiotic selection (hygromycin) until unsorted cells consistently expressed viperin. Isolation of single clones of viperin expressing cells was done by limiting dilutions. The expression levels were determined using Western blot (Figure 6).

### 3.3 Duck viperin inhibits influenza A virus.

To examine the antiviral properties of duck viperin, I generated DF-1 clones stably expressing V5 tagged duck viperin, challenged them with A/chicken/California/431/00 (H6N2) at MOI 0.05, and determined the percentage of infected cells by high content fluorescence microscopy. Using the Operetta microscope, cells infected with IAV appeared in green while uninfected cells appeared in blue (Figure 7a). I observed a seven-fold decrease in the percentage of virus-infected cells expressing viperin (viperin A clone) compared to the cells expressing an empty vector pcDNA3.1/Hygro+3.4 (Figure 7b).

For plaque assays, I used two different clones, clone 1 named viperin A and clone 8 named viperin B expressing V5 tagged and compared them to a DF-1 clone expressing an empty vector. Cells were infected with LPAI H6N2 for 48 hours at MOI 0.05. There was a significant decrease in the virus titres in DF-1 cells expressing viperin compared to the one expressing an empty vector, the mean virus titre for vector only was $5.9 \times 10^4$ pfu/ml, viperin A was $1.2 \times 10^4$ pfu/ml and viperin B was $2.6 \times 10^4$ pfu/ml (Figure 8).

To observe the effect of viperin on IAV during different time points, DF-1 expressing viperin or empty vector were infected with H6N2 at MOI 0.05 and the supernatant were collected at 24, 48 and 72 hours. This experiment was done twice and in each time there was a significant decrease in the virus titre in the infected cells expressing viperin compared to the infected cells expressing an empty vector (Figure 9).

To determine whether duck viperin is functional in human cells, and can decrease titre of the mouse adapted human IAV strain PR8 (A/Puerto Rico/8/1934 (H1N1)), we expressed viperin in HeLa cells. HeLa cells were transiently transfected with duck V5 tagged viperin and the expression of duck viperin was confirmed by Western blotting (Figure 10a). Transfected HeLa cells either with V5 tagged viperin or with an empty vector were infected with PR8 for 1hour, then washed and left for 47 hours with the addition of infectious media. Compared to HeLa cells transiently transfected with an empty vector and infected with PR8 for 48 hours. Using plaque assay, there was a decrease in the virus titre in HeLa cells transfected with viperin compared to cells with an empty vector (Figure 10b).

**3.4 Duck viperin perturbs the lipid rafts.**

During influenza virus infection, mouse viperin alters the cell plasma membrane through disruption of the lipid rafts (Wang et al. 2007). Lipid rafts are plasma membrane subdomains that contain high concentrations of cholesterol and glycosphingolipids. They are essential for the budding of influenza virus from the cell (Scheiffele et al. 1999; Leser & Lamb 2005). In order to determine how conserved duck FPPS was to other species, I generated a sequence alignment of duck FPPS with FPPS from other vertabrate species. Amino acid sequence alignment between duck and chicken FPPS revealed 89.1% identity in the amino acids sequence while mouse and human was 84.42% (Figure 11). To investigate the effect of duck viperin on the lipid rafts, DF-1 cells stably expressing viperin and DF-1 cells expressing an empty vector were stained with CTB-FITC (green) to stain the lipid rafts, anti-V5 Dylight650 (red) to stain V5 tagged viperin and Hoechst

33324 (blue) for the nucleus. Cells were imaged using the Leica TCS SP5 confocal microscope (Figure 12). Images showed a decrease in the lipid raft abundance when cells were expressing viperin, while, cells transfected with an empty vector do not show any decrease in the lipid rafts.

To confirm the effect of viperin expression on the lipid rafts, DF-1 cells were transiently transfected with a V5-tagged viperin. Cells were then stained with CTB-FITC to stain the lipid rafts (green), and cells were then fixed and stained for V5 tagged viperin with mouse anti-V5, then goat anti-mouse Alexa 594 (red) and the nucleus stained with Hoechst 33324 (blue). Cells were imaged using the Zeiss AxioObserver confocal microscope (Zeiss LSM 710) (Figure 13). Two different types of cells were shown; one expressing duck viperin and one not expressing duck viperin. As a general trend the lipid rafts decreased within the cells expressing duck viperin compared to the ones that were not expressing viperin.

To quantify the decrease in the lipid raft abundance during viperin expression, DF-1 cells stably expressing viperin and DF-1 cells stably expressing an empty vector were stained with CTB-FITC that stains the lipid rafts, and then using flow cytometry, the percentage of cells with lipid rafts was determined (Figure 14). The experiment was repeated and there was a consistent decrease in percentage of cells with lipid rafts in the cells expressing viperin compared to the cells expressing an empty vector.

Previously, Cresswell and his coworkers showed that viperin interacted with FPPS and decreased its activity (Wang et al., 2007). To confirm whether duck viperin interacts with FPPS and decreases its activity, I used a final concentration 10 μM of farnesol to DF-1 cells stably expressing viperin for 10 days. These cells were infected with H6N2 for 48 hours at MOI 0.05 (Figure 15). The addition of exogenous farnesol to DF-1 cells stably expressing viperin reversed their antiviral activity against IAV.

**3.5 The N-terminal domain mutation of duck viperin.**

The viperin N-terminal domain is important for the attachment of viperin to the cytoplasmic side of the endoplasmic reticulum (Hinson & Cresswell, 2009). To examine whether antiviral activity required the N-terminal end, I used overlapping PCR to generate the N-terminal truncation mutant (33 amino acids) and confirmed its sequence (Figure 16a). The viperin N-terminal domain truncation mutant was V5 tagged and the transfection efficiency was confirmed by the Western blot (Figure 16b). We then tested the viperin mutants in comparison to wild types for antiviral activity measured by plaque assay. There was a significant difference between viperin and the N-terminal truncated viperin mutant. The N-terminal truncated mutant lost its antiviral effect compared to the wild type (Figure 17).

**3.6 The central domain of duck viperin is essential for its antiviral activity.**

Viperin central the central domain consists of four radical SAM (S-Adenosyl methionine) motifs including the canonical CxxxCxxC in motif 1, this motif forms an iron-sulfur cluster with the help of the other SAM motifs in the central domain, they form

the [4Fe-4S] cluster and slowly cleave the radical SAM to the Ado• (Duschene & Broderick 2010). I mutated the first and last SAM motifs (Figure 16a). The mutants blocked the SAM motifs ability to form the [4Fe-4S] cluster. To determine whether the SAM mutants would have antiviral functions, DF-1 cells were transiently transfected with SAM1 mutant, SAM4 mutant, V5 tagged viperin and an empty vector, then infected with H6N2 at MOI 0.05, supernatant was collected after 48 hours and the virus titres were determined using plaque assay. The experiment was repeated and the results show two times increase in the relative fold change of the virus titres in both of SAM1 and SAM4 mutants compared to the DF-1 cells transfected with viperin (Figure 17). Both SAM1 and SAM4 mutants were V5 tagged and their expression was confirmed by Western blot (Figure 16.b).

**3.7 The C-terminal domain of duck viperin is not essential for its antiviral activity.**

Viperin C-terminal domain is important for the viperin dimerization and is responsible for crystalloid ER formation (Hinson & Cresswell, 2009). It has an important antiviral activity against dengue virus and HCV (Helbig et al. 2013; Helbig et al. 2005; reviewed in Helbig & Beard 2014), however, it is not important for the antiviral activity against HIV (Nasr et al., 2012). In order to investigate the effect of the C-terminal domain, DF-1 cells transiently expressing short and long C-terminal truncation mutants were infected with H6N2 at MOI 0.05, supernatant was collected after 48 hours, and compared to DF-1 cells transiently transfected with V5 tagged viperin or an empty vector (Figure 17). Viperin C-terminal short truncation lost its antiviral effect, however, viperin C-terminal long truncation did not lose its antiviral activity (Figure 17). Both viperin C-

terminal short and long truncation mutants were V5 tagged and their expression levels were confirmed using Western blot (figure 16.b).

