

Identification of mutations in the NS1 gene that control influenza A virus virulence in the mouse model

by:

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Thesis submitted to

The Faculty of Graduate and Postdoctoral Studies
in partial fulfillment of the requirements for the degree of
Doctor of Philosophy

Department of Biochemistry, Microbiology and Immunology
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Abstract

The genetic requirements for Influenza virus to infect and adapt to new species is largely unknown. To understand the evolutionary steps required by a virus to become virulent, a human virus (A/HK/1/68) (HK), avirulent in mice, was subjected to 20 and 21 serial lung-to-lung passages in mouse. Sequence analysis revealed the emergence of eleven mutations within the NS1 gene of the new virulent strains, many of which occurred in binding sites for transcriptional and translational cellular factors. In the present study we have rescued viruses containing each of the NS1 mouse adapted mutations onto A/PR/8/34 (PR8) backbone.

We found 9 of 16 NS1 mutants were adaptive by inducing mortality, body weight loss in BALB/c mice and enhanced virus replication in MDCK cells with properties of host cell interferon transcription inhibition. Sequence comparisons with the highly pathogenic A/Hong Kong/156/1997 (H5N1) and the most severe pandemic A/Brevig Mission/1/1918 (H1N1) NS1 genes showed convergent evolution with some of the mouse adapted viruses for F103L plus M106I and V226I plus R227K mutations respectively. The F103L and M106I mutations in the HK NS1 gene were shown to be adaptive by assessment with respect to replication, early viral protein synthesis, interferon- β antagonism and tropism in the mouse lung. We extended the study and proved increased virulence associated with F103L+M106I mutations in their respective H5N1 NS1 gene on the PR8 and HK backbones, as well as the PR8 NS1 gene and the H9N2 (A/Ck/Bj/1/95) gene in the PR8 and A/WSN/33 backbones

respectively. However the V226I and R227K mutations in their respective HK and 1918 NS1 genes slightly enhanced virulence and viral growth at later stages of infection.

This study demonstrates that NS1 is a virulence factor; involved in multiple viral processes including interferon antagonism and viral protein synthesis. Furthermore, NS1 mutations acquired during mouse adaptation are proven to be adaptive in human, mouse and avian NS1 genes.

Acknowledgments

I would like to take this opportunity to thank my supervisor Dr. Earl Brown not only for giving me the opportunity to work in his laboratory but also for being a great mentor and a very understanding person. Thank you Dr. Brown for your wisdom, your valuable comments and continuous encouragement. I have been very lucky to learn from your immense experience. Your passion for research and love for biking are a legacy I will proudly carry.

I would also like to thank the Faculty of Graduate and postdoctoral Studies at the University of Ottawa for granting me the “Bourse Internationale de la Francophonie” that covered my tuition fees and allowed me to pursue my graduate studies.

I want to thank my thesis advisory committee: Dr. Kathryn Wright, Dr. Ken Dimock and Dr. Alain Stintzi for their valuable evaluations and great feedback.

I have been very lucky to spend my graduate studies in an amazing Lab with wonderful labmates and lab-neighbors. I would like to thank each and every one of you for creating a very friendly environment and making it easy to ask the simplest questions. I will never forget our continuous jokes, coffee breaks on the floor outside the lab, pranks and games that released the stress of research and made my life in Ottawa very enjoyable. Thanks (in no order of preference) Trevor Beaudoin, Jay Majithia, Jihui Ping, Liya Keleta, Samra Uzicanin, Nicole Forbes, Shuai Wang, Will Stecho, Sujeeve Jeganathan and Mohammed Salman.

I also want to thank my special roommate and fellow PhD mate, Abir Yamak. Thank you for being there, for always listening to me and for continuously packing me delicious lunches. I wish you all the luck in your future steps. I will miss you and miss apartment 709.

I can't find the words to thank my sisters Sana, Ghazwa and Hala for their precious support, encouragement and for listening to my continuous whining throughout my PhD studies. I also would like to thank my amazing nieces and nephews for teaching me how to show my love. You are pieces of my heart, I am sorry for being away from you.

I would like to take this opportunity to thank my two special sisters Fida and Maysoun Dankar. Thank you Fida and Wissam El Cheikh ALi for offering me a home away from home. I am delighted I got the chance to spend time next to you and your amazing family. Thank you Ibrahim, Ahmed and Manessa for showing me your love at times of utmost need. Maysoun, thank you for your immense support, your time away from your family I want you to know that without you I wouldn't have been able to complete this journey.

I would like to dedicate my work to the closest people to my heart my parents Kamal Dankar and Najah Ghamrawi for believing in me and for transferring their love of education to me. I am so grateful you didn't stand in my way when you didn't agree with my choices I hope I have made you proud.

And lastly I would like to specially thank my very special someone, Sharif Khaled. I am deeply grateful you are mine.

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List of Abbreviations

DPI	Days post infection
dsRNA	Double stranded RNA
ELISA	Enzyme Linked Immunosorbant Assay
GFP	Green Fluorescent protein
HA	Hemagglutinin
HK	A/Hong Kong/1/68 (H3N2)
HPI	Hours post infection
IAV	Influenza A virus
IFN	Interferon
IL	Interleukin
IRF	Interferon regulatory factor
LD ₅₀	Lethal dose 50
M1	Matrix 1
M2	Matrix 2
MA	Mouse adapted
MA20	Mouse adapted strain-passage 20
MA21	Mouse adapted strain-passage 21
MEM	Minimum essential medium
MOI	Multiplicity of infection
mRNA	Messenger RNA

NA	Neuraminidase
NEP	Nuclear export protein
NP	Nucleoprotein
NS1	Non-structural protein 1
PA	Acidic polymerase
PB1	Basic polymerase 1
PB2	Basic polymerase 2
PFU	plaque forming units
PI	post infection
PR8	A/Puerto Rico/8/34 (H1N1)
RBD	RNA binding domain
RNP	Ribonucleoprotein
SA	Sialic Acid
ssRNA	Single stranded RNA
TLR	Toll like receptor
WSN	A/WSN/1933
WT	Wildtype

1. Introduction

1.1 Viral classification and host range

Influenza viruses belong to the family Orthomyxoviridae, which consists of five genera: *influenza A virus*, *influenza B virus*, *influenza C virus*, *thogotovirus* and *isavirus*. Influenza A viruses (IAV) are further subdivided into subtypes, according to antigenic differences in the surface glycoproteins: hemagglutinin (HA) and neuraminidase (NA). There are currently 17 HA (H1-H17) and 9 NA (N1-N9) subtypes with the newest virus isolated from bats in 2012 [1]. Two different species belong to the *thogotovirus* genus: *Thogoto* and *Dhori* viruses, while the *isavirus* genus contains only one species: Infectious Salmon Anemia Virus (ISA) [2].

Host range is a distinctive factor among these viruses: influenza B and C viruses are almost exclusively restricted to humans while thogoto viruses have been isolated from humans and ticks however isaviruses are only known to infect salmon. The natural reservoir for IAV is waterfowl and aquatic birds and all known subtypes have been isolated from these species; however they also infect a very wide range of mammals and birds including swine, poultry and horses [2] (Figure 1).

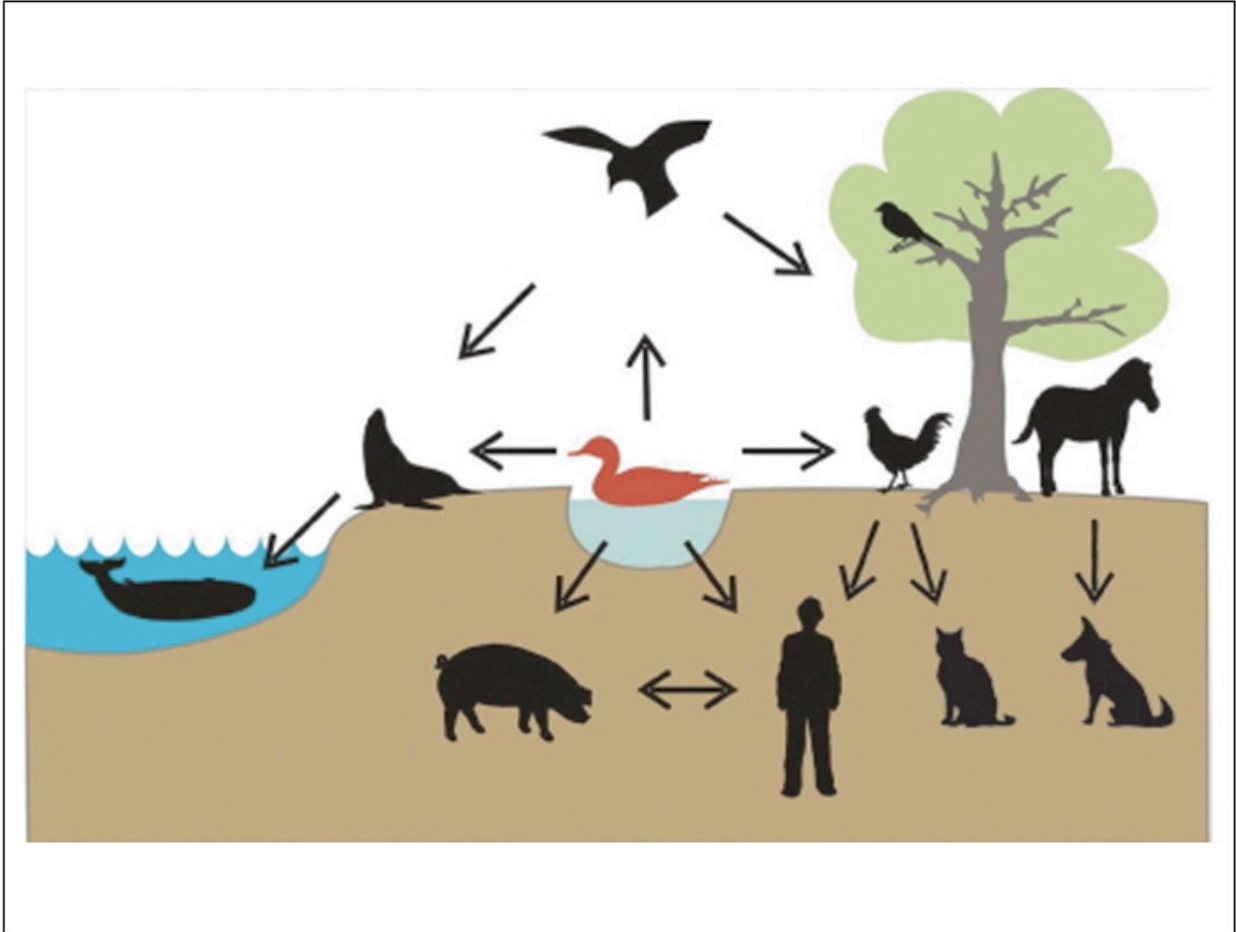


Figure 1 Influenza A virus hosts Influenza A viruses ' main reservoir are waterfowl and wild aquatic birds. Influenza A viruses can also transmit to multiple hosts including poultry, sea mammals, pigs, horses, dogs and humans (Fields Virology 5th edition [2]).

The four influenza A subtypes, H1N1, H1N2, H2N2 and H3N2 have established lineages in humans since the beginning of the past century with H1N2 reassortants arising within the last 10 years. However, after 1997 avian (H9N2, H5N1, H7N3 and H7N7) subtypes have sporadically emerged in humans [3-6]. Highly pathogenic H5N1 subtype, which causes the most severe disease, has expanded its host range to include domestic cats, tigers and leopards, fed infected poultry carcasses; species which generally have not been considered susceptible to influenza A [7, 8]. In humans, highly pathogenic H5N1 viruses cause a severe disease with 59% mortalities reported [9]. H5N1 still poses a global threat with a strong pandemic potential.

According to the World Health Organization Guidelines, the nomenclature of influenza virus consists of: the type of the strain, the host species of origin (omitted for human viruses), geographic site of isolation, the strain number, the year of isolation, and the subtype as shown in Figure 2.

Human Viruses:

Virus Strain/Site of Isolation/Strain Number/Year of Isolation (HA and NA subtype)

For example: A/Hong Kong/1/1968 (H3N2)

Non-Human Viruses:

Virus Strain/Species of Origin/Site of Isolation/Strain Number/Year of Isolation (HA and NA subtype)

For example: A/Chicken/Beijing/1/1994 (H9N2)

Figure 2 Influenza A virus nomenclature

1.2 Pathogenesis of influenza A viruses

Influenza A viruses cause seasonal epidemics occurring usually during the cold seasons of temperate climates, which can be associated with severe disease in the elderly, the very young and people with underlying health conditions. Acute influenza infections generally last for 7 to 10 days and clinical signs vary from asymptomatic to a very severe disease and death. Typical IAV symptoms are characterized by a high fever, tracheobronchitis, muscle ache, dry cough, headache and malaise [10]. Depending on the severity of the flu season in Canada, around 2000 to 8000 people die each year due to influenza and its complications [11].

Influenza A virus infects and replicates in the respiratory epithelium causing an inflammation of the upper respiratory tract and trachea resulting in acute respiratory disease [2]. In contrast, infections of the lower respiratory tract i.e. alveoli result in a more severe disease associated with necrosis and destruction of the bronchiolar and alveolar tissues as well as extensive lung infiltration by immune cells and tissue fluids [12, 13].

Influenza A virus infection not only causes rare instances of primary viral pneumonia but is often associated with secondary bacterial infections in high risk populations (such as *Streptococcus pneumoniae*, *Staphylococcus aureus* and *Haemophilus influenzae*) that increase disease severity and result in secondary bacterial pneumonia [14]. A recent study suggested that the type I interferon innate immune response following viral infections downregulates caspase-1 dependent cytokines (IL-1 β , IL-18 and IL-33) which therefore favors growth of secondary bacterial infections [15]. Rare but severe complications associated with influenza include gastrointestinal problems, toxic shock syndrome, renal

failure, myocarditis, pericarditis and encephalopathy [2]. Immunocompromised patients such as people with cardiovascular problems, diabetes, pregnant women as well as the elderly and the children are at higher risk of severe complications [10].

Historically humans have been infected with H1N1, H2N2 and H3N2 strains. In the late 20th century several avian viruses (H9N2, H7N7, H7N3 and H5N1) crossed the species barrier and infected humans causing mild respiratory symptoms with limited mortalities with the exception of H5N1 [3, 5, 6, 16]. However these avian viruses have caused serious mortalities, morbidities and enormous economic losses in poultry farms worldwide. The H9N2 subtype was first isolated from two children in Hong Kong in 1999 [3] with mild respiratory disease and seasonal flu like symptoms [3]. Similarly, the H7N7 and H7N3 subtypes cause mild conjunctivitis in most people infected but a few fatal cases were reported [5, 6, 17]. However, highly pathogenic H5N1 viruses are posing a pandemic threat by infecting 578 number of humans and causing 59 % mortalities in humans [9]. The disease severity ranged from typical mild upper respiratory illness to acute pneumonia and respiratory distress. Unlike previous influenza pandemics, H5N1 mortalities did not result from secondary bacterial pneumonia and severe complications were often observed in healthy young individuals due to primary viral pneumonia [12]. Mortalities were often associated with multi-organ failure. Post-mortem analysis of human fatalities revealed severe alveolar damage, pulmonary congestion, hemorrhage and interstitial fibrosis [12]. These complications have been linked to a high viral load in the respiratory tract, a severe hypercytokinemia and extensive infiltration of immune cells in the respiratory tissues which progress into hemophagocytic syndrome described as phagocytosis of major immune cells

[4, 12]. Although IAV replication is usually restricted to the lung, the highly pathogenic H5N1 viruses possess a unique ability for systemic spread in infected poultry as well as in some patients; in rare instances the virus has been isolated from the placenta, feces, blood and cerebrospinal fluid [16]. Viral transmission however is restricted in humans; the majority of human cases are associated with direct contact with infected poultry, while limited human-to-human transmission has been reported [16].