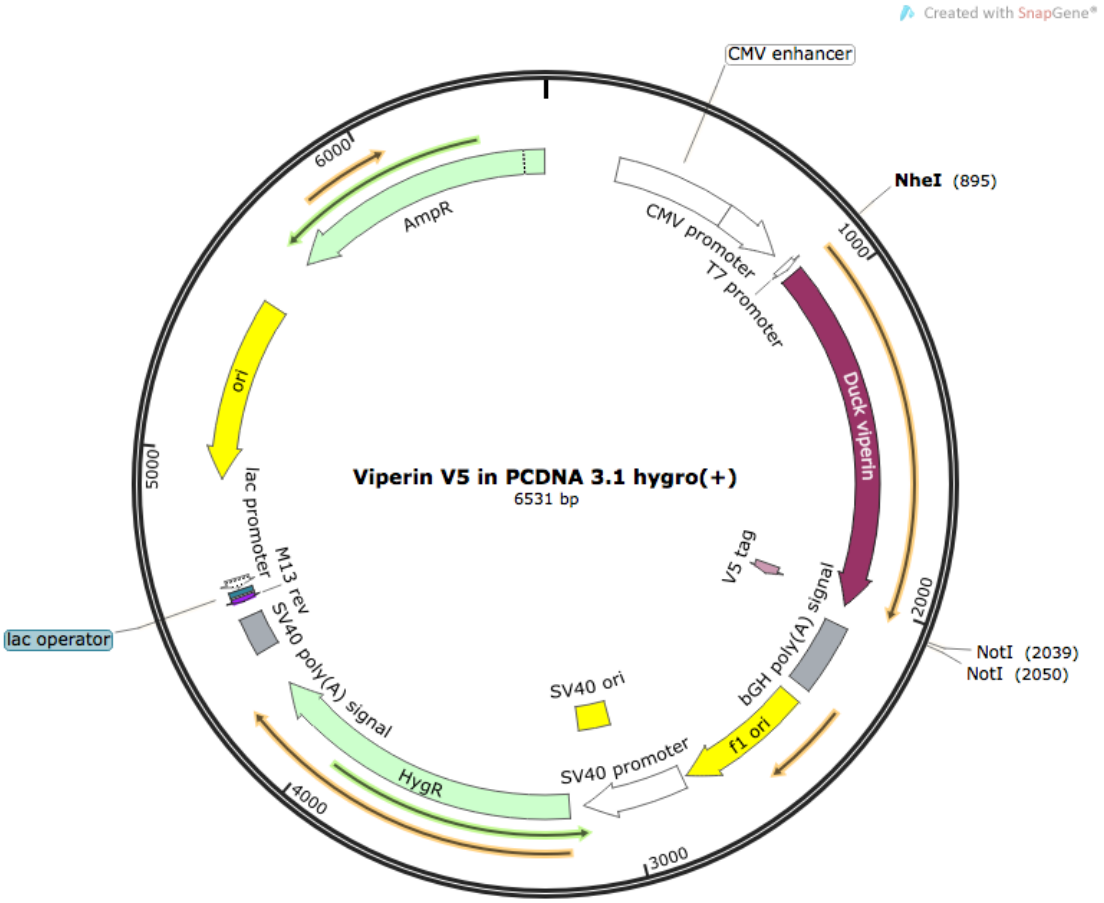



**Figure 4: Vector map of pcDNA3.1/Hygro+ with C-terminal V5 epitope tagged duck viperin insert.**

A


**Figure 5: Amino acid sequence alignment of duck, chicken, human, and mouse viperin.** The N-terminal domain of viperin is highlighted in blue, the C-terminal domain is highlighted in yellow while the central domain is not highlighted but the SAM motifs are in red (a).

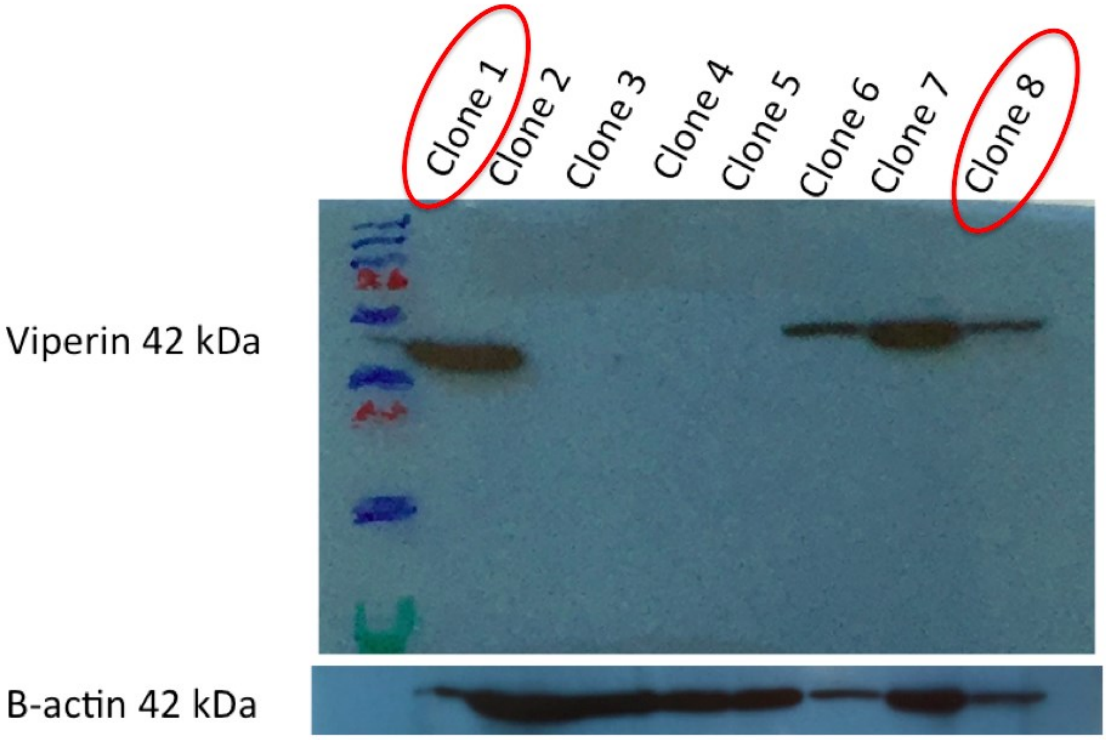
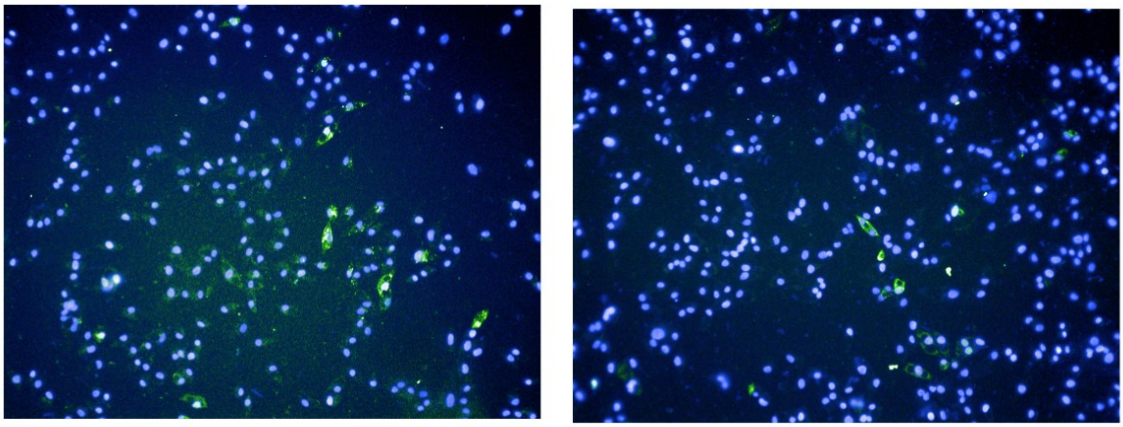



**Figure 6: The level of viperin expression determined by Western blots.** The level of viperin protein expression of each stably expressing DF-1 cell line was determined by Western blot.

A




B

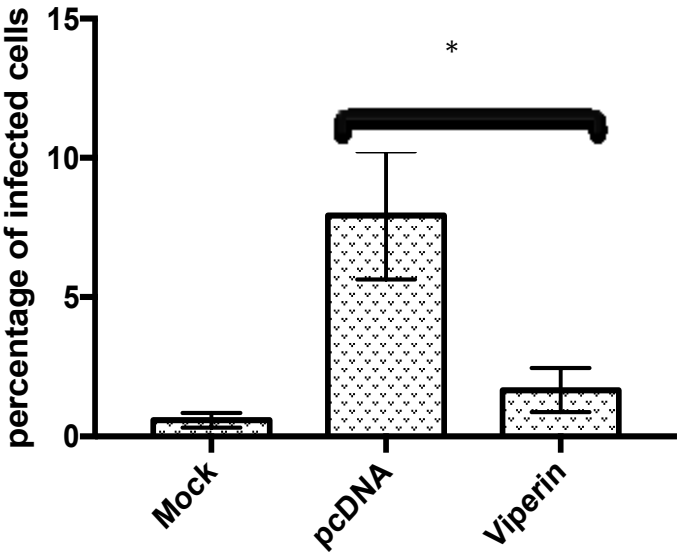



**Figure 7: DF-1 cells stably expressing duck viperin show decreased IAV infection.** DF-1 cells expressing viperin or an empty vector were infected with H6N2 at an MOI=0.05. Forty-eight hours post infection cells were fixed and stained with anti-nucleoprotein-FITC antibody (green) and Hoechst 33342 (blue), representative figures of the image taken are shown (A). DF-1 cells were infected with H6N2 at MOI=0.05, and percentage of infected cells determined by counting the percentage of nucleoprotein positive cells using operetta microscopy (B). (*, P= 0.04776;).

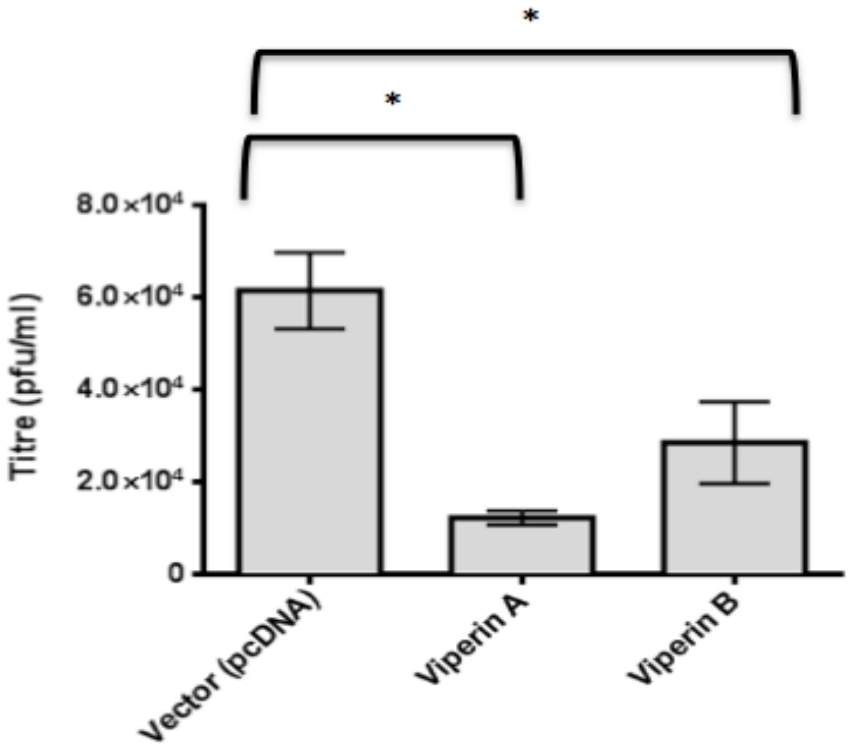
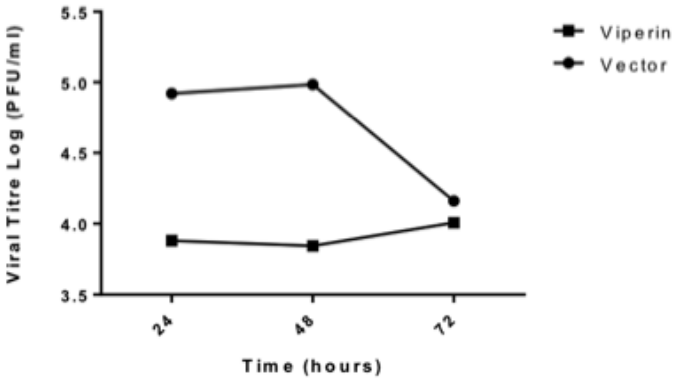



**Figure 8: The antiviral effect of the duck viperin against IAV.** Two different clones of DF-1 cells stably expressing viperin were infected with H6N2 at MOI=0.05, supernatant was collected after 48 hours, then the viral titres were determined using plaque assay. A decrease in the virus titres in DF-1 cells expressing viperin compared to DF-1 cells expressing an empty vector is observed. (*, $P \leq 0.05$)

A




B

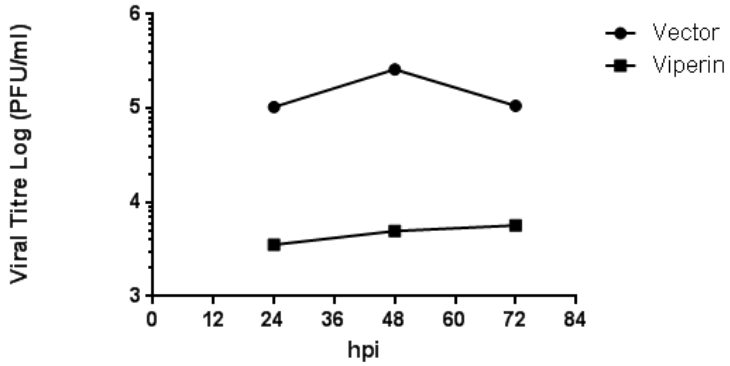
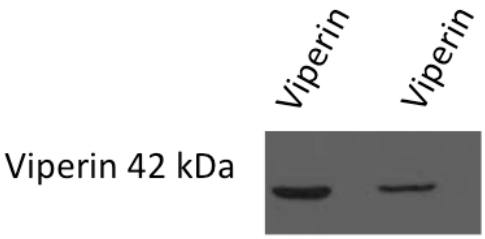



**Figure 9: The antiviral effect of the duck viperin against IAV at different time points.** Virus titres were determined for DF-1 expressing duck viperin and DF-1 expressing an empty vector using plaque assay after being infected with H6N2 at different time points 24 hours, 48 hours and 72 hours. The experiment was repeated three times and it showed a consistent decrease in the antiviral titre during the expression of viperin compared to empty vector in the DF-1 cells. Plaque assays were repeated in duplicates and the results of each experiment are shown. (*=$P \leq 0.05$;). Experiment 1 (A), Experiment 2 (B)

A




B

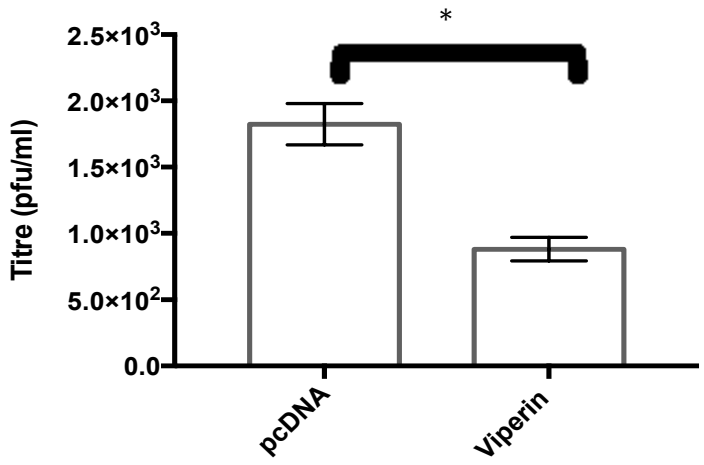



**Figure 10: The antiviral effect of the duck viperin against IAV expressed in HeLa cells.** Viperin protein expression of two different plasmid preps transiently transfected HeLa cell line determined by Western blot (A). HeLa cells were transiently transfected with an empty vector or duck viperin and infected with PR8 for 48 hours, virus titres were determined using plaque assay (B). ($n \geq 2$, *, $P \leq 0.05$;)


**Figure 11: Amino acid alignments of duck, chicken, human, and mouse FPPS**. FPPS amino acids sequence alignments show that FPPS is conserved among species.