1.3 Influenza pandemics

In addition to local and regional outbreaks, IAV is also capable of causing severe worldwide epidemics. Novel avian viruses can emerge in humans causing pandemics associated with serious illness and resulting in a huge number of mortalities globally. In the past century IAV were the causative agent of three pandemics; namely the 1918 (H1N1), 1957 (H2N2), and the 1968 Asian flu (H3N2). The most lethal one being the 1918 Spanish Flu, where around 20% of the world population was infected and 50 million people died [18]. The H1N1 was of avian origin and had acquired multiple mutations to fully adapt to humans [18]. Following this pandemic, the circulating human H1N1 virus evolved until 1957 when it acquired three avian-like genes (HA, NA and basic polymerase 1 (PB1)) and the resulting novel H2N2 virus caused the second 1957 pandemic that resulted in 2 million deaths worldwide [19]. The third pandemic was caused by the H3N2 virus, which originated from the previously circulating human H2N2 virus and obtained two genes of avian origin: hemagglutinin (H3) and PB1 [19]. The H3N2 Hong Kong flu caused approximately 1 million deaths.

The most recent influenza A virus pandemic occurred in 2009 and was caused by a

H1N1 virus of swine origin. Although of relatively typical or low virulence there were higher numbers of atypical pneumonia especially in children [20]. Although of animal origin, the H1N1 virus was capable of efficient human-to-human transmission and was able to spread to 208 countries infecting more than half a million people in less than a year [21]. The virus is a reassortant of four swine viruses. Six genes (basic polymerase 2 (PB2), PB1, acidic polymerase (PA), HA, nucleoprotein (NP) and non-structural protein 1 (NS1)) were derived from three North American swine viruses while the NA and M genes were derived from an Eurasian swine lineage [21]. Unlike typical seasonal flu infections, people hospitalized with the pandemic H1N1 2009 flu were mainly children and individuals < 65 years of age. The virus replicated in the upper and lower respiratory tract as it is capable of binding both host cellular receptors α -2,6 and α -2,3 sialic acid (SA) receptors causing mild to severe viral pneumonia [22]. Although the virus lacks almost all identified influenza virulence markers [21] the 2009 pandemic showed that pandemics can still occur in spite of widespread awareness and surveillance [23].

1.4 Virion structure

Intact viral particles are pleomorphic; the spherical particles have a diameter of 100 nm while the filamentous particles have about the same diameter but are longer (some are more than 20 μ m in length) [24]. Although studies have shown that both filamentous and spherical influenza particles contain one copy of the viral genome [25], filamentous forms of influenza particles are predominant in the lung tissues and upper respiratory tract of patients [26]. Further research is required to elucidate differences in viral entry between spherical and filamentous influenza particles, although experimental studies have shown

that a switch to spherical morphology result in increased virus yields [24].

Influenza A virus particles are composed of 70% protein, 20% lipid, 5 to 8% carbohydrates and 1% RNA. Influenza A virus is an enveloped negative-sense RNA virus with a segmented genome comprised of eight gene segments encoding for ten to eleven proteins, namely three surface proteins, 6 internal and 2 nonstructural proteins. The HA and NA surface glycoproteins, as well as matrix 2 (M2) protein are integrated into the lipid envelope that is host-derived. The internal proteins are polymerase complex proteins (PB1, PB2 and PA), matrix protein 1 (M1), nucleoprotein (NP), and nuclear export protein (NEP previously known as NS2) (Figure 3). The nonstructural proteins are non-structural protein 1 (NS1) and PB1-F2 [2, 27]. Each viral mRNA encodes for one protein with the exception of M and NS genes: M1 and NS1 proteins are encoded by the full transcript, respectively and the second protein (M2 and NEP, respectively) is encoded by a spliced mRNA [2]. In addition to the PB1 protein, the PB1 gene segment codes for an additional protein through a second open reading frame +1: the PB1-F2 protein that is only expressed in some of IAVs [27].

Influenza virus

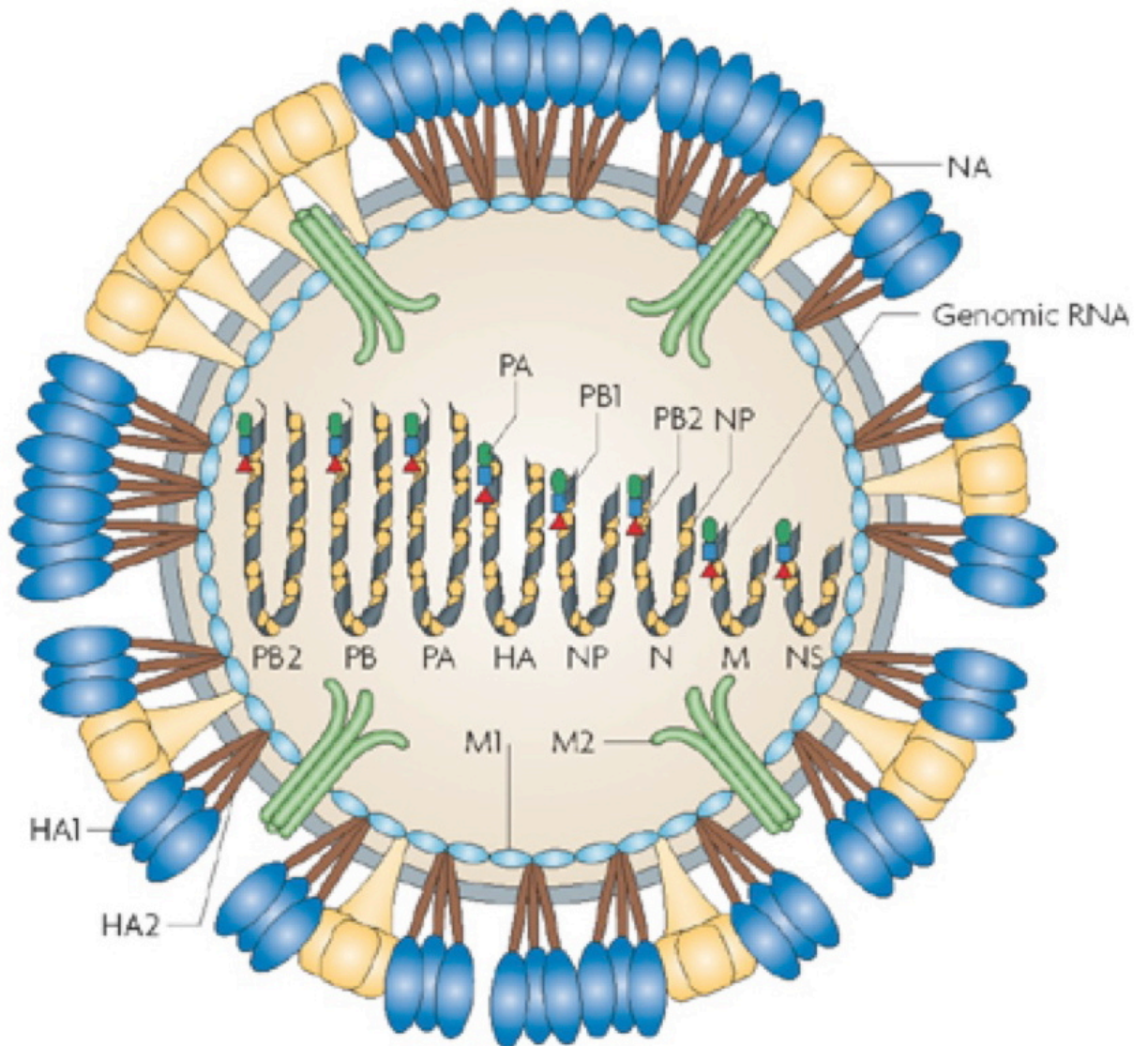


Figure 3 **Influenza A virus structure:** Influenza A virus consists of eight RNA segments that encode 10 to 11 proteins among which 10 are structural proteins (Modified from [28])

1.4.1 The RNA-dependent RNA complex

The influenza polymerase complex is composed of three proteins encoded by the three largest RNA transcripts: PB2, PB1 and PA. Since the viral genomic RNA (vRNA) are negative sense RNAs, they require the viral polymerase to initiate their transcription and replication; therefore in intact viral particles the ribonucleoprotein complex (RNP) is composed of one heterotrimer polymerase complex associated with each of the eight NP encapsidated vRNAs situated at both the RNA duplex formed by the 5' and 3' conserved and complementary ends [2]. The association between the polymerase complex and the vRNA facilitates the role of the viral polymerases in viral transcription and replication.

PB2. The basic polymerase subunit 2 encoded by gene segment 1 is involved in cap binding required to prime the vRNA transcription initiation process. Earlier studies used cross-linking methods to identify amino acid (a. a.) residues 242-252 and 533-577 as cap-binding regions in the PB2 subunit [29, 30]. However, another study contradicted this as it identified a central domain that consists of two PB2 aromatic residues Phe 363 and Phe 404 required to sandwich the N methyl-guanine present in the cap structures [31]. These aromatic residues act similarly to other cap binding proteins (i.e. eIF4E) [31]. However, recent studies using a new technique ESPRIT, a robotic random library-based construct screening process and x-ray crystallography expanded the cap binding site to a fragment (aa 318- 483), which crystallized in the presence of the methylated guanine mGTP showing its exact position [32].

In addition, PB2 is an essential virulence determinant of Influenza A virus with significant involvement in host adaptation and virus transmission [33, 34].

PB1. The basic polymerase 1 subunit encoded by gene segment 2 is the only influenza viral polymerase subunit that shares a high homology with other RNA polymerases. PB1 contains the four conserved motifs (I-IV) that are present in RNA-dependent RNA polymerases [35]. The polymerization active site defined as S-D-D at positions 444-446 is present in motif III, which enables it to catalyze the sequential addition of nucleotides during chain elongation of vRNA, complementary RNA (cRNA) and mRNA [36]. In intact viral particles, PB1 is considered to be the central polymerase as it interacts with both the C-terminus of PA and the N-terminus of PB2 subunits through its N- and C-terminal domains respectively [37-39]. Recently, a new open reading frame in the PB1 gene segment has been identified, leading to the discovery of a new viral protein PB1-F2 (87 a.a.) that is only expressed in some influenza viruses and is found to localize to the inner and outer mitochondrial membrane receptors: the adenine nucleotide translator (ANT3) and the voltage-dependent ion channel 1 (VDAC-1), respectively. PB1-F2 sensitizes the cells to apoptotic stimuli mainly through releasing cytochrome c in a cell-specific manner [27, 40]. A recent study discovered a new function for PB1-F2 in modulating the type I interferon (IFN) response. A specific mutation N66S is responsible for a delayed type I IFN response *in vivo* accompanied by an increase in cytokine and chemokine secretion as well as monocyte and neutrophil infiltration in the lungs, which have been shown to exacerbate disease severity [41].

PA. PA is the third largest polymerase subunit and is the only viral protein encoded by gene segment 3. Early studies had not identified a specific function for the PA subunit. Only

recently, using purified protein complexes, researchers determined the structure of the N-terminal domain of PA and proved that PA is the viral polymerase subunit that possesses the endonuclease activity (which was previously ascribed to the PB1 subunit). The role for the endonuclease function was confirmed by mutagenesis of the endonucleolytic active site, where the transcription activity was aborted while the endonuclease-independent replication process was maintained [42]. Therefore, the main role that is known for PA is the cap cleavage 10 to 13 nucleotides downstream of its 5' end [42, 43]. The 5' cap cleavage from the cellular pre-mRNA frees the cap primer, which primes vRNA transcription. PA is also involved in virus assembly; this activity is mediated through PA residues 507 and 508 [44].

Recent functions have been ascribed to the three polymerase genes in limiting the innate immune response by directly blocking interferon production and degrading the host RNA polymerase II protein (POL II) leading to a general inhibition in host mRNAs. The three polymerase subunits of A/PR/8/34 were demonstrated *in vitro* to be involved in inhibiting IFN- β promoter activation through binding the mitochondrial antiviral protein signaling molecule (MAVS) [45, 46]. The PB2 subunit was the most efficient at inhibiting endogenous IFN- β transcription following infection with IAV *in vitro*, a function independent of NS1 IFN- β inhibition (detailed in section 1.8) [45, 46]. This can be explained by a recently described interaction between the three viral polymerase subunits and the host POL II at its active transcription site, a finding supported by co-immunoprecipitation, co-purification and co-localization studies [47]. This association triggers the ubiquitylation and proteasomal

degradation of the host POL II resulting in overall transcription inhibition of host mRNAs [47, 48].

However, recent research employing protein overexpression from plasmids showed that the viral polymerases are sufficient to induce POL II ubiquitylation and inhibition *in vitro* while POL II degradation was only achieved following viral infection. Therefore, other – yet to be identified- viral component(s) are required and/or associated with the viral polymerases to induce host transcription shut off [48]. It remains to be proven whether the ability of viral polymerases to induce host mRNA shut off correlates with replication or virulence of influenza viruses.

1.4.2 Viral surface glycoproteins

Hemagglutinin. HA is the fourth largest viral protein and the main surface antigen determinant, and together with the neuraminidase glycoprotein, determines the IAV subtype. HA is classified into 17 different HA subtypes grouped in two classes based on amino acids sequence similarity: group 1 contains H1, H2, H5, H6, H8, H9, H11, H12, H13 and H16 and group 2 contains H3, H4, H7, H10, H14, and H15 while H17 forms a third more ancient group [49, 50]. HA is responsible for the initial step in viral replication including viral attachment to host cell sialic acid receptors, and entry into host cells. HA is also involved in viral budding, and viral particle formation, the later steps of replication [2]. The HA₀ glycoprotein is a trimer with each molecule composed of two regions, a globular head (HA1) that attaches to the cell surface and a hydrophilic stalk (HA2) protruding from the host cell membrane [51]. Cleavage of the HA₀ into the two functional proteins HA1 and HA2 is a prerequisite for virus fusion and infectivity [52]. The receptor-binding site lies in the HA1

protein, while HA2 is mainly responsible for fusion of the viral envelope with the endosomal membrane; a process required for the release of the RNPs into the host cells, where replication and viral gene transcription follows [51].

The cleavage site of HA₀ is also a major determinant of pathogenicity in avian influenza viruses. For avian strains, it is well established that the presence of Q/E-X-R amino acids at the HA₀ cleavage site results in a low pathogenicity virus while a multibasic (R/L-X-R/L-R) HA₀ cleavage site is a determinant of a high pathogenicity. Increased virulence of avian highly pathogenic avian influenza (HPAI) H5 and H7 IAVs require the presence of a multi-basic amino acid HA₀ cleavage site [53]. This property has been explained by the fact that multibasic cleavage sites are recognized by ubiquitous intracellular furin-like proteases, which results in systemic multi-organ infection. In contrast, low pathogenicity viruses possess a cleavage site that is recognized by trypsin-like enzymes, which are only found in epithelial cells, restricting viral entry and replication to respiratory and enteric cells [51, 54].

Moreover, HA is a major immune determinant resulting in the formation of neutralizing antibodies following infection and replication; therefore, HA has been an important target in vaccine development [2].

Neuraminidase. NA, the viral protein encoded by genome segment 6, is the second major surface glycoprotein. There are 9 different NA subtypes that are divided phylogenetically into two groups: group 1 containing N1, N4, N5 and N8 and group 2 contains N2, N3, N6, N7 and N9 [54]. Similar to the HA structure, the NA protein is composed of two parts, a globular head and a hydrophobic transmembrane [55, 56]. This protein is found as a

tetramer in viral particles; its enzyme-active site is located at the center of each of the tetramer subunits [55, 57].

NA has a major role in virus release and spread from the infected cells. Temperature-sensitive mutant viruses possessing a non-functional NA were observed by electron microscopy to form aggregates on the membrane of infected cells [58]. This highlighted the role of NA, which was later described as an enzyme mainly involved in cleaving and removing SA from the newly synthesized viral glycoproteins thus preventing self-aggregation [54]. NA also removes SA from host cell receptors and the neighboring mucus, which facilitates the release of the newly formed viral particles from infected cells and allows the spread of progeny virions [58]. NA also plays a role in activating the transforming growth factor (TGF- β), which results in apoptosis [59].

Being a surface protein, it also serves as an important target of the adaptive immune system that blocks NA's enzymatic activity [60]. NA of both groups 1 & 2 is also the target of the classical anti-influenza drugs (Oseltamivir and Zanamivir families); however, numerous studies draw attention to the continuous emergence of drug-resistant viruses [61].

1.4.3 Nucleoprotein

The nucleoprotein is expressed early and encoded by genome segment 5. NP is one of the major proteins that are associated with the RNP complex to encapsidate RNA. This interaction is not sequence-specific; however the abundant arginine residues in NP reflect its positively charged nature, which supports the ionic binding to the phosphate-rich, negatively-charged RNA [62, 63]. It has been described that each NP monomer binds to

approximately 24 RNA nucleotides [64] with the RNA exposed to the exterior [65]. NP has the ability to homo-oligomerize, which generates a higher-order structure of the RNP [66]. The large RNP complex relies on an active transport machinery to move into the nucleus. It has been suggested that NP is involved in RNP's active nuclear localization through its nuclear localization signal (NLS). So far, two NLS domains have been identified in the NP protein, the N-terminal unconventional NLS [67, 68] and the bipartite NLS (198-216 a.a) [69]. Mutagenesis studies suggest the possibility of a third NLS in the NP protein, since blocking both the conventional and the bipartite NLS still allowed partial nuclear localization of the RNP [2]. The NP is also known to bind three members of the human importin family ($\alpha 1$, $\alpha 3$ and $\alpha 5$) [67, 68, 70]. The tight association of NP with the RNP, the two identified NLS signals and the described interaction with human importin proteins, all strongly suggest that NP is involved in RNP localization. However, all polymerase subunits contain NLS domains; therefore it is not clear yet whether NP alone, or in complex with some or all of the polymerase proteins is involved in nuclear localization of RNP [71].

In addition to its abundance in the nucleus, NP is also localized in the cytoplasm. Studies have shown that it is capable of binding chromosome region maintenance protein-1 (Crm-1), which suggests another possible role in RNP export [72].

Studies have demonstrated that NP contains another trafficking signal known as a nuclear accumulation/retention signal (336-345 a.a) [73]. The abundance of NP in the nucleus facilitates the nuclear retention of the newly made RNPs until later in infection when M1 and the viral NEP protein associate with RNP to then interact with the nuclear export machinery to trigger nuclear export for particle assembly and budding [2]. The viral

protein can also undergo host-dependent phosphorylation, which allows host range restriction [74].

1.4.4 Matrix protein

Two proteins, matrix protein 1 (M1) and matrix protein 2 (M2), which results from alternative splicing are encoded by genome segment 7. Matrix protein 1, the most abundant protein in the influenza virus, contains three domains with the internal domain mediating oligomerization and RNP association [75]. In the viral particle, the M1 protein has been described as a “helical net” [24], which lies right under the viral envelope. It provides support and structure to the viral envelope and links the surface proteins through their cytoplasmic tails to the core of the virus by interacting with the NP protein [2, 24]. Release of M1 from the RNP plays a major role in nuclear import of the vRNP after uncoating and this release is prevented by the antiviral drugs adamantanes [71]. Different strategies have been described to explain the process of RNP export to the cytoplasm, but all hypotheses agreed on the involvement of interaction between M1 and the NEP through their C-terminus and nuclear export signal (NES) respectively in RNP export. This process is irreversible, as the NLS of M1 and the NEP binding site on M1 protein overlap [76], which suggests a possible role of NEP in masking the reversible RNP nuclear import [2]. Another strategy is suggested by the interaction through RNP binding of the heat shock protein 70 (hsp 70) at the M1 binding site, which is another irreversible method to keep the RNPs in the cytoplasm [77, 78]. M1 is also involved in virus assembly where M1 binds to lipid rafts and the cytoplasmic tails of the surface viral glycoproteins in the host membrane and

thereby recruits the newly formed viral proteins to the plasma membrane at the site of assembly [24, 79].

Both described roles of M1 (virus assembly and RNP nuclear export) are late processes in virus replication. As M1 is expressed later in infection, the virus then must undergo a full round of replication before RNP is exported to the cytoplasm [2].

The M2 protein is a 97 a.a. integral homotetramer protein that consists of an external domain, a transmembrane domain and an internal domain [2]. The transmembrane domain (19 a.a) forms the pore of the ion channel and is involved in virus acidification [80, 81]. Oligomerized M2 protein serves as a proton-selective ion channel; its main function is to pump protons (H^+) from the acidic endosomal environment to the interior of the virus. The protonation of the virus leads to two essential steps required for viral entry: 1) a conformational change in HA necessary for fusion, and 2) the disruption of protein-protein interactions, leading mainly to an important dissociation between M1 and the RNP, freeing the RNPs to be further imported to the nucleus [82]. The M2 also regulates the pH in the Golgi apparatus during HA processing and export to the plasma membrane. It prevents premature acid-induced conformational changes of the HA protein before being transported to the budding site [83].

The membrane-spanning region of the cytoplasmic tail of M2 is an amphipathic helix that has recently been shown to be involved in cholesterol binding and localization to the lipid raft-enriched site of the plasma membrane, suggesting a possible role of M2 in virus assembly and budding [84]. Both M1 and M2 proteins can associate together and influence

the structure of influenza virus by modulating the ratio of filamentous to spherical particles formation (reviewed in [24]).

The ion channel is the target of a class of drugs that has been used in treatment and prophylaxis of influenza A individuals for over 30 years, the M2 class of inhibitors the adamantanes. Additionally, the highly conserved region comprises an exterior exposed domain of M2 and thus serves as a target to some influenza preventive vaccines [85].

1.4.5 Non-structural protein-1 (NS1) and nuclear export protein (NEP)

NS1 and NEP are encoded by gene segment eight and they share the first 10 N-terminal amino acids [86]. NS1 is expressed from the full-length transcript of genome segment eight and is highly variable in length; isolates vary from 124 to 237 a. a. [87]. NS1 is a 26 kDa protein that is divided into two major functional domains: the N-terminal RNA-binding domain (RBD) (1-73 a.a.) and the C-terminal effector domain (74-230 a.a.). These domains allow the NS1 protein to exhibit both protein-protein and protein-RNA interactions [88]. The coding region of NS1 has been shown to contain two nuclear localization signals (NLS1 and NLS2) and one nuclear export signal (NES), which localize the protein in the nucleus and the cytoplasm at different times [89]. The dual localization of NS1 contributes to its multifunctional role.

NS1 is a multifunctional protein that binds both ssRNA and dsRNA [90, 91] and interacts with a number of host cellular proteins [88, 92]. NS1 is a major player in viral replication and functions as an IFN antagonist to inhibit IFN production and signaling (reviewed in [88]). NS1 functions are provided in more detail in subsequent sections.

Nuclear export protein (NEP or NS2) plays a major role in RNP nuclear export. Due to its small size (14.5 kDa), the NEP can diffuse freely through the nuclear pores and translocate to the nucleus [71]. NEP is thought to be involved in nuclear export of the newly formed RNP through its nuclear export signal (NES) that lies in the N-terminal domain (residues 1-54) [93]. The current model for RNP export is the formation of the complex RNP-M1-NEP. The M1 protein binds the RNP complex through its C-terminal domain with high affinity, leaving its N-terminal domain, which contains the NLS signal free. The NEP binds to the NLS of the M1 protein through its C-terminal domain; therefore blocking the reversible nuclear import of the complex. In this scheme, the N-terminal domain of the NEP protein that contains the NES is free to bind to the host export machinery exportin-1 or chromosome region maintenance protein-1 (Crm-1) [76].

1.5 Viral replication

Influenza A virus replication is initiated by the attachment of the viral HA protein to the host cell receptor, SA. HA binding to various sialic acid moieties is dependent upon the viral host lineage. Human viruses preferentially bind to SA that are linked to galactose sugar via α -2,6 linkage (SA α -2,6 Gal), while avian viruses preferentially bind SA with α -2,3 linkages (SA α -2,3Gal). The varying distribution of SA receptors in different hosts (avian vs. humans), organs (respiratory vs. intestinal) and cell types (ciliated vs. non ciliated) restricts viral infection and replication to specific hosts and cell types (reviewed in [94]). Once bound, the virus is internalized via clathrin-dependent endocytosis [95]. However, a non-clathrin dependent pathway that does not require an acidified environment has also been described [96]. The low pH in the late endosome is required for a conformational change in

the HA protein that exposes its HA2 fusion peptide [51]. The exposed fusion peptide is then inserted into the endosomal membrane facilitating the fusion of the viral envelope with the endosomal membrane thus creating pores through which the viral contents are released into the cytoplasm of the host cell [51]. The M2 ion channel protein, which pumps the endosomal H⁺ ions into the virus particles, facilitates the uncoating process and also results in the dissociation of M1-RNP, which frees the RNP to be released into the cytoplasm [82]. The free RNP is then transported into the nucleus through an active nucleocytoplasmic process involving the NLS of RNP protein complex and the host nuclear trafficking machinery [71, 97].

Once in the nucleus, the negative sense vRNA is transcribed into mRNA, which is then capped and poly-adenylated. The transcription process requires all three viral polymerase subunits. Transcription initiation involves the PB1 subunit bound to the 5' end of the vRNA, which activates the PB2 subunit to recognize and bind the host cap of cellular pre-mRNAs [98, 99]. This enhances the interaction between PB1 and the 3' end of the vRNA thus forming a stable vRNA loop [2, 98, 100]. Simultaneously the PA subunit cleaves the cellular pre-mRNAs through a cap-snatching process required to prime vRNA transcription [43]. Transcription initiation starts when PB1 adds a guanosine at the 3' end of the cap primer, which is complementary to the penultimate cytosine at the 3' end of the vRNA template [101]. Subsequently, PB1 continues to catalyze mRNA chain elongation until it encounters a polyuridine rich residue in the vRNA template, which along with a steric hindrance PB1 blockade signal for polyA addition at the 3' end of the newly formed mRNA [102-104].

The vRNA also serves as a template for replication and generation of complementary RNA (cRNA). Unlike mRNA, cRNA are neither capped nor poly-adenylated therefore replication is independent of cap binding and snatching activities of PB2 and PA respectively. The positive sense cRNA in turn serves as a template for the generation of the negatively oriented genomic vRNAs. Newly synthesized vRNAs and cRNAs are encapsidated and consequently it has been hypothesized that their synthesis is dependent on the availability of the newly produced NP viral proteins however the switch from transcription to replication is poorly understood [105, 106].

The encapsidated vRNA associates with the polymerase complex and forms the mature RNP complex, which in turn associates with the M1 protein [107]. The RNP-M1 complex interacts with the NEP protein, which in turn recruits the host nucleocytoplasmic trafficking machinery and the complex is then exported to the cytoplasm [71, 76].

The RNPs along with the surface proteins HA, NA and M2 are directed to the virus assembly site in the apical plasma membrane where they associate with lipid rafts [24]. The surface proteins are directed first to the endoplasmic reticulum where the surface glycoproteins get glycosylated. The HA protein is assembled into trimer, and NA and M2 as tetramers, before being transported to the assembly site on the apical plasma membrane (reviewed in [24]). After assembly, the M1 shell causes extrusion of the plasma membrane leading to the budding of the newly formed viral particles from the infected cell [24, 108]. The final step in viral replication is the release of the new virions attached to the sialic acid receptors on the infected cells. The release is mediated by the enzymatic activity of NA [58]. The newly formed viral particles require further HA cleavage by trypsin-like enzymes (low

pathogenic avian influenza (LPAI) viruses) that liberate the fusion protein and the new infectious progeny can start a new replication cycle [51, 54] (Figure 4).

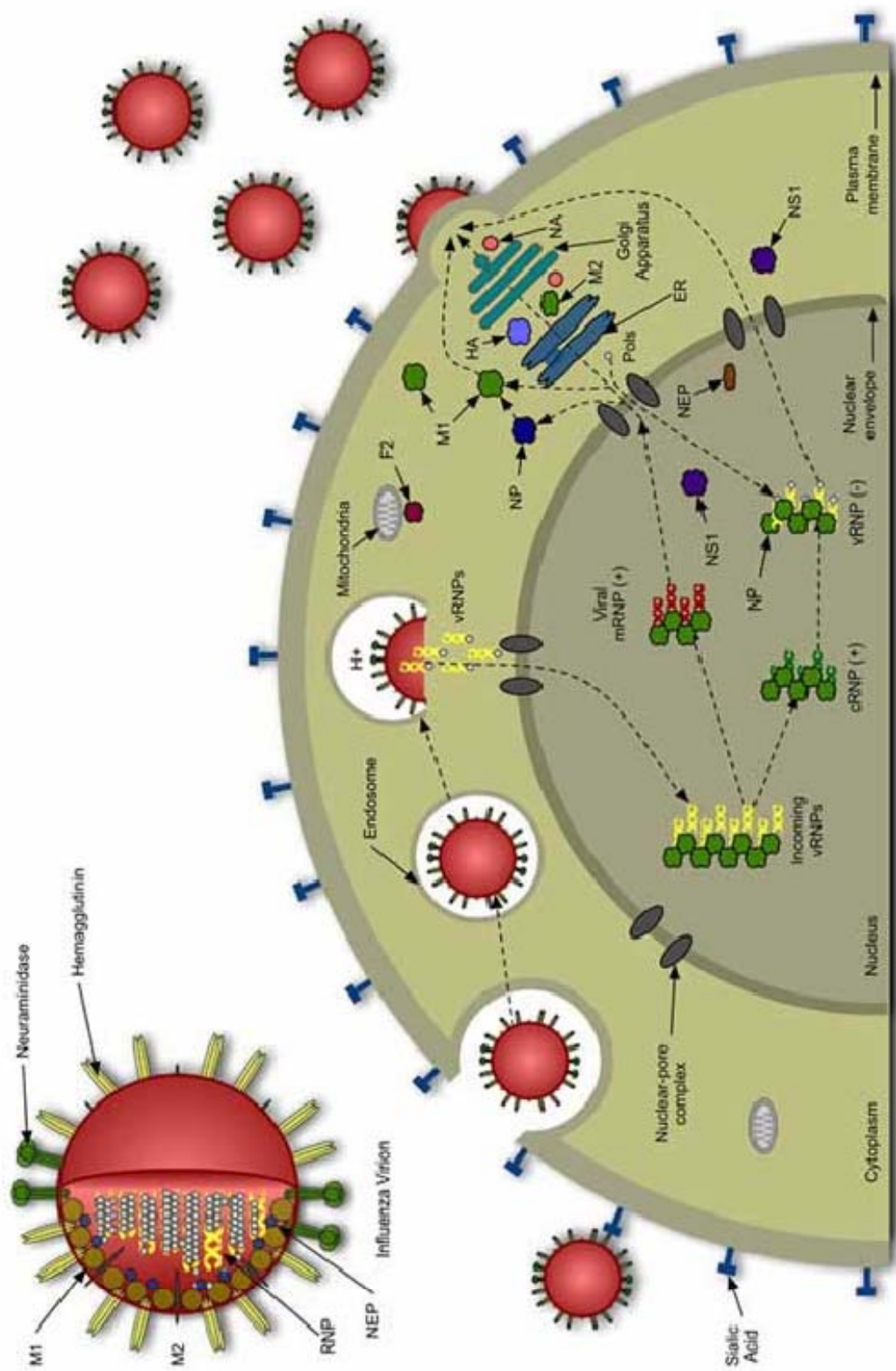


Figure 4 Influenza A virus life cycle Influenza A virus binds the SA receptors and is then internalized by receptor mediated endocytosis. The low pH in the endosome triggers fusion of the viral and endosomal membranes followed by the release of the RNPs, which are then directed into the nucleus. Once in the nucleus, the vRNAs are transcribed into mRNAs, which are then translated into viral proteins. Replication of vRNA occurs through a positive sense intermediate cRNA. The newly formed vRNA are then encapsidated and directed to the assembly site where viral budding and release occurs.