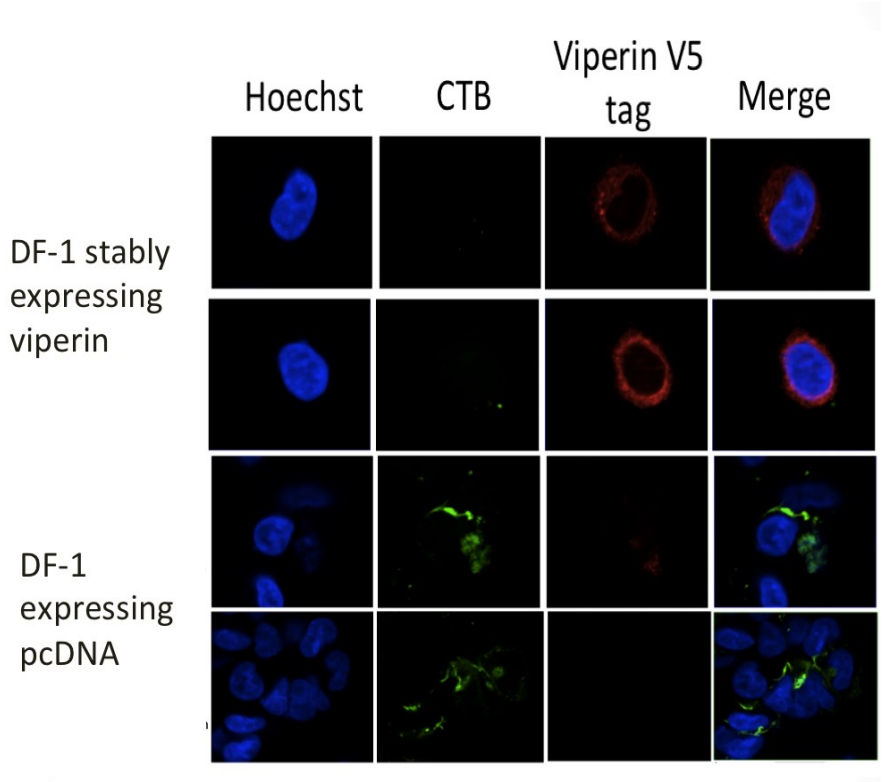
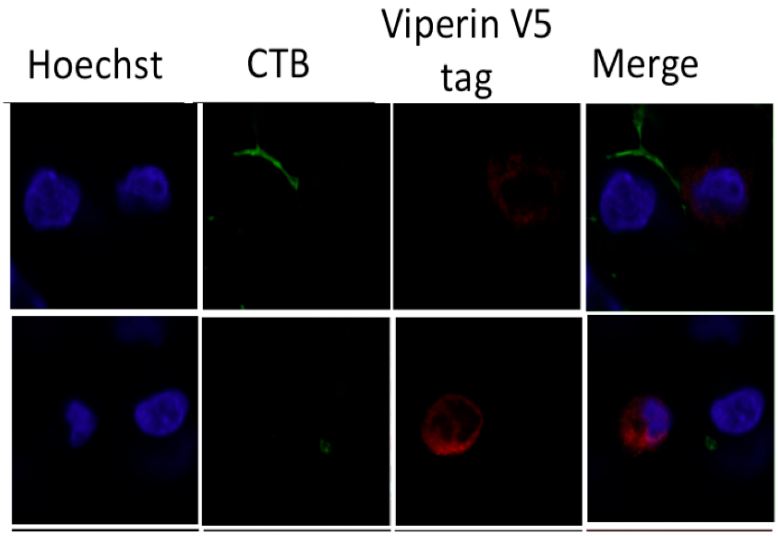



**Figure 12: Inhibition of lipid rafts in DF-1 cells stably expressing viperin.** DF-1 cells stably expressing duck viperin, and DF-1 cells transfected with an empty vector were fixed, stained, and imaged using confocal microscopy Leica TCS SP5. Panels show staining for the nuclei using Hoechst 33324 (blue), for the lipid rafts using cholera toxin B- FITC (green), V5-epitope tagged Viperin (red) stained with anti-V5 dylight650, and a merged image

A




B

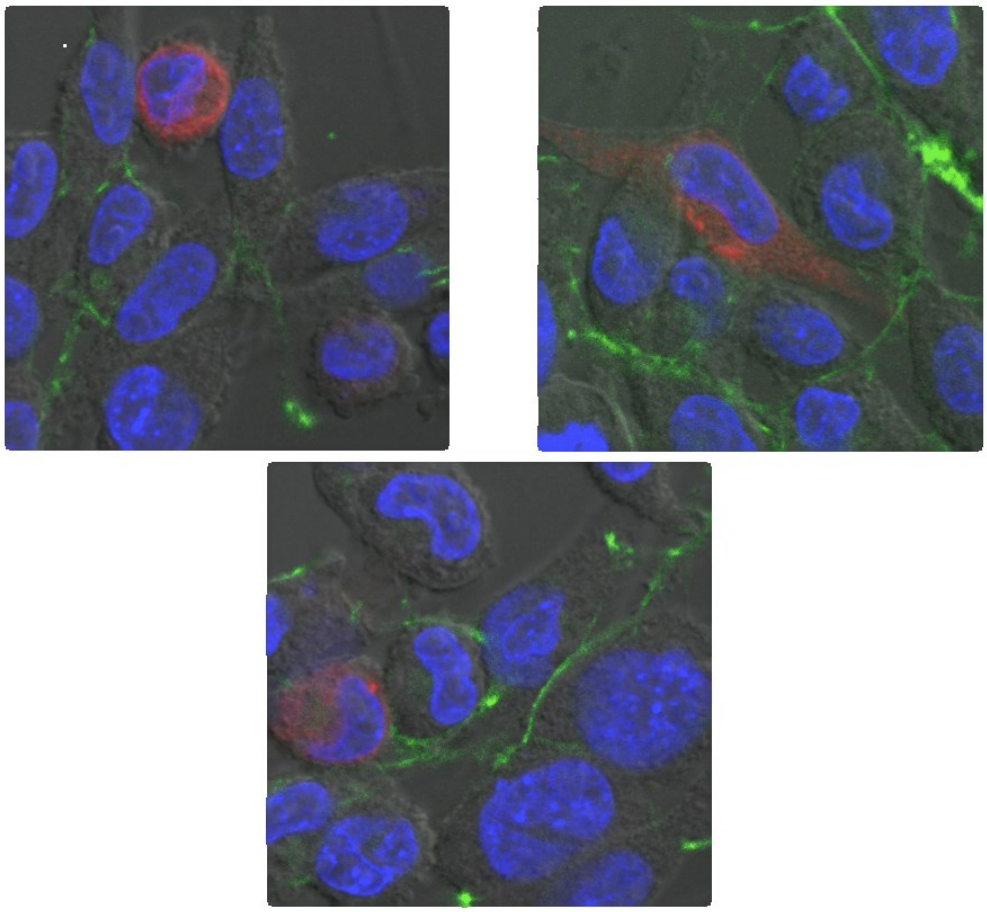
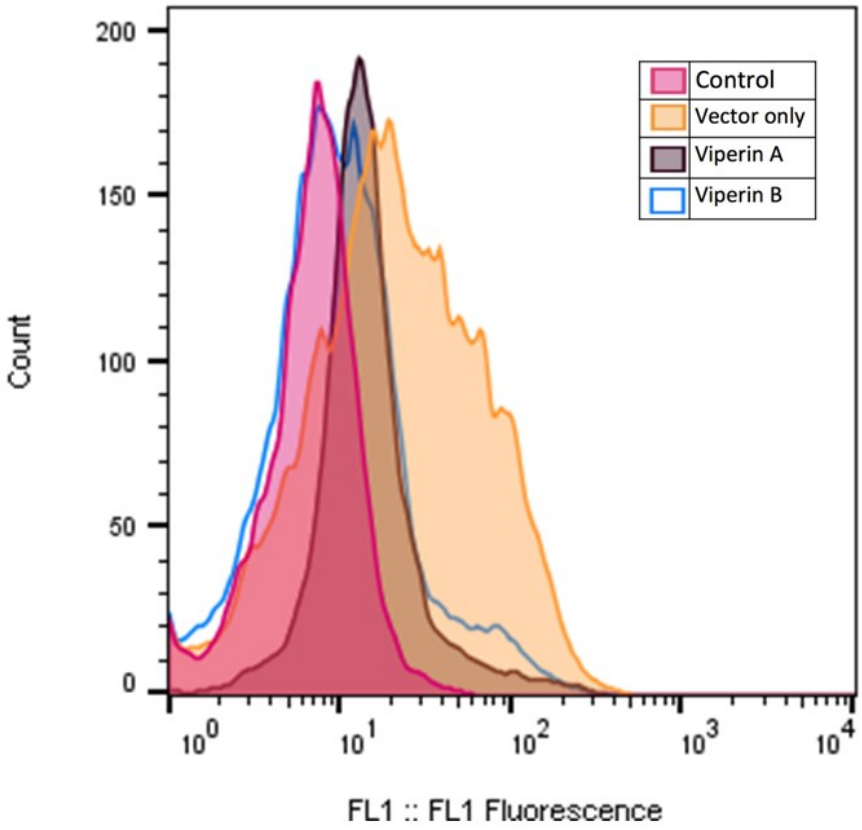
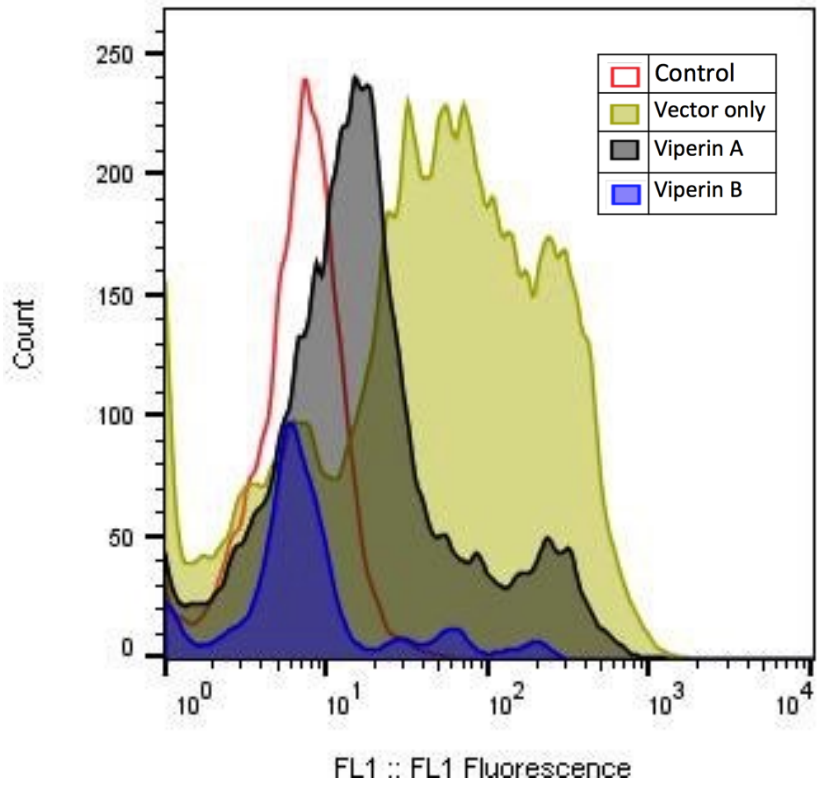