1.6 Type I interferon response to influenza A viruses

To date, three classes of interferon have been identified: interferon type I, II and III according to differences in the receptor complex they bind to and signal through. Type I IFNs are well characterized and are known for their robust host response against viral infections. More specifically, type I IFN (α/β) are considered “the most powerful innate immune defense mechanism” against influenza virus-infection and replication [109]. Pathogen recognition receptors (PRR) including toll-like receptors TLR-3 and -7, RNA helicase retinoic inducible gene-1 (RIG-I) and the NOD-like receptors (NLRs) recognize influenza viruses and trigger a complicated cascade of intracellular events. Depending on the nature of the stimulus and the equivalent PRRs, distinct signaling pathways including kinases and transcription factors are induced directly in the infected cell (autocrine) [109, 110] or induce signaling in neighboring uninfected cells (paracrine) [111].

RIG-I, the major PRR during IAV infection, recognizes both ssRNA and dsRNAs through its C-terminal domain [91, 112], which leads to a conformational change exposing the two-caspase activation and recruitment domains (CARDs) in its N-terminal region [113]. The first N-terminal CARD domain of RIG-I is ubiquitinated by the ubiquitin ligase tripartite motif (TRIM-25) [114] as a requirement for the second N-terminal CARD domain of RIG-I to interact with the CARD domain of its downstream effector: the mitochondrial IFN- β promoter stimulator-1 (IPS-1) (also termed as MAVS, VISA, Cardif) [115]. This binding interaction leads to the subsequent activation of interferon regulatory factor 3 (IRF-3) and nuclear factor - κ B (NF- κ B) transcription factors [116].

TLR-3, which is expressed in respiratory epithelial cells, recognizes dsRNA of influenza

viruses, while TLR-7, which is expressed in dendritic cells, senses the anti-genomic ssRNA possessing a non-self signature, the 5' triphosphate group [117-119]. Once activated, TLR-3 leads to the phosphorylation of the transcription factors IRF-3, IRF-7 and NF- κ B [109]. Following TLR-7 activation by ssRNA, the myeloid differentiation factor (MyD88) is recruited, which then associates with a complex of kinases [120]. The MyD88-kinase complex binds directly to IRF-7 and therefore translocate together to the nucleus and associate with the IFN- β promoter (reviewed in [121]).

Following PRRs activation, the downstream transcription factors get activated in the cytoplasm and subsequently translocate into the nucleus where they interact with the IFN- β promoter. The C-terminus of the stimulated IRF-3 undergoes phosphorylation in the cytoplasm causing a conformational change leading to its dimerization and the unmasking of its NLS signal, which allows the translocation of the active IRF-3 into the nucleus [122, 123]. The NF- κ B is held in complex with its inhibitor NF- κ B inhibitor (I κ B) in the cytoplasm [124]. Upon stimulation, I κ B is phosphorylated and then ubiquitinated, which leads to its subsequent degradation by proteasomes. The accessible NLS of the free NF- κ B therefore directs it to the nucleus [124].

In the nucleus, the dimerized NF- κ B, IRF-3, and IRF -7 assemble within the IFN- β promoter along with the heterodimer activator transcription factor-2 (ATF-2/c Jun) forming the enhanceosome [109]. This complex is aided by the co-activator CREB-binding protein (CBP)/p300, which recruits the transcription machinery and drives IFN- β transcription (reviewed by [121]). Studies have shown that the recruitment of IRF-3 and/or 7 are indispensable for IFN- β induction, while the contribution of both factors ATF-2/c Jun and NF-

κB may depend on the cell type, enhanceosome structure and the type of stimulus [121].

Thereafter, the mature IFN-β protein is synthesized and secreted to the extracellular environment where it binds its receptor IFNAR (IFN α/β receptor) on the surface of the infected cell or its neighboring cells. IFNAR is composed of two extracellular subunits IFNAR1 and IFNAR2, which associate with two intracellular tyrosine kinases TYK2 and JAK1 respectively. The binding of type I IFN (α/β) to IFNAR results in a signaling cascade that starts with the phosphorylation and activation of TYK2 and JAK1, which leads to the recruitment and phosphorylation of the signal transducers and activators of transcription (STAT) STAT1 and STAT2. The heterodimer STAT1/STAT2 translocates into the nucleus and associates with the DNA binding protein IRF-9 to form the ISGF-3 heterotrimer. ISGF-3 binds the IFN-stimulated response element (ISRE) present in the promoters of more than 300-IFN stimulated genes implicated in the antiviral response and drives their transcription [110, 121]. Many of the stimulated genes have been directly implicated in the IAV antiviral response such as RIG-I and STATs, resulting in increased IFN-β production via an amplification loop. In addition to its role as a 'primary' inducer of IFN-β, IRF-7 has a major role in exerting a positive feedback loop enhancing the IFN-α/β response following viral infections to sustain the antiviral state [125, 126].

The most prevalently studied IFN inducible genes implicated in counteracting influenza virus replication are the RNA dependent protein kinase R (PKR), 2'-5' oligo (A) synthetase (2'-5'OAS), RNase L, viperin and the orthomyxovirus resistance genes (Mx) [109]. Analyses of viral infections and replication in mice with targeted gene knockouts elucidated the role played by these gene products in counteracting viral replication. PKR is

constitutively expressed in a non-activated form in cells but its level of expression is increased following IFN stimulation. PKR is activated by either dsRNA or PKR-activating protein (PACT) [127] and then undergoes phosphorylation and dimerization, which activates the PKR kinase domain [110]. PKR phosphorylates its substrate the eukaryotic translational initiation factor 2 α (eIF2 α), which acts by inhibiting gene translation, leading to cell content degradation and autophagy [128]. The OAS proteins are also constitutively expressed in cells at a basal level. They undergo activation and oligomerize upon detection of IAV dsRNA in the cytosol. The oligomerized OAS synthesize the unique 2'-5'oligoadenylates oligomers that activate the constitutively expressed, inactive ribonuclease L (RNase L). The active homodimeric RNase L enzyme further recognizes and cleaves viral and cellular ssRNA into short RNA [129, 130] thereby impairing viral gene translation and replication [109]. MxA, the human homologue of Myxovirus-resistance (Mx) genes is expressed following IFN secretion. MxA can trap viral proteins in the cytoplasm early after infection, degrade them and therefore block viral genes transcription and replication [131, 132]. Studies have shown that MxA targets NP and PB2 proteins of the thogoto virus [110, 133] and therefore prevents the nuclear transport of the RNPs [69, 131]. The expression of viperin (virus inhibitory endoplasmic reticulum associated, interferon inducible), a newly discovered protein, can disrupt lipid rafts at the plasma membrane and therefore blocks IAVs assembly and budding [134].

The type I IFNs also perform an immunomodulatory role by upregulating molecules implicated in antigen presentation such as major histocompatibility complex-1 (MHC-I) [109, 135]. The IFN response can also induce pro-inflammatory cytokines (IL-1 (α/β), IL-6,

IL-8, and TNF- α) [136] and chemokines (MIP-1 α , MIP-1 β , MCP-1 [137]) that attract cells to the site of infection, which in turn attracts innate immune cells (such as macrophages and neutrophils) in the early stages as well as lymphocytes and monocytes in the later stages of infection [10]).

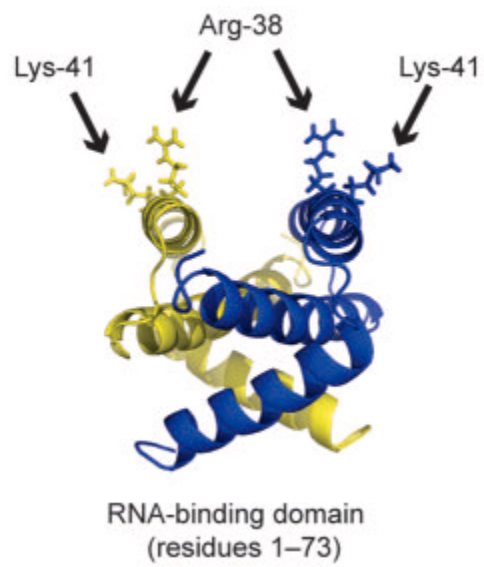
As the type I IFN response results in robust pro-apoptotic and anti-proliferative signaling, other regulators of the antiviral immune response are required to limit the damaging effects of type I IFN on host cells [111]. Upon sensing a danger signal within the cytoplasm, the third group of the PRR family members, NLRs, oligomerizes forming doughnut-shaped inflammasomes, which activate pro-caspase-1. Active caspase-1, also known as IL-1 β converting enzyme regulates the inflammatory response by processing and activating the IL-1 β , IL-18 and IL-33 cytokines [138]. It has been reported that inflammasome formation regulates the extent of lung damage and epithelial necrosis in IAV infection [139, 140].

1.7 Structure of the non-structural protein 1

As previously described in section 1.4.5, NS1 is formed of a N-terminal and a C-terminal domain, which mediate RNA binding and protein-protein interactions, respectively. The NMR and crystal structures indicate that NS1 functions as a symmetric dimerized protein. This finding has explained how NS1 can perform multiple tasks (interaction with RNA of various lengths through its RBD and numerous host protein associations) at the same time [141]. The polypeptide chains of the RNA binding domain (a.a 1-73) form three- α -helices (helix 1: a. a 4-24, helix 2: a. a 31-50 and helix 3: a. a 54-70). Dimerization is crucial for dsRNA binding; therefore, the six-helices structure of the RBD is

thought to contribute to dsRNA binding in a 1:1 stoichiometry [142]. Although several residues have been implicated in the NS1-dsRNA interaction as reviewed in [88] it has been proven that two critical sites in helix 2, Arg 38 and Lys 41 are essential for this interaction (Figure 5a). Studies have characterized the NS1-dsRNA binding as a unique and a very critical interaction, since both the RNA binding domain of NS1 and the dsRNA conserve their initial conformation [143].

a



b

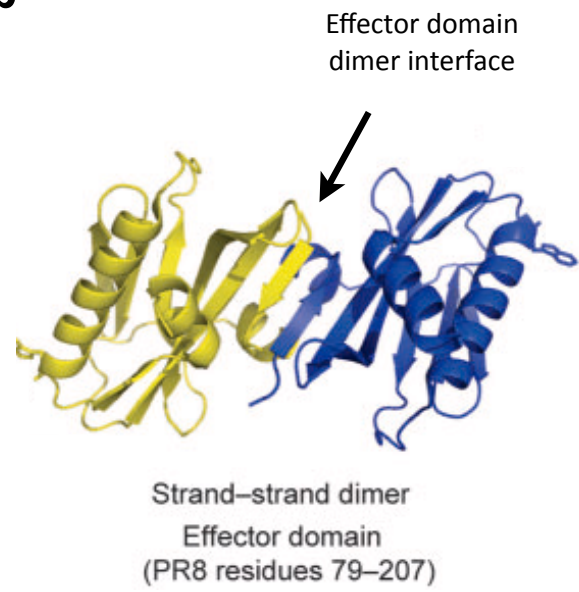


Figure 5 **Non-structural protein-1 dimer structure** **a.** The RNA-binding domain dimer structure consisting of amino acids 1 to 73 **b.** The effector domain dimer structure consisting of amino acids 79 to 207 (modified from [144])

The effector domain of NS1 is formed of three- α -helices and seven- β strands. Six anti-parallel β -strands (1-6) surround one long and central α -helix (helix 2) described as “ α -helix, β -crescent form” [143] (Figure 5b). Through its effector domain, NS1 interacts with a number of host cellular proteins including the poly-A-binding protein 1 (PABPI), poly-A-binding protein nuclear I (PABPNI), the nuclear RNA export factor 1/tip-associated protein (NXF1/TAP), the eukaryotic initiation factor 4G1 (eIF4GI), PKR, the cleavage and polyadendylation specificity factor 30 (CPSF 30), P85 β (the regulatory subunit of class 1 phosphoinositide-3 kinase), importin- α , phosphatase inositol 3 kinase (PI3K) and hStaufen [9] (Figure 6). The effector domain is also important for stabilizing the RBD-dsRNA interaction [143, 145].

Little is known about NS1 protein post-translational modifications. Few reports have demonstrated that NS1 of some strains are phosphorylated in their C-termini (positions 195, 197 and 215 a.a) [146, 147]. Although A/Udorn/1/72 viral replication is altered when phosphorylation is abrogated, the biological functions of NS1 phosphorylation remain unclear [146]. A recent study showed that NS1 of most IAVs viruses possess two SUMO1 (small ubiquitin-like modifier-1) acceptor sites (219 and 221 a.a) in their C-terminus and therefore can be SUMOlated. Mutagenesis substitution proved that NS1 SUMOlation enhances viral replication and prevents early NS1 degradation, however the 2009 H1N1 pandemic virus does not possess this post-translational modification due to a C-terminal truncation that removes this region [148].

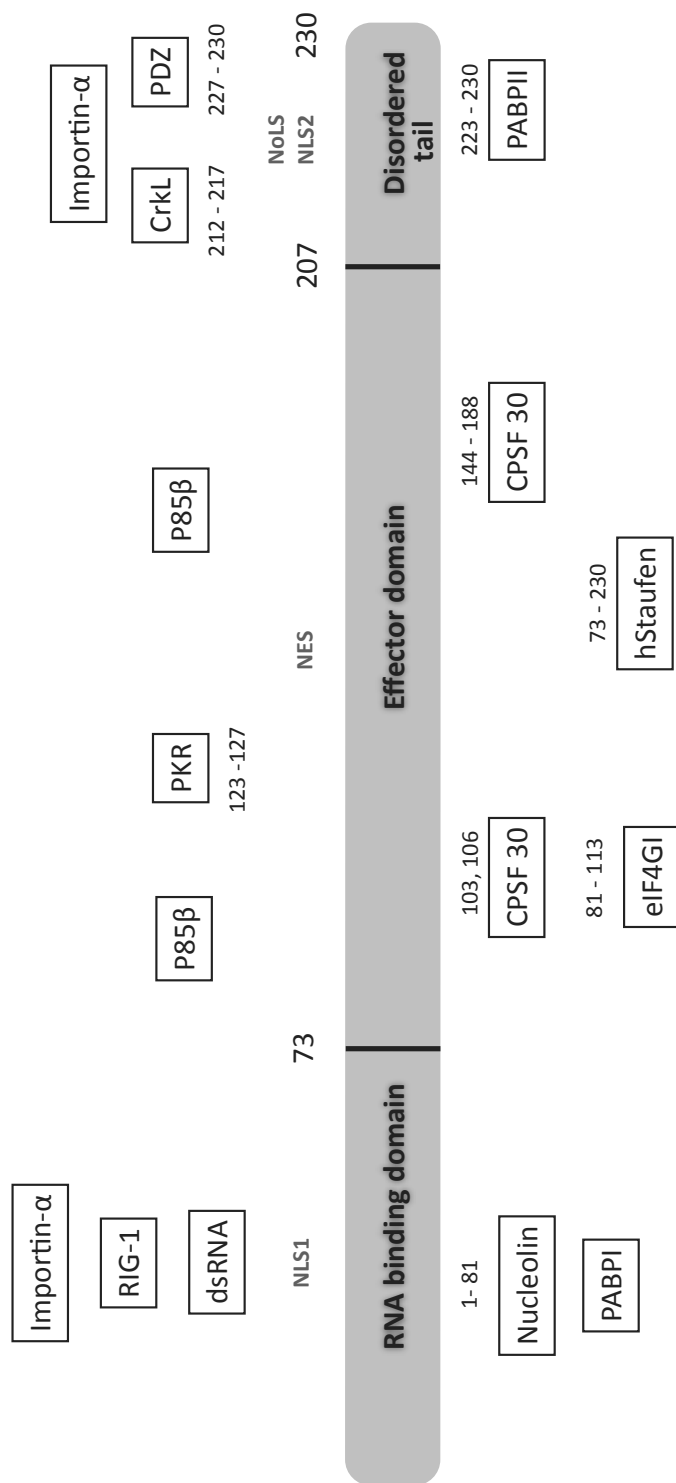


Figure 6 Non-structural protein-1 binding partners NS1 is 125 to 230 a.a. long depending on the strain. NS1 mediates RNA binding through its RBD (1-73 a.a) and host protein interactions through its effector domain (73 to 207 a.a). More host protein interactions can be mediated through the 207 to 230 a.a. disordered tail of NS1. NS1 protein possesses two NLS (NLS1 and NLS2) and one NES (modified from [88]).

1.8 NS1 an IFN antagonist that functions by inhibiting IFN induction and IFN effectors

A major function of NS1 is its involvement in IFN antagonism. This function was first demonstrated by the construction of a virus lacking the NS1 gene and only expressing NEP in segment eight, which was only able to replicate in IFN deficient systems including Vero cells, embryonated eggs (i.e. 6 day old eggs) and STAT1^{-/-} mice [149]. Previous studies using truncated or mutant forms of NS1 genes have demonstrated the involvement of NS1 protein in limiting IFN induction at both the pre-transcriptional and post-transcriptional levels as well as limiting the IFN induced signaling pathway [88].

1.8.1 Pre-transcriptional inhibition of IFN induction by NS1 (cytoplasmic level)

NS1 binds to viral pathogen associated molecular patterns (PAMPS) such as dsRNA and ssRNA with a 3' triphosphate group and therefore prevents their recognition by RIG-I, TLR-3 and TLR-7. Therefore IFN induction is altered at the pre-transcriptional level by preventing the phosphorylation of the transcription factors IRF-3, IRF-7, ATF-2/c-Jun and NF- κ B [150-152]. Another strategy employed by NS1 is the direct binding to RIG-I resulting in inhibition of the RIG-I/IPS-1 mediated activation of the downstream effectors IRF-3 and NF- κ B [117]. It has been shown that RIG-I-NS1 binding is mediated through the N-terminal region of NS1, and this interaction is mainly dependent on the Arg-38 and Lys-41 residues within the RBD [91]. NS1 can also inhibit RIG-I activation by binding TRIM25 (at a region including E96, E95 residues of NS1) thereby inhibiting TRIM25 ubiquitination of RIG-I and ultimately blocking RIG-I downstream signalling [153]. By interfering with the PAMP-PRR interactions at different levels, NS1 can thus prevent IFN- β transcription and therefore

limits the resultant IFN cascade.

1.8.2 Post-transcriptional inhibition of IFN induction by NS1 (nuclear level)

NS1 also acts as an IFN antagonist in an indirect post-transcriptional mechanism. NS1 inhibits the 3'-end processing of host mRNAs by binding to the F2F3 domain of CPSF-30 [154, 155], PABPNI [156] and U6 snRNA [157], therefore downregulating host mRNA splicing and maturation, including type I IFN mRNA. The C-terminal residues of NS1 (223-227) are required for binding to PABPNI [156] whereas NS1 binds to CPSF-30 through the residues 184-188, 103, 106 and 144 [155, 158, 159]. The CPSF binding site in NS1 protein has been recently determined to involve interactions with residues spanning a.a. 89-180 [160]. By binding the two nuclear proteins CPSF-30 and PABPNI, NS1 prevents the formation of mature host mRNAs by blocking their splicing and poly-adenylation [154, 155]. Research using recombinant viruses has shown the involvement of the polymerase subunits PA and NP in stabilizing the NS1-CPSF-30 interaction [161]. Poly-adenylation inhibition is limited to host mRNA since the viral mRNAs processing is mediated through the viral polymerases [2]. NS1 also inhibits pre-mRNA splicing by binding U6 small nuclear RNA (snRNA) and therefore limiting pre-mRNA splicing and poly-adenylation [157].

In addition to possessing multiple strategies to prevent the maturation of the poly-adenylated cellular mRNAs NS1 also sequesters them in the nucleus. NS1 can directly bind poly-adenylated mRNA through its 113 a.a. N-terminus [162, 163] and consequently the fully processed host mRNAs are retained in the nucleus [156]. Additional proteins involved in the nuclear export of host mRNA (e.g. NXF1/TAP, p15, Rae1, E1B-AP5 and Nup98) have also been identified as NS1 binding partners, suggesting another method for sequestering

host mRNA in the nucleus that involves inhibition of cellular transport [164]. Therefore, by inhibiting both host mRNA maturation and nuclear export, NS1 severely downregulates the abundance of IFN- β and ISRE containing mRNAs, thereby inhibiting the production of the mature IFN- β and IFN inducible genes at the post-transcriptional level. At the same time, the partially formed mRNAs retained in the nucleus also promote viral replication by providing the host-derived 5'-cap necessary to prime viral transcription [154, 156].

1.8.3 NS1 limits IFN induced signaling

NS1 also blocks IFN induced STAT-1 signaling to prevent IFN stimulated gene transcription [165]. NS1 interferes with two essential IFN signaling effectors, 2'-5' OAS and PKR and therefore alters two fundamental host defense mechanisms implicated in limiting viral transcription and translation respectively [166]. Following viral infection, NS1 inhibits PKR activation by preventing the formation of dsRNA- PKR complex in two different manners: direct binding of NS1 (through the 123-127 residues) and PKR [127, 167] or through the ability of NS1 to bind dsRNA through its RBD [168]. Both activities inhibit the latent PKR from undergoing dimerization and phosphorylation, which in turn limits the phosphorylation of eukaryotic initiation factor eIF2 α thereby allowing cellular and viral protein synthesis to ensue [169]. PKR activation might also be inhibited through a possible interaction between NS1 and p85 β , which in turn activates p85 β , a PKR inhibitor [170]. NS1 can also block the activity of 2'-5' OAS by preventing its activation by viral dsRNA and therefore disrupting the OAS/RNase L pathway. Such activity prevents viral RNA degradation by OAS and thus promotes viral replication [166]. In addition, it has been shown that inhibition of viral RNA degradation also reduced RIG-I activation and therefore

limits IFN induction [171].

Alternatively, NS1 can also inhibit IFN signaling by inhibiting STAT1 and STAT2 tyrosine phosphorylation as well as STAT2 translocation into the nucleus and the formation of the ISGF-3 complex. In addition, the same study showed also a decrease in IFNAR1 and IFNAR2 expression on infected cells *in vitro*. The process is mediated by the upregulation of the JAK1 and TYK2 inhibitors: suppressor of cytokine signaling SOCS1 and SOCS3 [165].

The currently described counteractive measures mediated by NS1 have allowed IAV to escape host innate immune responses and enhance viral replication in multiple hosts [88]. In addition, it has been reported that NS1 also limits the adaptive immune response by inhibiting antigen presentation and dendritic cell (DC) maturation [172]. However, infections with highly pathogenic viruses such as HPAI H5N1 and the Spanish flu 1918 strains resulted in acute respiratory distress caused by an aberrant innate immune response [173, 174]. Although the HPAI H5N1 NS1 mediated an important inhibitory effect on IFN and IFN inducible gene production [175], the large extent of tissue damage has been linked to the high levels of cytokines and chemokines produced as well as the extensive macrophage and neutrophil infiltration in infected lungs [173, 175, 176]. The role of this combination of an effective immunosuppressive NS1 with a robust and sustained immune reaction is not well understood in highly pathogenic influenza virus infections [111].

1.9 The role of NS1 in viral replication

Although reports have shown that NS1 is an accessory protein in virus infection [149], it is thought to play an important role in enhancing viral replication at different levels. NS1 can limit early apoptosis and therefore allow viral replication to ensue [88]. It is thought

that NS1 uses the host translation machinery to selectively enhance viral protein translation and therefore replication [177]. Alternatively, NS1 also mediates viral polymerase activity and therefore enhances viral gene transcription. NS1 can also undergo post-translational modifications to inhibit its degradation and therefore increase viral replication [148].

NS1 can suppress early-induced apoptosis through its direct binding (through residues 89-93, 164-167, and 141-142) to the p85 β regulatory subunit of the heterodimeric kinase PI3K [178]. This interaction leads to PI3K activation and the subsequent phosphorylation of its downstream factor, Akt [178]. Alternatively, avian NS1 can also mediate PI3K activation by binding Crk/CrkL signaling proteins [179]. This process delays apoptosis and prolongs cell growth and proliferation as well as IAV viral entry, infection and replication [180, 181]. The direct binding of NS1 to PKR as well as the inhibition of OAS/RNaseL pathway can also lead to suppression of cell death [166, 182].

NS1 binds two translation initiation proteins, eIF4GI (through the residues 81-113) [183] and PABPI through its N-terminal residues [180]. In addition, NS1 is also capable of binding the 5'untranslated region (UTR) of the viral mRNA [184] and the viral polymerase complex [92, 185]. It is therefore suggested that NS1 selectively enhances viral mRNA translation by recruiting the host translation initiation machinery to form a heterotrimeric complex NS1-eIF4GI-PABPI at the 5'UTR of viral mRNAs [180]. hStaufen, a dsRNA binding protein involved in microtubular and host mRNA transport and polysome formation, is another binding partner of NS1. It has been hypothesized that NS1 interacts with hStaufen to also enhance viral protein translation through directing viral mRNA into polysomes [186, 187]. Therefore, NS1 can increase viral protein production in transfected cells [177] and

simultaneously inhibit the host innate immune response by sequestering the cellular translational machinery away from host mRNA transcripts.

Recently, studies have shown that NS1 can also influence viral replication by affecting viral gene transcription. A recent report showed that NS1 genes harboring gain-of-function mutations selected upon mouse adaptation improved polymerase activity *in vitro* which also correlated with an increase in viral replication *in vitro* and in mouse lungs [160]. In addition, the report by Shapira et al. (2009) demonstrated that NS1 interacts with each of the three viral polymerases, however the nature of this interaction needs to be further elucidated [92]. Another recently reported interaction between NS1 and the RNA helicase A (RHA) also affected viral replication. The knockdown of RHA *in vitro* altered viral polymerase activity, RNA replication and transcription and therefore showed a significant impact on viral replication [188]. This study proved that NS1 and the viral polymerase interaction might involve additional host factors such as RHA.

Recent *in vitro* co-immunoprecipitation and co-localization studies showed that NS1 interacts with the poly ADP-ribose (PARP)-10 protein, a member of the PARP family, in the nucleus. In the absence of PARP-10, cell cycle was arrested at the G2-M phase and viral replication was greatly reduced [189].

Although some of the functions attributed to NS1 can vary among strains, these mechanisms (and presumably their modulation) allow NS1 to escape the innate immune response and increase viral replication and growth [88, 111].

1.10 Roles of influenza viral proteins in virulence

It has been observed that virulence is polygenic and requires multiple mutant genes [190]. It is also stated that pathogenicity and virulence is controlled by the combination or “constellation” of particular influenza viral genes in a specific host [191]. Mutagenesis and reverse genetics studies have determined some key mutations in different influenza viral genes that contribute to high pathogenicity and host adaptation through improving multiple aspects of the viral replication cycle [191].

The HA protein is considered to be a critical determinant of virulence. As mentioned above, the single or multiple basic receptor sites contributed to low and high pathogenic (H5 & H7) viruses in poultry respectively [192]. Adaptive changes in the HA receptor site are also considered pivotal for a virus to bind the new hosts’ sialic acid receptors and transmit efficiently within the new species [191]. Mutations in the avian H1 (E190D and G225D) and the avian H2 and H3 (Q226L and G228S) have been selected in all three pandemic viruses 1918, 1957 and 1968 [193]. By gaining these mutations, avian H1, H2 and H3 acquired the ability to bind the human α 2,6- linked sialic acid moieties and transmit efficiently between humans [194, 195]. Other key mutations selected upon mouse adaptation in H3 HA1 and HA2 subunits adjacent to the receptor site, G218W and T156N respectively, have been shown to affect both growth and virulence in the mouse [196].

Several studies have identified host signatures in the polymerase subunit PB2 that contribute to the adaptation of avian viruses to mammalian species. One notable host-range determining residue, the PB2 627, is a glutamic acid (E) in most avian viruses and a lysine (K) in all human viruses. It has been proven that a single E627K mutation is required

for an avian virus to adapt to humans by enhancing viral growth and possibly vRNA replication in the upper respiratory tract, which exhibits lower temperatures [23, 197]. This mutation E627K is also associated with increased viral transmission possibly due to the increased growth in the upper respiratory tract [34]. Little is known about the mechanism by which the PB2 627K affects growth at lower temperatures. It has been suggested that possible interactions with NP are disrupted in the presence of 627E [198] and/or the presence of a host inhibitor (yet to be identified) in human cells that impairs viral replication of avian viruses [199, 200].

Several studies suggested an important role for the PB2 D701N mutation that was also selected in several H5N1 viruses containing a PB2 627E mutation [4, 34]. The PB2 D701N mutation increases viral replication [33] and the nuclear localization of the PB2 subunit in human cells possibly through an enhanced interaction with importin α [201]. The 701N mutation also compensates for the reduced transmissibility induced by 627E in mammalian hosts [34]. These two PB2 mutations seem to be strong determinants for a virus to adapt to new species and have been widely selected after serial passage in mice [33, 202-204].

However, the 2009 H1N1 virus that exhibited high replicative efficiency in human tissues and high transmissibility between humans did not possess either of these two key PB2 mutations described above, but instead possessed two basic residues 590S and 591R that have been shown to play similar roles to that seen for the 627K mutation in humans [23]. It is suggested that the double mutations re-created a similar basic patch in the PB2 subunit, which seems to be required for efficient human infectivity and transmission [205].

The newly discovered viral protein PB1-F2 is also implicated in virulence by causing apoptosis in immune cells and therefore delaying viral clearance and enhancing secondary bacterial infections [27, 40, 206, 207]. It has been shown that this protein contributes to the pathogenicity of 1918, 1957 and 1968 pandemic viruses as well as H5N1 HPAI virus [40, 208, 209]. However, the addition of the full length PB1-F2 protein to the H1N1 2009 pandemic virus (which does not express the PB1-F2 protein) did not increase virulence [210]. In addition, a recent mutation identified in the PB1-F2 protein N66S contributed to increased viral replication and delayed innate immune response in the mouse lungs [41].

With respect to the role of the NS1 protein in virulence, previous studies of pathogenic avian A/HK/156/97-like H5N1 showed that the NS1 gene mediated increased virulence in the mouse model [211] and swine models [175]. Furthermore other studies of virulence identified single mutations in the NS1 gene (P42S, D92E and V149A) as well as multiple mutations in the PDZ ligand domain that increased viral pathogenicity [175, 212-214]. The single mutation P42S in H5N1 NS isolated from ducks increased virulence in mice and reduced host IFN induction by preventing its dsRNA-mediated transcription activation [212]. The D92E mutation increased viral growth in the presence of IFN *in vitro* and increased pathogenicity in pigs [175]. The NS1 mutation V149A also occurring in H5N1 viruses isolated from geese was associated with increased viral replication, virulence in chickens and reduced IFN protein levels *in vitro* [213]. A naturally occurring 5 a.a. deletion of NS1 residues 191-195 of H5N1 swine viruses contributed to reduced pathogenicity in mice and chicken due to reduced NS1-CPSF binding and IFN antagonism [215]. The NS1 PDZ binding domain (consisting of four amino acids at the C terminus) termed the PDZ ligand

(PL) also represents a virulence determinant not by decreasing IFN production, but by affecting binding to proteins with PDZ domains [214]. Studies have shown that the NS1 PL of human viruses show weak binding to human PDZ domains, while the NS1 PL of avian viruses show strong binding which may interfere in several signaling pathways [214].