**Figure 13: Duck viperin perturbs the lipid rafts in DF-1 cells transiently expressing viperin.** DF-1 cells transiently transfected with duck viperin were fixed, stained, and imaged. Images show staining for nuclei using Hoechst 33324 (blue), for the lipid rafts using cholera toxin B- FITC (green), V5-epitope tagged Viperin (red) stained with Alexa Fluor® 594. Images taken using the confocal microscopy Leica TCS SP5 (A).Images taken using the confocal microscopy Zeiss LSM 710 (B).

A

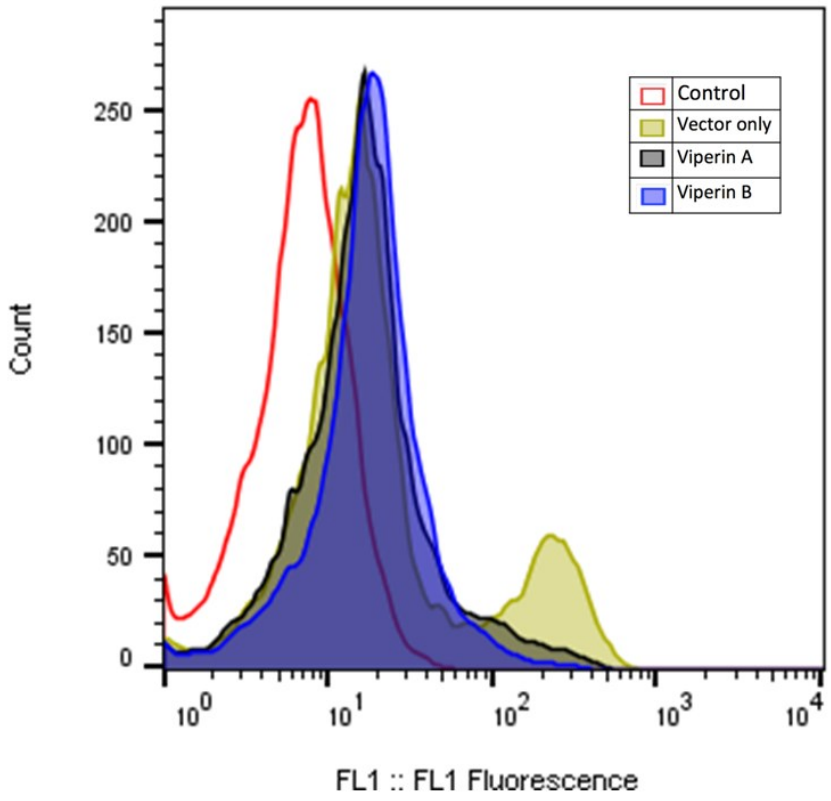

Control
Vector only
Viperin A
Viperin B
Count
200
150
100
50
0
$10^0$
$10^1$
$10^2$
$10^3$
$10^4$
FL1 :: FL1 Fluorescence


Control
Vector only
Viperin A
Viperin B
Count
250
200
150
100
50
0
$10^0$
$10^1$
$10^2$
$10^3$
$10^4$
FL1 :: FL1 Fluorescence

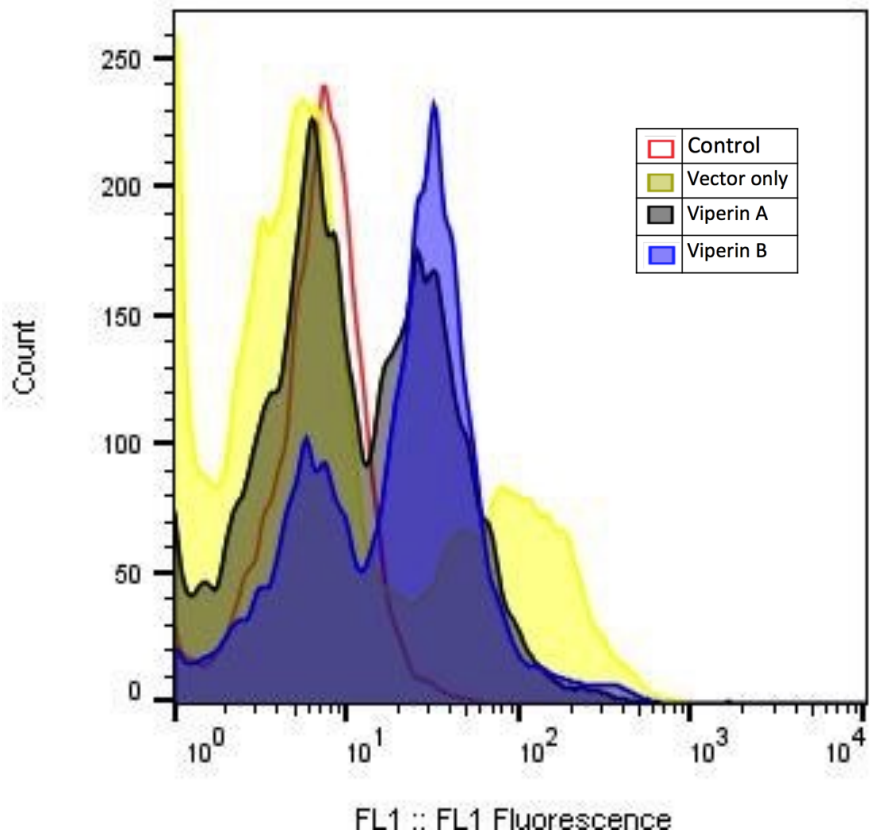


B

C


Control
Vector only
Viperin A
Viperin B
Count
250
200
150
100
50
0
$10^0$
$10^1$
$10^2$
$10^3$
$10^4$
FL1 :: FL1 Fluorescence

D


Control
Vector only
Viperin A
Viperin B
Count
250
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$10^1$
$10^2$
$10^3$
$10^4$
FL1 :: FL1 Fluorescence

**Figure 14: Flow cytometric analysis of the lipid rafts stained with CTB-FITC during viperin expression.** Cells were stained with CTB-FITC then trypsinized then fixed with 1%PFA (A). Cells were stained with CTB-FITC then scrapped from the plate using cell scraper and fixed with 1% PFA (B). Cells were scraped from the plate using cell scraper then stained with CTB-FITC and fixed with 1%FPA (C). This experiment was repeated where cells were stained with CTB-FITC then scraped from the plate using cell scraper and fixed with 1%PFA (D).

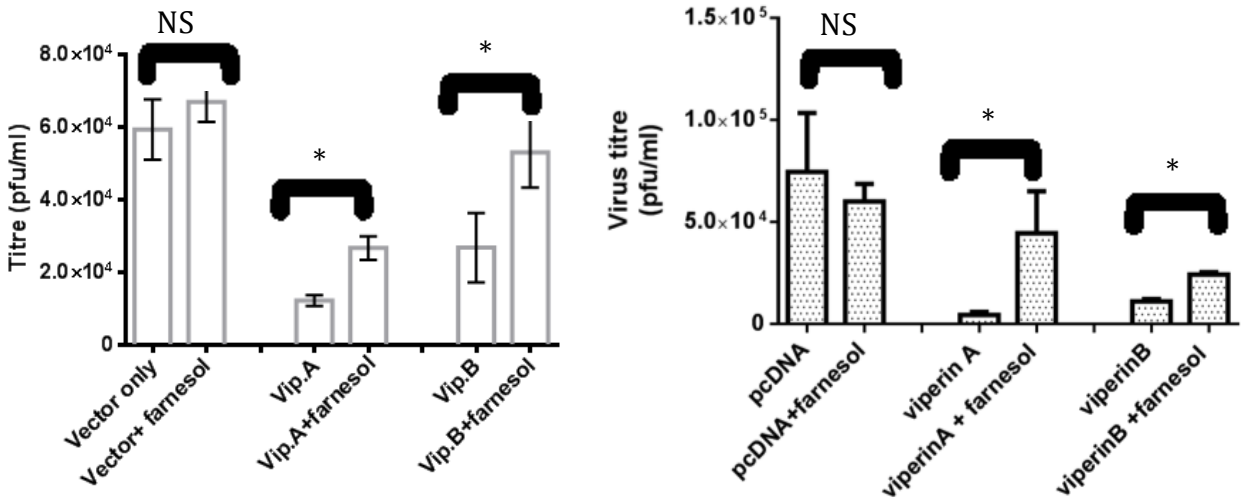
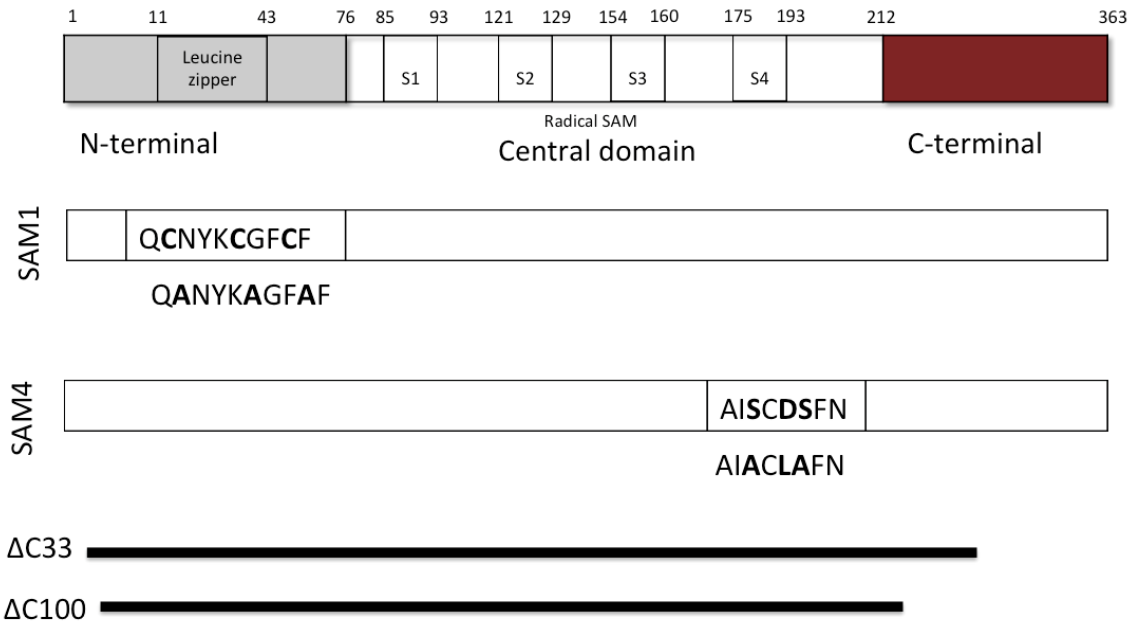



**Figure 15: Exogenous farnesol reversed viperin-mediated inhibition of IAV.** Two different DF-1 clones expressing viperin and DF-1 expressing an empty vector, were infected with H6N2 for 48 hours, the virus titres were determined using plaque assay. The experiment was repeated twice in duplicates. Statistical significance in comparison to vector control cells was analyzed using an unpaired two-tailed Student's t-test ($n \geq 2$; **, $P \leq 0.01$; ***, $P \leq 0.001$) (NS, not significant).

A




B

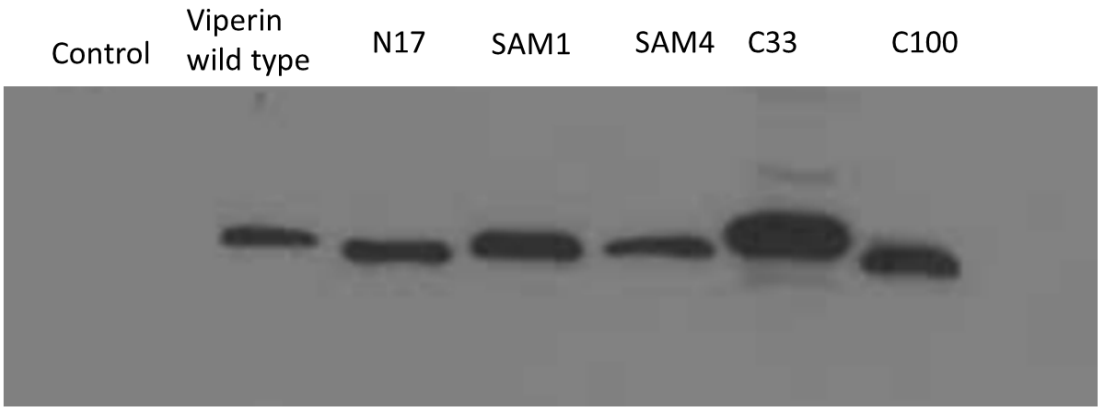



**Figure 16: Viperin mutant design.** Schematic representation of duck viperin showing the truncation mutants and SAM domain mutants (A). Western blot showing viperin protein mutant expression (B)