In contrast, other studies have identified F103L and M106I mutations in the H5N1 NS1 gene that results in a loss in virulence, a loss of the ability to bind CPSF and a loss in the inhibition of IFN induction when transferred into A/Udorn/1/1972 (H3N2) virus [216]. When studied in the H5N1 genetic background, the mutations also decreased virulence [217].

1.11 Epistatic interactions in influenza A viral proteins

As described in the previous section, some specific IAV mutations have been described as gain-of-function mutations and have been associated with enhanced virulence in various hosts. However, several studies have shown that beneficial mutations in RNA viruses can sometimes have a negative or no effect if expressed on a different genetic background [218]. The process is termed epistasis where the effect of one gene is affected by another gene or a combination of genes that regulate its expression either negatively or positively. In literature, epistasis is widely studied in terms of interactions between deletrious mutations. Normally, the organism's fitness decreases linearly with every addition of a deletrious mutation [219]. However, epistasis can determine the direction shift of the linear correlation between the number of deletrious mutations and log fitness [219]. When the deletrious effect of multiple mutations is greater than the effect of the single mutations combined epistasis is synergistic or negative and therefore the organism fitness is greatly reduced [220]. Conversely, when the product of single deletrious

mutations is decreased, the robustness of these mutations on fitness is reduced and therefore epistasis is antagonistic or positive [221]. Epistatic interactions have been reported in animals, bacteria as well as DNA and RNA viruses including influenza A viruses [218]. Positive epistasis has been reported in the NA surface glycoproteins' evolution where the effect of H274Y NA residue that confers oseltamivir resistance but reduces viral fitness is alleviated by two NA mutations that are not in close proximity to H274Y residue in the folded NA structure [222]. Another example of positive epistasis has been observed in the NP protein. The NP R384G mutation leads to viral escape from cytotoxic T cell lymphocyte, however this activity is compromised by a reduced viral fitness. The presence of four additional NP mutations alleviated the detrimental effect of NP R384G on viral fitness [223]. These examples prove that epistatic interactions are common in influenza A viruses and therefore it might not be enough to determine the effect of single viral genes or point mutations on viral fitness. Consequently, it is imperative to consider the “added value” of the genomic context when studying viral evolution [224].

1.12 Evolution of influenza A virus

How influenza virus evolves to become capable of infecting new species is poorly understood. So far, adaptive evolution acquired by point mutations, reassortment and recombination have been implicated in the emergence of new viral strains [225]. The rapid adaptive evolution of influenza viruses has been shown to be driven by selective pressures such as drug treatment, global vaccination programs and intensive closed systems in animal farming [226]. As a result viruses acquire new pathogenic phenotypes that are often associated with adaptation to new hosts or an increase in virulence and pathogenicity.

In general, RNA viruses are known for their extremely high mutation rates of 10^{-4} misincorporations per nucleotide that are estimated to result in one mutation per genome per replication [226, 227]. Influenza polymerases lack proofreading capability; therefore errors are not repaired during viral replication, which allows mutations to persist in the viral genome. Many of these mutations are phenotypically weak or deleterious and thus not selected upon natural selection but due to genetic drift, however numerous are stable and are incorporated in the genome when selected under conditions of selective pressure [226, 228]. This form of adaptive evolution is frequently observed in the surface HA and NA genes, termed as antigenic drift, resulting in new variants that are no longer neutralized by the pre-existing host antibodies. The high frequency of antigenic drift in seasonal IAVs elucidates the imperative annual surveillance for the predominant influenza strains circulating and subsequently the need to develop the closest vaccine strain on a frequent basis [229, 230]. It is possible that the 1918 pandemic virus of avian origin had jumped to humans through direct transmission. This event would only be possible if the virus had rapidly acquired point mutations through an antigenic drift process [231]. A counter hypothesis suggested a transmission through an intermediate host (swine), which facilitated the challenge of adaptation to a new host [232, 233].

In addition, influenza viruses possess a segmented genome, which facilitates the exchange of viral gene segments upon co-infection of two or more viruses in the same host cell. The process results in the generation of progeny viruses with novel gene combinations, and is termed reassortment or antigenic shift when it involves the surface proteins [228]. Reassortments result in the emergence of new viral strains that often cause serious

infections mainly because the novel host is immunologically naïve to these newly emerged viruses [233]. At least two pandemics (1957 and 1968) in the 20th century and the recent 2009 pandemic strain are resultants of viral gene reassortments of two or more viruses (detailed in section 1.2).

The currently circulating H5N1 lineages are the result of both reassortment and antigenic drift [225]. The first HPAI H5N1 strains circulating in humans in 1997 are the descendents of H5N1 strains isolated from geese in China (A/goose/Guangdong/1/96) [234]. The virus has evolved through multiple reassortment events and had acquired internal genes from a quail H9N2 virus and NA gene from a teal H6N1 virus [235]. In addition, H5N1 viruses further accumulated multiple mutations in the HA gene that have given rise to extensive antigenic drift to form multiple clades [236]. Currently, the H5N1 sub-lineages varying in their HA sequences are classified into ten clades and have spread geographically throughout China, Vietnam, Indonesia, Africa, Europe and the Middle East.

Although rare, recombination also contributes to viral evolution through the production of a genome with deleted, inserted or mosaic genome segments [225]. These processes have only been witnessed a few times in the history of IAV evolution when a LPAI H7N3 virus acquired a portion of NP into their HA cleavage site through non-homologous recombination resulting in a HPAI virus that caused an outbreak in chickens [237]. In addition, H9N2 viruses circulating in China possessed hybrid PA segments that resulted from homologous recombination between H9N2 and H5N1 viruses [238].

All of these evolutionary mechanisms allow the generation of new viral genome sequences which provide the genetic variants that allow for the selection of the strongest,

most adaptive mutations when selected under novel host or environmental conditions [226].

1.13 Background to this project

It is commonly known that viruses can acquire virulence traits through adaptation. Therefore experimental evolution has been widely used to understand the genetic basis for virus adaptation in a new host. The mouse has been the most studied animal model in influenza pathogenesis. In addition to its low cost and easy husbandry, the mouse exhibits similar lung pathology due to viral pneumonia caused by IAV compared to humans. The immune cells' infiltrations as well as the cytokine prevalence in the bronchoalveolar regions are also observed in the mouse model following IAV infection [239].

Previously in our laboratory, mouse adaptation has been used to identify the evolutionary steps required by a human strain to adapt to a new species. Our studies involved mouse adaptation of the prototype A/FM/1/47 (H1N1) (FM) a descendent of the 1918 H1N1 virus. The parental strain is non-virulent in the mouse with a median lethal dose (LD_{50}) = $10^{6.5}$ pfu. After 12 passages, the mouse adapted variant FM-MA became virulent and the LD_{50} was $10^{2.0}$ pfu [240]. Genomic sequencing analysis identified mutations in the PB2, PB1, HA, NA and M genes that are responsible for the increased FM-MA virulence in the mouse [241]. Genetic analysis employing reassortants proved that each of these mutations enhanced virus pathogenicity and replicative fitness in the mouse [241]. However the FM strain was previously passaged in the mouse following its isolation in 1947 and may not have been the most ideal genetic background for further analysis [202]. Therefore another prototype virus, A/Hong Kong/1/68 (H3N2) (A/HK/1/68, HK or HK-wt), a human virus that

doesn't have any history of mouse adaptation with a LD₅₀ of $>10^{7.7}$ pfu in mice was used to generate virulent mouse-adapted variants. After twenty lung-to-lung passages, mouse-adapted viruses (MA20's) were selected that had increased in virulence where the LD₅₀ of the passage population of viruses decreased by greater than 5 logs to $10^{2.7}$ pfu [202]. Additional mouse-adaptation experiments were also performed where ten sister clones of HK-wt that were derived from stock HK virus by plaque purification were independently passaged 21 times in mouse lungs. The LD₅₀ of each of the ten selected mouse-adapted variants (MA21) was decreased compared to the HK parental strain with a minimum of a LD₅₀= $10^{1.1}$ pfu for one of the variants MA63 [160].

Genomic sequence analysis of MA20 and MA21 virus genomes respectively revealed mutations in different viral segments within each variant [203] suggesting again that virulence in IAV is multigenic. Further research assessed the roles of the MA mutations identified in the HA and PB2 segments and demonstrated their involvement in virus adaptation through increasing replicative fitness and virulence in the new host [33, 196].

1.14 NS1 variants

Sequence analysis of the NS genes of 42 mouse-adapted clones was performed and the results are summarized in Table 1. In total, eleven different mutations were observed in the NS1 gene and four overlapping mutations in the NEP gene (Table 1). When mapped, the adaptive mutations occur in major transcriptional and translational controlling sites (Figure 7). Interestingly, some of these mutations were convergent; in that they have been observed independently in different populations. Some mutations are parallel where identical mutations are selected (i. e. D125G was observed in MA 102 and MA 111) (Table

1) or directional involving the same position but mutated to different a.a. (i.e. M106 was mutated to a Valine in MA 41, MA 42, and MA 43 and to an Isoleucine in MA 51, 52, 53) (Table 1). Sequence comparisons of the MA NS1 genes with the highly pathogenic influenza virus A/Hong Kong/156/97 (H5N1) identified two groups of variants MA20B, C and D [202, 242] as well as MA51, 52 and 53 [196, 242] (Table 1) that possessed the F103L and M106I mutations respectively and thus were convergent with highly pathogenic H5N1 IAV isolated from humans in Hong Kong in 1997 (A/Hong Kong/156/97-like viruses) [243]. The NS1 mutations V226I and R227K selected in the two variants MA103 and MA112 respectively have also been selected in A/Brevig Mission/1/1918 (H1N1) Spanish flu [244, 245]. This indicated that these mutations were under positive selection in mouse adapted as well as in human and avian adapted viruses and were therefore functionally important such that they may be involved in increasing replicative fitness and virulence of the mouse adapted variants. Although the F103L and M106I mutations have been associated with increased IFN- β induction in reassortants of the A/Hong Kong/156/97 (H5N1) NS1 gene on the A/Udorn/1/1972 (H3N2) backbone [216] the IFN- β induction by the parental HK-wt, the MA20c (F103L) and MA51 (M106I) mutant viruses in mouse M1 cells were measured. Using an MOI of 2, the MA20c and MA51 viruses induced low levels of IFN- β after 24 h of incubation that were not significantly different from HK-wt (12 $\pm$ 8 pg/ml) (Unpublished data).

Table 1 NS1 mutations in MA variant clones of A/HK/1/68 (HK-wt) H3N2

VIRUS		NS1										NEP			
		2	23	98	103	106	124	125	180	226	227	2	23	70	108
HK-wt		D	V	L	F	M	M	D	V	V	R	D	S	G	E
MA20	HKMA20 (MA20)	-	A	-	-	-	-	-	-	-	-	-	-	-	-
	HKMA20a (MA20A)	-	A	-	-	-	-	-	-	-	-	-	-	-	-
	HKMA20b (MA20B)	-	-	-	L	-	-	-	-	-	-	-	-	-	-
	HKMA20c (MA20C)	-	-	-	L	-	-	-	-	-	-	-	-	-	-
	HKMA20d (MA20D)	-	-	-	L	-	-	-	-	-	-	-	-	-	-
MA21	HK4MA21-1 (MA41)	-	-	-	-	V	-	-	-	-	-	-	-	-	-
	HK4MA21-2 (MA42)	-	-	-	-	V	-	-	-	-	-	-	-	-	-
	HK4MA21-3 (MA43)	-	-	-	-	V	I	-	-	-	-	-	-	-	-
	HK5MA21-1 (MA51)	-	-	-	-	I	-	-	-	-	-	-	-	-	-
	HK5MA21-2 (MA52)	-	-	S	-	I	-	-	-	-	-	-	-	-	-
	HK5MA21-3 (MA53)	-	-	S	-	I	-	-	-	-	-	-	-	-	-
	HK6MA21-2 (MA62)	-	-	-	-	-	-	-	A	-	-	-	P	-	-
	HK6MA21-3 (MA63)	-	-	-	-	-	-	-	A	-	-	-	P	-	-
	HK9MA21-3 (MA93)	N	-	-	-	-	-	-	-	-	-	N	-	-	K
	HK10MA21-2 (MA102)	-	-	-	-	-	-	G	-	-	-	-	-	-	-
	HK10MA21-3 (MA103)	-	-	-	-	-	-	-	-	I	-	-	-	-	-
	HK11MA21-1 (MA111)	-	-	-	-	-	-	G	-	-	-	-	-	-	-
	HK11MA21-2 (MA112)	-	-	-	-	-	-	-	-	-	K	-	-	S	-

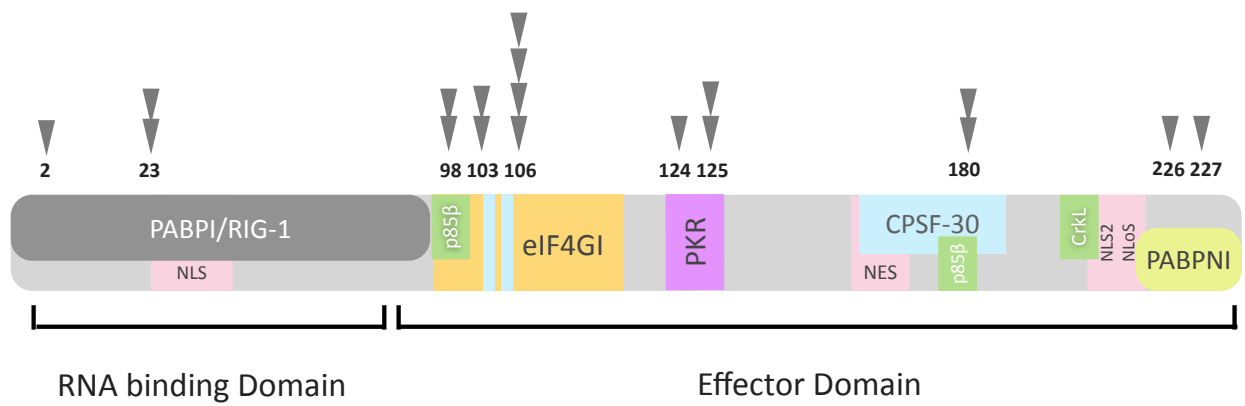


Figure 7 Non-structural protein-1 mouse adaptive mutations localize to binding sites for cellular transcriptional and translational factors. NS1 mutations are indicated in grey arrows on a structural map for NS1 protein and its binding partners

Hypothesis

Mutations in the NS1 multifunctional protein are expected to contribute to host adaptation and therefore to increased virulence and pathogenicity. These viruses may increase virulence through changes in their properties leading to enhanced replication *in vitro* and *in vivo*, viral protein synthesis and decreased IFN- β sensitivity.