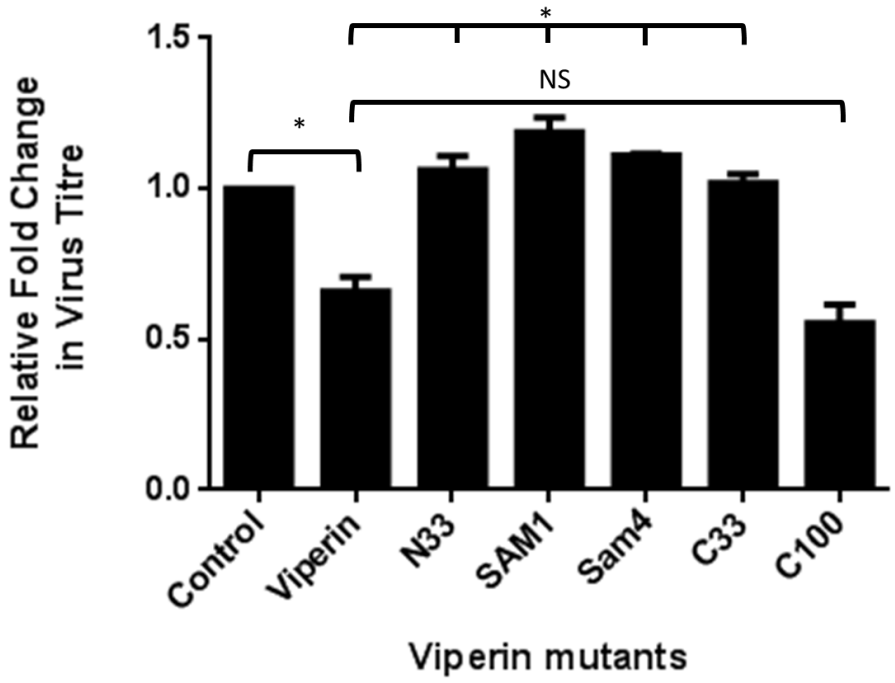



**Figure 17: Relative fold change in the virus titre of expressing viperin or viperin mutants.** DF-1 cells were transfected with empty vector as a control, viperin and viperin mutants. Cells were infected with H6N2 at MOI=0.05, and supernatant was collected after 48 hours. The viral titres were determined using plaque assay. (*, $P \leq 0.05$;), (NS, not significant).

## Chapter 4. Discussion

Ducks are the natural host of IAV and they show little to no signs of infection. During IAV infection in ducks, *viperin* was found to be the most up-regulated gene in their lungs. As the most upregulated gene during IAV infection, in the virus' natural host made it a good candidate for further investigation. Here, I have generated stable DF-1 chicken fibroblast cell clones expressing duck viperin. I showed that viperin has an antiviral effect against IAV. I demonstrated the ability of duck viperin to perturb the lipid rafts and that the addition of exogenous farnesol reversed viperin anti-influenza activity. Finally, I have shown that the SAM motifs, at the central domain, and N-terminal domain are important for viperin antiviral activity.

### 4.1 Duck viperin has anti-influenza activity

When DF-1 cells stably expressing duck viperin were exposed to IAV H6N2, a decrease in the IAV titres was observed 24, 48 and 72 hours after infection compared to DF-1 cells expressing an empty vector. This experiment was repeated three times and the antiviral activity of duck viperin was observed in each time. These results are consistent with Wang et al., where they found that mouse viperin can inhibit the influenza A/WSN virus at an MOI of 0.02. They determined the viral titers at 24, 48, and 72 hours post-infection by plaque assay (Wang et al., 2007). In order to confirm our results we used the Operetta microscope to count the number of infected cells after 48 hours of infection with H6N2 and found a decrease in the infected DF-1 cells expressing viperin compared to the ones expressing an empty vector. We have also attempted to see if duck viperin had an anti-influenza effect in HeLa cells using a mouse-adapted strain PR8. Interestingly, with the help of the plaque assay technique, HeLa cells transiently expressing duck viperin had

less viral titres compared to cells transfected with an empty vector. These results confirmed the antiviral activity of the duck viperin against IAV. During expression of mouse viperin in HeLa, viperin was found to interact with FPPS and decreased the release of the virus from the cells (Wang et al., 2007).

### 4.2 Duck viperin perturbs the lipid rafts

Influenza A virus begins its infection process through binding to a sialic acid receptor on the cell surface. Once binding starts, endocytosis of the bound virus takes place. The acidic endosomal environment triggers membrane fusion releasing the viral genome into the cytosol to make its way into the nucleus. In the nucleus, the viral genome is replicated and mRNA is synthesized. Viral mRNA enters the cytosol and viral protein synthesis starts. These proteins are then transferred to the lipid rafts to egress the cell. Here, I have shown that during duck viperin expression, the lipid rafts are perturbed. When lipid rafts were stained with CTB-FITC in DF-1 cells expressing duck viperin, I noticed a decrease in the lipid rafts abundance compared to the non-viperin expressing cells. These results confirmed the previous findings by Wang et al., where they have used fluorescence recovery after photobleaching (FRAP) studies to demonstrate that viperin expression results in an elevated lateral mobility of HA as a consequence of increased membrane fluidity, HA is normally associated with the lipids rafts and the increase in the fluidity indicated that HA was not associated with the lipid rafts due to a decrease in the lipid rafts abundance (Wang et al., 2007). These results are consistent with the model that viperin disturbs lipid rafts, which impairs the viruses ability to bud from the plasma membrane.

To further confirm the duck viperin effect on the lipid rafts, external farnesol was added to the media. Farnesol is produced through FPPS activity to produce FPP in the cell. FPP is a precursor of cholesterol, farnesylated and geranylated proteins, which are the main component of the lipid rafts (Szkopińska & Płochocka 2005). Our addition of farnesol to the media for ten days reversed the viperin inhibition effect on IAV. These results are consistent with the findings of Wang and coworkers, where they identified FPPS to be a potential target for mouse viperin using a yeast two-hybrid screen (Wang et al, 2007). They confirmed through a pull down assay that mouse viperin was attached to FPPS. When FPPS activity was measured during mouse viperin expression using FPPS activity assay, they found a decrease in its activity ( Wang et al., 2007). The FPPS in chickens is highly conserved compared to the that of ducks. When exogenous farnesol was added to monocyte-derived macrophages cells expressing viperin, its anti-HIV activity was reversed (Nasr et al., 2011)`. The lipid rafts are important for the entry and budding of HIV (Carter et al. 2009; Campbell et al. 2001). These experiments suggested the importance of the lipid raft for viperin antiviral activity. When viperin was expressed, there was a decrease in the lipid rafts abundance, which decreased the IAV titres. When farnesol was added, it acted downstream of FPPS which restored the activity of the lipid rafts, reversed viperin inhibition effect and increased the IAV titres. Theses findings prove that FPPS activity is important to viperin function. FPPS might be affecting an important farnesylated or geranylated protein, which is important for IAV egress from the cells.

### 4.3 Duck viperin N-terminal domain and SAM motifs are important for its anti-influenza effect

Viperin consists of three regions; the N-terminal domain, the C-terminal domain and the central domain. Each of them is necessary for the anti-viral activity of viperin in different species against different viruses. The N-terminal domain is not conserved among species. When the first 17 amino acids of the duck viperin N-terminal domain is deleted, viperin loses it antiviral activity. This deleted part consists of an α helix and first part of the leucine zipper, which are important for viperin attachment to the ER. When attached to the ER membrane, viperin aggregates to the extent of inducing a lattice like dramatic change in the shape of ER (crystalloid ER) that may inhibit the trafficking of proteins essential for viral replication of some viruses. Hinson and Creswell demonstrated the N-terminal amphipathic α-helix localizes to the ER but is not sufficient to cause crystalloid ER formation, suggesting that the C-terminal is important for the formation of the crystalloid ER (Hinson & Cresswell 2009). This crystalloid ER formation is formed only when viperin is attached to the ER through the N-terminal domain. When the N-terminal domain was deleted viperin was localized in the cytoplasm (Hinson & Cresswell, 2009). N-terminal domain truncation moves viperin to a homogenous cytoplasmic distribution, coinciding with a loss of antiviral effect. This suggested that viperin attachment to the ER is necessary to perturb the lipid rafts and this might explain why N-terminal truncation leads to the loss of viperin anti-influenza activity. Viperin N-terminal domain is important for localizing viperin to the ER and lipid droplets during HCV infection and is important for CHIKV antiviral effects (Helbig et al., 2011; Teng et al., 2012).