Objectives

- 1) In order to determine the contribution of each of the mouse adapted NS1 mutations to virulence, synthetic viruses will be generated that differ due to these individual mutations. These viruses will possess different A/HK/1/68 MA20 or MA21 NS1 genes on A/Puerto Rico/8/34 (H1N1) backbone. These viruses will have the same structural proteins of A/Puerto Rico/8/34 origin and only after infection will express the different NS1 proteins possessing different adaptive mutations.
- 2) Examine the effect of each of the NS1 mutations on viral growth, disease severity (virulence) and the ability to inhibit IFN- β transcription.
- 3) Focus on the F103L+M106I and V226I+R227K mutations observed in the highly pathogenic influenza virus A/Hong Kong/156/97 (H5N1) and the A/Brevig Mission/1/1918 (H1N1) Spanish flu respectively. Determine their contribution to viral growth in mouse lungs, IFN- β production *in vitro* and *in vivo* as well as viral protein synthesis.
- 4) Evaluate the effect of the same mutations F103L+M106I and V226I+R227K in

their respective NS1 genes A/Hong Kong/156/97 (H5N1) and A/Brevig Mission/1/1918 (H1N1) Spanish flu respectively using different genetic backbones.

2. Materials and Methods

2.1 Cells

Mardin-Darby canine kidney (MDCK) cells, human embryonic kidney cells (293T), mouse kidney epithelial cells (M1), mouse embryonic fibroblasts (MEF), human lung epithelial cells (A549) were maintained in minimum essential medium (MEM) or Dulbecco's Modified Eagle Medium (Gibco, Carlsbad, USA) supplemented with L-glutamine (2 mM), penicillin (100U/ml), streptomycin (100µg/ml) and fetal bovine serum (10% v/v). All cultured cells were stored in a 37°C incubator with 4% CO₂.

2.2 Cell Storage

Confluent cells grown in a T75 flask were trypsinized using 0.5% trypsin-EDTA (Gibco, Carlsbad, USA) and suspended in 10 ml MEM. The cells were spun at 1500 rpm for 5 min (IEC DPR-6000 centrifuge) and re-suspended in freezing medium containing: MEM with 40% FBS and 10% Dimethyl sulfoxide (DMSO). The cells were aliquoted and stored at -80°C overnight before being transferred to liquid nitrogen.

2.3 Viruses

The prototype A/HK/1/68 (H3N2) (obtained from the Laboratory Center for Disease Control, Health Canada) having an LD₅₀ of $>10^{7.7}$ pfu/ml in mice was used to generate virulent mouse-adapted strains as described and performed previously [196, 202, 242]. Briefly, the virus was plaque-purified twice in MDCK cells. The purified virus was grown in 10 day old specific pathogen free (SPF) embryonated chicken eggs (Canadian Food Inspection Agency, Ottawa, Ontario). The virus was then passaged in CD-1 mice (Charles

River Laboratories, Quebec, Canada). Initially, groups of 3 CD-1 mice were infected intranasally with 50 μ l of 10^6 pfu under halothane anesthesia. Three days following infection, mice lungs were collected, pooled, diluted in 3 ml of phosphate buffer saline (PBS) (1ml/lung) and homogenized by sonication. Lung homogenates were then diluted (1:10) and 50 μ l were used for subsequent infections. Twenty lung-to-lung passages were performed (MA20) and six clones were purified following twenty passages. The isolated clones are referred to as follows: HKMA (mouse adapted passaging # (20)- clone # (A-E)).

Another independent mouse-adaptation experiment was also performed, that employed ten sister clones that were first derived from the same stock A/HK/1/68 virus by plaque purification. The NS1 genes of all ten sister clone stocks were identical in sequence to the original HK wild type (HK-wt) stock. Stocks of each of the ten clones were grown in embryonated eggs and then independently subjected to 21 lung-to-lung passages in CD-1 mice (MA 21). Mouse passaging was performed under the following conditions: For the first 10 passages, one mouse/group was infected for 1 day. For the next 5 passages, 2 mice/group were infected for 2 days while for the last 6 passages, 3 mice/group were infected for 3 days. At the end of the experiment, three clones were isolated from each of the ten passages. The isolated clones are referred to as follows: HK (sister clone #) MA (mouse adapted passaging # (21))- clone # (1-3)). All the passaging experiments were performed previously by others [202, 203].

2.4 Viral RNA extraction

Viral RNA was extracted from 140 μ l of virus grown in allantoic fluid stock (as described in section 2.13) using QIAamp Viral RNA Mini Kit (Qiagen, Mississauga, Ontario)

according to the manufacturer's instructions.

2.5 Reverse transcription and polymerase chain reaction

The RNA stored in AVE buffer was reverse transcribed using 1 µl of primer RTA (0.1 µg/µl), 1 µl of primer RTG (0.1 µg/µl), 8 µl of 5X first strand buffer, 4 µl of 0.1M Dithiothreitol (DTT), 1.6 µl of 25mM dNTP (Fermentas, Burlington, ON. Canada), 1 µl of RNase inhibitor (40 units/µl) (Invitrogen, Burlington, Ontario) and 2 µl of Moloney Murine Leukemia Virus Reverse Transcriptase (200 units/µl) (Invitrogen, Burlington, Ontario) for a total volume of 40µl. The components were mixed together and the reverse transcription (RT) reaction was performed using a thermal cycler (Progene Techne) under the following conditions: 21°C for 10 min, 33°C for 10 min, 37°C for 10 min and 42°C for 1 hr. The complementary DNA (cDNA) was stored at -20°C.

The NS1 gene was then amplified by polymerase chain reaction (PCR). The reaction mixture contained 3 µl of cDNA, 1µl of forward primer Kc19 (0.1 µg/µl), 1 µl of reverse Kc11 (0.1 µg/µl), 1 µl of 10 mM dNTP (Fermentas, Burlington, ON. Canada), 5 µl of 10X PCR buffer, 10 µl of 25 mM MgCl₂, and 1 µl of Taq DNA Polymerase (5 units/µl) (Fermentas, Burlington, ON, Canada) for a total volume of 50 µl. The samples were mixed and the reaction was performed using a thermal cycler (Progene Techne) under the following conditions: 1 cycle (94°C for 4 min), 30 cycles (94°C for 1 min, 52°C for 30 sec, 72°C for 2 min 30sec) and 1 cycle (72°C for 10 min). The samples were analyzed by electrophoresis on an agarose gel and stored at -20°C.

2.6 PCR purification

The PCR products were purified using the QIAquick PCR Purification Kit (Qiagen, Mississauga, Ontario) according to the manufacturer's instructions. The purified PCR products were then sequenced at the Stem Core Facility (OHRI, Ottawa, ON) to assure that the product was free of unwanted mutations and was then used for ligation-independent cloning into the pLLB(A) vector as described below.

2.7 Restriction digestion

Single restriction digestion was used to linearize the pHH21 and pLLB vectors using Stu1 enzyme. The plasmids (1 µg) were mixed with sterilized water to reach a total volume of 17 µl. Then 2 µl of the appropriate buffer and 1 unit of the Stu1 restriction enzymes (New England Biolabs, Pickering, ON) were added to a total reaction mixture of 20 µl. The samples were mixed and incubated at the recommended temperature for 1 hr. Then the samples were analyzed on an agarose gel. If digestion was incomplete, the samples were re-incubated for a longer period of time.

2.8 Cloning of the viral NS genes

2.8.1 Ligation-independent cloning

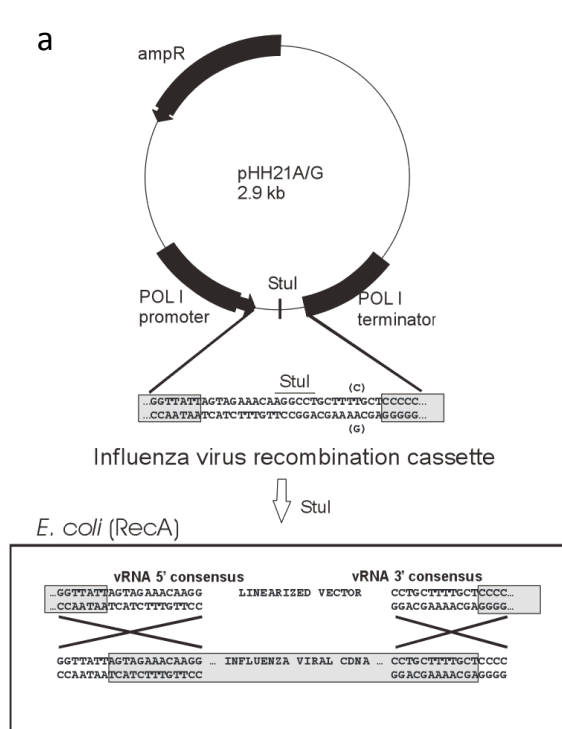
Two vectors, the unidirectional pHH21 plasmid and bidirectional pLLB plasmids constructed in collaboration with Qinfang Liu and Jinhua Liu at the China Agricultural University were employed in our studies [246, 247]. Briefly, influenza viral cDNA was inserted between the human RNA POL I promoter and mouse RNA POL I terminator separated by a Stul restriction site (Figure 8a,b). All influenza viral genes have a conserved

sequence of thirteen nucleotides at their 5' end and twelve nucleotides at their 3' end with only one nucleotide difference of A/G at position four at the beginning of the viral gene sequence. The recombination cassette employed in the cloning is comprised of both the plus and minus strand of the influenza virus genome with either an A or G in the plus sense strand separated by the *StuI* restriction site [246, 247]. The pHH21A and pLLBA are complementary to the following influenza gene segments: HA, NP, NA, M and NS while the pHH21G and pLLBG are complementary to PB2, PB1 and PA.

For the pLLB plasmids, the recombination cassette is also nested between an opposing RNA POL II promoter and a poly A sequence (Figure 8b), therefore the pLLB plasmid can express both the viral RNA and the mRNA of each influenza virus gene segment [247]. By restriction digestion with *StuI* enzyme, the plasmid is linearized and the viral cDNAs are cloned into the recombination cassette by ligation independent homologous recombination.

The cloning was performed using 500 ng of the NS PCR product mixed with 100 ng of the plasmid (pLLB or pHH21) linearized by *StuI* (described above in section 2.7). Further, the mutated NS pLLB vector was treated with T4 DNA polymerase (New England BioLabs, Pickering, ON.) before transformation. T4 DNA polymerase has a proofreading activity to create single stranded ends in the insert and the vector. This step allows easier assembly between the insert and the vector and increases the efficiency of recombination [248]. The mixture is further incubated with 50 μ l of DH5 α Library Efficiency Cells (Invitrogen, Burlington, Ontario) and transformation was performed as described below in section 2.10.

The NS gene of A/Chicken/Beijing/1/95 (H9N2) (A/Ck/Bj/1/95) (obtained from China Agricultural University) was cloned into the pHH21A while the NS genes of A/HK/1/68 (H3N2), A/Hong Kong/156/97 (H5N1) (A/HK/156/97) (Genbank AF036360) (synthesized by Biomatic Corporation, Cambridge, Ontario) and A/Brevig Mission/1/1918 (H1N1) (synthesized by Biomatic Corporation, Cambridge, Ontario) were cloned into pLLBA plasmids by homologous recombination [246]. The A/Puerto Rico/8/34 (A/PR/8/34) NS gene was previously cloned into pHW2000 bidirectional plasmid (obtained from R. Webby, St Jude's Hospital, Memphis).



in situ recombination between linear vector and viral cDNA in *E. coli*

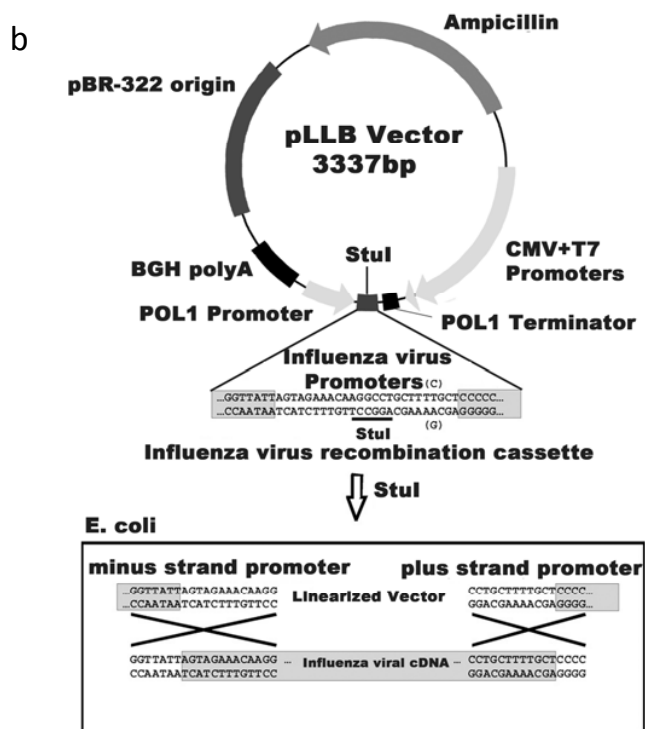


Figure 8 **Ligation-independent cloning strategies for influenza viral genes in pLLB (A and G) and pHH21 (A and G) recombination plasmids.** **a.** The pHH21 (A&G) plasmids contain a recombination cassette comprised of the influenza virus promoters joined naturally by a *stu1* restriction site. Once linearized by *stu1*, viral cDNAs can be mixed with the vector and transformed into *E.coli*. **b.** The recombination cassette of the pLLB (A&G) plasmids is also nested between an opposing T7 promoter and a polyA sequence. The pLLB (A&G) plasmid can express both the viral RNA and the viral mRNA required for the generation of synthetic influenza viruses by reverse genetics [246, 247].

2.8.2 Site-directed mutagenesis

Site-directed mutagenesis was used to introduce point mutations into the A/HK/1/68 (H3N2), A/PR/8/34 (H1N1), A/Ck/Bj/1/95 (H9N2), A/HK/156/97 (H5N1) and A/Brevig Mission/1/1918 (H1N1) NS genes (previously inserted into either pLLB or pHH21 plasmids by ligation independent method as described above). Polymerase chain reaction was performed using 50 ng of the NS gene of interest mixed with 1µl of each of the self-complementary primers (0.1µg/µl) containing the mutated site of interest (Appendix 1), 1µl of 10mM dNTP (Fermentas, Burlington, ON.), 5 µl of 5x Phusion buffer containing 7.5 mM MgCl₂, 0.5 µl of Phusion DNA polymerase (5 units/µl) (Thermo Fisher Scientific, Ottawa, ON.) in a 25 µl reaction using a thermal cycler (Progene Techne) at the following conditions: 1 cycle (98°C for 30 sec), 30 cycles (98°C for 10 sec, 52°C for 30 sec, 72°C for 2 min 30sec) and 1 cycle (72°C for 8 min). To remove the input plasmid, the PCR product was treated with 1µl of the DPN1 enzyme (New England BioLabs, Pickering, ON.) for 1 hour at 37°C to digest the HK-wt DNA template. The mixture was then transformed as described below in section 2.10.

2.9 Gel extraction

Following restriction digestion, the samples were run on an agarose gel (0.8%) and the fragment of interest was excised using a hand held UV light (BLAK-RAY long wave ultraviolet lamp 21). Gel extraction was performed using QIAquick Gel Extraction kit (Qiagen, Mississauga, Ontario) according to the manufacturer's instructions.

2.10 Transformation

A 50µl aliquot of Library efficiency DH5α competent cells (Invitrogen, Burlington, Ontario) were mixed with 3µl of the proper plasmid (>0.1 µg) and incubated for 30 min on ice. The mixture was then heat shocked for 45 sec at 42°C and chilled for 2 min on ice. The mixture was diluted with pre-warmed 500 µl of S.O.C medium (Invitrogen, Burlington, Ontario) and incubated in a bacterial shaker at 225 rpm at 37°C for 1 hr. Samples were then centrifuged for 30 sec at 8000 rpm. The bacterial pellet was re-suspended with 100 µl of fresh S. O. C. medium and then plated on Luria Bertani (LB) agar plates containing ampicillin (100 µg/ml). The plates were incubated at 37°C overnight.