Viperin central domain contains 4 radical SAM motifs. The first one contains the canonical CXXXCXXC motif and coordinates a [4Fe–4S]-cluster formation, while the three other motifs support the [4Fe–4S]-cluster formation. When the cysteine amino acids were mutated into alanine, the first SAM motif lost its ability to form the [4Fe–4S]-cluster due to the absence of sulfur. This mutation reversed viperin inhibition effect on IAV and increases infectivity. When last SAM motif in the central domain is mutated, duck viperin loses its inhibition effect on IAV. These results confirm the importance of SAM motif in duck viperin anti-influenza activity. When viperin loses its ability to form the iron-sulfur cluster and formation of the Ado•, it loses its antiviral activity against IAV. It is unclear what is the exact role of SAM domain in viperin. However, SAM domain and the formation of the Ado• are important in many chemical transformation steps. An example of the importance of Ado•, during anaerobic growth in the bacteria, pyruvate is converted with CoA into acetyl-CoA and formate in a reaction catalyzed by pyruvate-formate lyase (PFL). PFL is only activated by Ado• which is also generated through SAM motif forming the iron sulfur cluster (Frey et al., 2008; Wagner, et al., 1999). SAM motifs are also important in the antiviral activity of viperin against HIV. Mutation in the SAM motifs affected viperin inhibitory effect on HIV in MDMs cells (Nasr et al., 2012). These conclude the importance of the radical SAM motifs in the central domain during viperin anti-influenza activity.

Viperin C-terminal domain is highly conserved among species; its exact function is still unknown. It is important for the antiviral activity of some viruses such as HCV.

During IAV infection, when the last 33 amino acids were deleted from the C-terminal domain of viperin, it lost its anti-influenza effect. Surprisingly, when the last 100 amino acids were deleted viperin restored its anti-influenza effect. The same results were also noticed during HIV infection of the monocyte-derived macrophages cell line during viperin C-terminal truncation mutants' expression. Nasr et al found that the C-terminal short truncation of viperin reversed viperin anti-HIV activity, however viperin C-terminal long truncation did not affect viperin anti-HIV activity (Nasr et al., 2012). These findings show that viperin C-terminal domain is not important for its anti-influenza effect. The short truncation might be causing instability to the viperin protein. This instability might be affecting the viperin anti-influenza effect negatively. Previous work done by Hinson and Cresswell showed that C-terminal domain is important for viperin dimerization (Hinson & Cresswell, 2009). Both the long and short C-terminal truncations are important for viperin antiviral activity against HCV (Helbig et al., 2011). This suggests that the mechanism of viperin inhibition of HIV replication in human MDMs is more similar to that of influenza than HCV. The significant inhibition of viperin activity with the short C terminal domain truncations was consistent but was not observed with the longer truncations, perhaps suggesting that short truncations may alter the tertiary structure of viperin and interfere with its antiviral activity.

Viperin inhibits different viruses in different ways, which suggest that the viperin antiviral effect is multifaceted. These interactions involve multiple mechanisms of action including alterations in viperin subcellular localization. For duck viperin N-terminal and SAM motifs in the central domain are important for its antiviral activity.

### 4.4 Future directions

Studies have shown that viperin inhibits several viruses *in vitro* and we have shown that duck viperin inhibits IAV infection done. Our work shows that duck viperin is active against IAV. To uncover the molecular mechanism by which viperin acts, it is important to identify the chemical reactions catalyzed by viperin. These chemical reactions might be catalyzed by the duck viperin through the radical SAM motifs in the central domain, which are responsible for duck viperin anti-influenza activity. The interaction between FPPS and duck viperin needs to be investigated further. Furthermore, the addition of farnesol, which works downstream of FPPS, highlights the importance of FPPS during IAV. Therefore the interaction between viperin and FPPS can help to solve the mystery behind viperin anti-influenza activity. Previous studies have shown that viperin co-precipitates with FPPS and decreases its activity (Xiuyan Wang et al., 2007), however the exact mechanism by which viperin interacts with FPPS is still unknown. This interaction might be the key to understand how duck viperin exerts its antiviral activity against influenza virus.

More broadly, the characterization of the antiviral activity of other duck ISGs against IAV will help our understanding of the innate immune response of the natural host of IAV. It is important to remember that viperin represents only one of the hundreds of ISGs that are upregulated by IFN. Other candidate duck ISGs have been previously identified such as OASL (Vanderven et al., 2012) which needs to be investigated.

## 4.5 Conclusions

Here, I have provided evidence of the duck viperin antiviral effect against IAV. I generated and isolated DF-1 cells stably expressing duck viperin. Significantly, I showed that duck viperin decreased the IAV titres in DF-1 cells stably expressing transiently transfected duck viperin as well as decreasing the virus titres in HeLa cells expressing duck viperin. I showed that the amount of infected DF-1 cells stably expressing viperin decreased using the Operetta microscope. I confirmed the perturbation in the lipid rafts due to viperin during IAV infection and I showed that the addition of exogenous farnesol that work downstream FPPS reversed viperin anti-influenza effect. Figure 18 shows IAV release upon viperin expression (Wang et al. 2007). I constructed domain deletion mutants and have demonstrated that the viperin N-terminal domain and SAM motifs are important for viperin anti- influenza effect.

From these results we can conclude that duck viperin during IAV infection is regulated by RIG-I (Loo et al., 2008), then it binds to FPPS and decreases it function. The decrease in the FPPS function leads to perturbation of the lipid rafts, the site of virus egress. This perturbation in the lipid rafts decreases the virus titres. The activity of viperin during IAV infection is controlled by the N-terminal domain and the SAM domains.

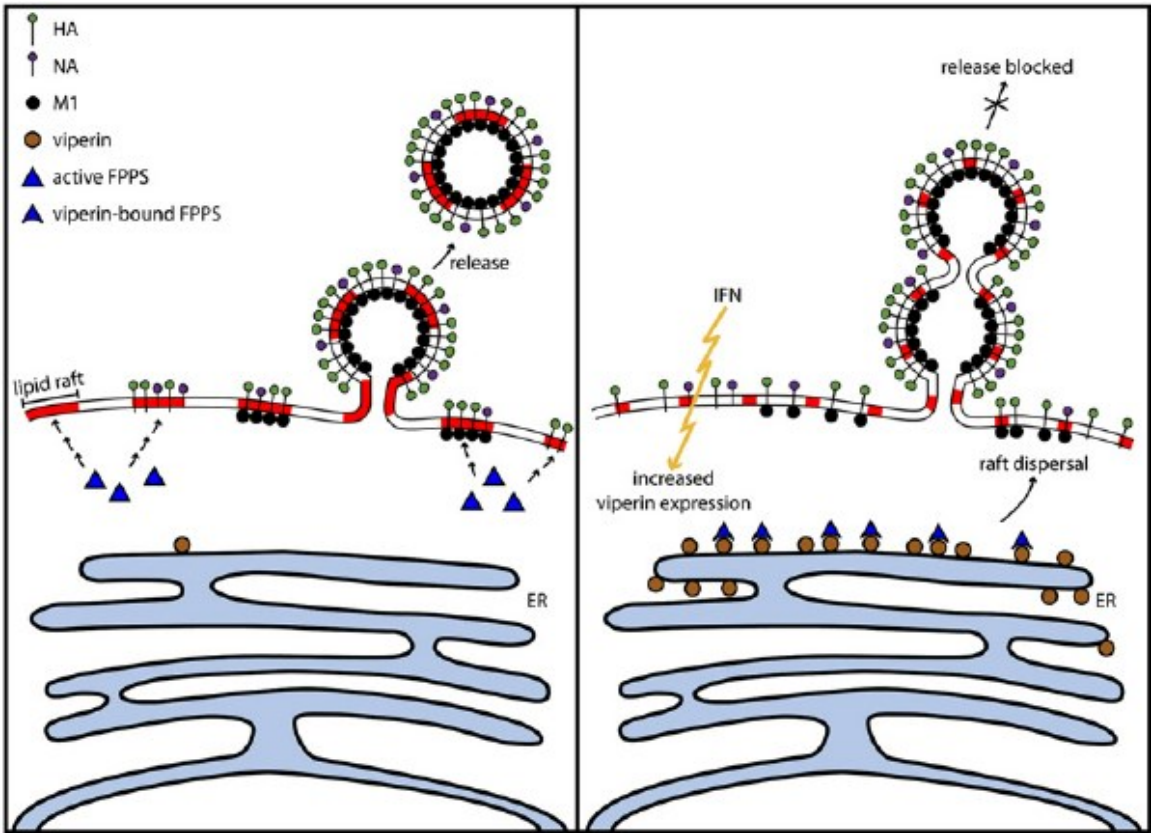



**Figure 18. Model showing influenza A virus release upon viperin expression and interaction with FPPS.** Left panel shows virus budding in the absence of viperin. Right panel shows IFN- induced viperin expression followed by its interaction with FPPS and disruption of lipid rafts and finally blockage of viral budding. (from Wang et.al. 2007)

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