2.11 Plasmid purification

Colonies were picked from the agar plate, grown separately in 3.5 ml LB broth containing ampicillin (100 µg/ml) 225 rpm overnight at 37°C. Following incubation, 3 ml of the LB broth was used for plasmid purification (Miniprep). For long term storage, 0.5 ml of the culture were mixed with an equal volume of 30% glycerol and stored at -80°C. These stocks were used to grow large volumes of bacterial cultures for large scale DNA production (Midiprep).

2.11.1 Miniprep

Following incubation, miniprep was performed using the QIAprep Spin Miniprep Kit (Qiagen, Mississauga, Ontario) according to the manufacturer's protocol. The samples were analyzed on an agarose gel and stored at -20°C.

2.11.2 Midiprep

An inoculation loop of the frozen glycerol stock was added to 2 ml of LB media containing ampicillin (300 µg/ml). The samples were incubated in a bacterial shaker at 37°C for 4 to 5 hrs. Five hundred µl of the shaken culture was then transferred to 100 ml of fresh LB media containing ampicillin (300 µg/ml). The cells were spun down at 8000 rpm for 15 min at 4°C. Midi prep was performed using the Qiagen plasmid Midi Kit (Qiagen, Mississauga, Ontario) in accordance with the manufacture's instructions. The samples were analyzed on an agarose gel and stored at -20°C.

2.12 Reverse genetics (8-plasmid system)

The influenza NS1 viral genes that were cloned by ligation-independent cloning or modified by site-directed mutagenesis can now be used to rescue influenza viruses containing our mutation of interest, which will enable us to determine their contribution to virulence and pathogenicity. The remaining 7 gene segments of A/PR/8/34, A/WSN/33 and A/HK/1/68 obtained from R. Webby (St. Jude's Children's Research Hospital, Memphis), Y. Kawaoka (University of Madison Wisconsin) and E. G. Brown (University of Ottawa, Ontario) respectively were previously cloned into pHW2000, pHH21 and pLLB respectively.

Each of the NS plasmid constructs along with the 7 plasmids encoding the viral structural genes of A/PR/8/34 (0.65 µg/plasmid) was diluted in 75µl of Opti-MEM-1 (Invitrogen, Burlington, Ontario) (Solution 1). Separately, 10µl of lipofectamine 2000 (1 µg of plasmid: 2 µl of lipofectamine) (Invitrogen, Burlington, Ontario) were diluted into 75 µl of Opti-MEM-1 incubated for 5 min at room temperature (RT) (Solution 2). Both solutions

were then pooled together, mixed gently by pipetting up and down and incubated for 30 min at RT. The plasmids and the transfection reagent were added into a 6 well poly (D) lysine-coated plates (as described in section 2.15) of 80% confluent 293T cells (grown in DMEM and washed twice with warm 1X PBS). Sixteen hours post transfection the transfection mix was replaced by Opti-MEM-1 (Invitrogen, Burlington, Ontario) supplemented with TPCK trypsin (1µg/ml) (Thermo Fisher Scientific, Ottawa, ON.). Forty-eight hours post-transfection the cell supernatant was collected and inoculated in 10-day old embryonated specific pathogen free embryonated eggs (Canadian Food Inspection Agency, Ottawa) for virus propagation at 37°C for 72 hours. The NS gene sequence of the rescued viruses was verified by sequence analysis. The H9N2-NS plasmid was rescued previously in the laboratory onto the A/WSN/33 backbone using the 12 plasmid system as described [249].

2.13 Virus propagation in embryonated chicken eggs

Fertilized chicken eggs were incubated in a 37°C egg incubator with automatic rocking (Twice/hr cycle). The ten day-old chicken eggs were candled and only the eggs with the living chicken embryos were used for virus propagation. Following sterilization with 70% ETOH, the air sac of embryonated eggs was infected with 100 µl of the desired dilution before sealing with melted paraffin wax. The eggs were incubated at 37°C for 72 hours. The embryos were euthanized by incubating the eggs at -20°C for 45min. Following surface sterilization, the hemagglutinin-positive allantoic fluid was collected and spun at 1500 rpm for 15 min (IEC DPR-6000 centrifuge). The samples were aliquoted and stored at -80°C.

2.14 Hemagglutinin assay

The assay was performed using round bottom 96-well plates. One hundred μl /well of the collected allantoic fluid was added to the first row in duplicates. Fifty μl /well of PBS was added to the remaining wells. Two-fold serial dilutions were performed. Once the dilutions were performed 50 μl /well of 0.75% chicken RBCs (Canadian Food Inspection Agency, Ottawa, ON) were added to each well. The samples were mixed and allowed to settle at room temperature for at least 45 min. The HA titers were determined based on the titration end point (represents 1 HA unit).

2.15 Poly-D-Lysine coating

For culturing 293-T cells, 6 or 96 well plates were coated with Poly-D-lysine hydrobromide (Sigma-Aldrich, Oakville, ON) to allow for a better cell to solid surface attachment. The empty plates were incubated with 0.1 mg/ml of poly-D-lysine diluted in sterile distilled water (1ml/25cm² surface equivalent to one well of 6-well plates or 100 μl /well of 96 well plates). The plates were agitated at 300 rpm for an hour at room temperature and then washed with 1ml of distilled water. The plates were then dried for a couple of hours before cells and media are introduced.

2.16 Plaque Assay

Virus samples were serially diluted in PBS. Six well plates of confluent MDCK cell monolayers were washed twice with warm 1X PBS and then infected with 100 μl of the different virus dilutions in duplicate for each dilution. The plates were incubated at 37°C for 30min to allow virus adsorption. Following adsorption, the cells were overlaid with 0.65% agarose (Invitrogen, Burlington, Ontario), in complete MEM supplemented with TPCK

trypsin (Thermo Fisher Scientific, Ottawa) (1µg/ml). The plates were incubated at 37°C, 4% CO₂ for 3 days and then fixed with 3 ml of Carnoy's fixative (three parts acetic acid to one part methanol v/v) for 45 min. The overlay was then washed away and the plates were stained using 2ml/well of 1% Crystal Violet for 45min. The plates were then washed, dried and plaques were counted (expressed as plaque forming units (PFU)). The stained plates were photographed and the diameter of at least ten plaques/virus was measured (mm).

2.17 Growth of the recombinant viruses *in vitro*

Monolayers of MDCK and A549 cells were washed twice with 1x PBS and infected with each of the rPR8 viruses at an MOI of 0.001. Viral infection was performed as follows: A volume of 100 µl of the required virus dilution was used to infect the pre-washed cells in triplicate. The infected plates were incubated at 37°C for 30min to allow virus adsorption and then serum-free MEM was applied with trypsin (1µg/ml) (Thermo Fisher Scientific, Ottawa). The cell supernatant was collected at 12, 24, 48 and 72 hours post infection (hpi) and viral titer was assessed by plaque assay in duplicate for each sample.

2.18 Growth of the recombinant viruses *in vivo*

Groups of eleven BALB/c mice (4-to-6-week-old females from Charles River Laboratories, Montreal, Quebec, Canada) or twelve CD-1 mice (19 to 21 gram-females from Charles River Laboratories, Montreal, Quebec, Canada) were infected intranasally under halothane anesthesia with 5×10^3 pfu of each of the viruses in a volume of 50µl [202]. BALB/c mice were sacrificed at days 1, 2, 3, 5 and 7 post infection. Lungs from two mice were collected for each of the days except for day 2 where three mice were sacrificed. CD-1 mice were sacrificed at 1, 3, 5 and 7 days post infection (dpi) and lungs from three mice

were collected for each day. Organs were weighed, diluted 1:4 in PBS and homogenized by CryoMill (Mixer Mill MM 200 Retsch) (for quantification by plaque assay). The same protocol was applied for the rWSN viruses. However, two mice were collected at each of 1, 3, 5 and 7 dpi.

2.19 Mouse survival assay

Groups of 5 BALB/c or 5 CD-1 mice (unless otherwise stated in the text) were infected intranasally under halothane anaesthesia. Mortality was monitored for 10 dpi. Body weight was recorded daily and the LD₅₀ was calculated by the Karber-Spearman method [250].

2.20 Luciferase assay

The reporter plasmids expressing firefly luciferase under the control of the following human promoters: IFN- β (p125), IRF-3, ISRE and NF- κ B (Obtained from Yan Zhou, University of Saskatchewan) were used to measure the relative ability of NS1 proteins to suppress transfection-induced IFN- β , IRF-3, ISRE and NF- κ B production. While a second reporter plasmid expressing *Renilla* luciferase under the control of the constitutive simian virus 40 promoter (pRL-SV40) (Obtained from Yan Zhou, University of Saskatchewan) was applied to measure general host expression.

Plasmid transfection was performed as follows: 60 ng of firefly reporter plasmids, 60 ng of pLLB NS (or empty pLLB vector as a negative control) plasmids and 30 ng of the internal control pRL-SV40 were diluted into 50 μ l Opti-MEM-1 (Invitrogen, Burlington, Ontario) (Solution 1). In a separate tube, 0.5 μ l lipofectamine 2000 (Invitrogen, Burlington, Ontario) (was added to 50 μ l of Opti-MEM-1 (Invitrogen, Burlington, Ontario), mixed gently by

pipetting up and down, and incubated for 5 min at RT (Solution 2). Both solutions 1 and 2 were then mixed gently and incubated for 30 min at RT.

The transfection mix was then transfected in a 96 well poly –D- lysine-treated plates of 293-T human embryonic kidney cells (washed twice with warm 1X PBS). Sixteen hours post-transfection, the previous transfection mix on cells was removed and the cells were transfected with 1000 ng of synthetic analog of dsRNA, Polyinosine-polycytidylic (Poly I:C) (Amersham Pharmacia Biotech) to induce IFN- β production. Briefly, solution A was prepared by mixing 50 μ l of Opti-MEM-1 with 1 μ l of Poly I:C (1 μ g/ μ l) (Amersham Pharmacia Biotech) while in tube B, 25 μ l of Opti-MEM-1 was incubated with 0.5 μ l of lipofectamine for 5min at RT. Both solutions were then mixed gently together, and the mix was then added to the 293T cells. At 40 h post-transfection, luminescence was measured according to the Promega Dual-Glo Luciferase Assay System protocol, using a Glomax Multi-Detection System Mode 9301-010 (Fisher Scientific, Nepean, Ontario). Relative luciferase activities were calculated as the average +/- standard deviation of the ratios of firefly and renilla luciferase luminescence for three internal replicates.

2.21 Immunofluorescence staining of BALB/c and CD-1 lungs

BALB/c and CD-1 mice were infected intranasally with 10⁵ PFU of each of the recombinant viruses and lungs were collected 2 and 3 dpi respectively. Lungs were inflated and fixed with 3.7% formaldehyde as described earlier [251]. Paraffin-embedded or frozen lung sections were stained as described previously [196]. Briefly, lungs were inflated and incubated overnight in 3.7% formaldehyde and only lungs to be treated as frozen lung sections were transferred into a solution of 20% sucrose. Both paraffin embedded and

frozen lung sections were fixed in acetone for 20 min at RT and then air dried for 45 min (only frozen lung sections were air dried prior to acetone fixing as well). The slides were incubated with 10 mM PBS for 5 min at RT and then treated with 20 μ l trypsin (0.25X) for 5 min at RT. The slides were then washed with 10mM PBS for 5 min followed by two washes for 10 min. Following washing, the slides were incubated with 100 μ l of the primary antibody (1:500 in antibody buffer) in a humid chamber at 4°C overnight. Rabbit anti-PR8 and anti-WSN primary antibodies that had been pre-adsorbed (as described in section 2.20) with mouse lung extract were used. Following primary antibody incubation, the slides were washed 3x as described above followed by incubation with 100 μ l of Cy3-labeled secondary antibody (Jackson conjugated donkey anti-rabbit secondary antibody ImmunoResearch laboratories Inc) (1:500 in antibody buffer) in a humid chamber for 1 hour at RT. Slides were washed 3x with 100 mM PBS and the nuclei were stained by applying 100 μ l Hoechst (0.2 μ g/ml) for 5 min in the dark at RT. The slides were washed 3x and then 20 μ l of anti-fade mounting medium was added before the cover slip is applied. The slides were stored in -20°C. Images were taken at 20x magnification using an Olympus BX50 microscope and processed in a parallel manner using Photoshop 7.0.

2.22 Adsorption of antisera with lung extracts

Uninfected CD-1 mouse lungs were collected and homogenized in 1 ml PBS using Cryomill machine. The homogenized lungs were mixed with 5V volumes of acetone and fixed for 5 min on ice. The samples were then pelleted and the acetone was evaporated in the warm room (37°C). The lung extract was mixed with 5 ml serum (to be used in immunostaining) and sonicated. The mixture was rotated overnight at 4°C and then

centrifuged to collect the antibodies. The serum collected was then aliquoted and stored at -20°C and used for lung staining.

2.23 Rate of viral protein synthesis

Monolayers of M1 and MDCK cells were infected (as described in section 2.16) with the different rPR8 viruses at MOI=3. Two, 4, 6 and 8 hpi, the cells were washed with 1x PBS and then pulse-labeled with 80 µCi/ml ³⁵S cysteine and methionine (Express tag, Perkin Elmer, Canada) for one hour in cysteine and methionine free media (Gibco, Carlsbad, USA). Cell lysates were then collected with 0.5 ml of 1x sodium dodecyl sulfate (SDS) sample buffer. The rate of viral protein synthesis was then assessed by SDS page gel electrophoresis and autoradiography as previously described [252].

2.24 Interferon-β ELISA Assay

2.24.1 *In vitro* ELISA assay

Monolayers of M1 cells were infected (as described in section 2.16) with each of the rPR8 and rWSN viruses at an MOI of 2. Twenty-four hours post-infection, the cell supernatants were collected and tested for IFN-β production. Infection of M1 cells with the VSV Indiana Δ 51 mutant (provided E.G. Brown, University of Ottawa, Ottawa, ON) that has a defect in the ability to block IFN induction was performed as a positive control using identical conditions [253]. Mouse IFN-β was titrated relative to mouse IFN-β standards by commercial Enzyme Linked Immuno Sorbant Assay (ELISA) as described by the manufacturer, PBL Biomedical Laboratories (New Jersey, USA) with subtraction of PBS mock-infected M1 cells control values.

2.24.2 *In vivo* ELISA assay

Groups of 6 CD-1 mice were inoculated with 5×10^3 pfu dose of the recombinant PR8 viruses expressing either A/HK/156/97 (H5N1) or A/Brevig Mission/1918 (H1N1) NS genes and their respective NS1 mutations 103F and 106I as well as 226V and 227R respectively, in a 50 μ L volume. Lungs were collected at 1, 3 and 5 dpi, suspended in 4X volume:weight cold sterile PBS, and homogenized by CryoMill machine (Mixer Mill MM 200 Retsch). Interferon- β concentration was determined by ELISA assay (PBL Biomedical Laboratories (New Jersey, USA)) after subtraction of PBS mock-infected control lung values.

2.25 Interferon sensitivity

Monolayers of M1 cells were treated with 200 U/ml of murine IFN- β (Sigma-Aldrich Oakville, Ontario) for 24 hours in triplicate assays. Twenty-four hours post-treatment, the cells were washed twice with 1x PBS prior to infection (as described in section 2.17) with the rPR8 viruses at MOI=1 (1.5×10^6 PFU per 35 mm dish) for 30 min at 37°C. Following infections, the cells were washed twice with 1x PBS, and serum free media was applied with trypsin (1 μ g/ml). The supernatant was then collected at 0, 8, 24 and 48 hpi and the viral titer was assessed by plaque assay (in duplicates for each individual infection). The same experiment was done in parallel but without prior IFN- β treatment.

2.26 CPSF-30 binding

The F2F3-FLAG tagged segment inserted in the pET17 vector under the control of the T7 promoter was previously constructed in our laboratory by N.E. Forbes. The following plasmids H5N1-NS and H5N1-NS-L103F, H5N1-NS-I106M and H5N1-NS-L103F+I106M as

well as 1918-NS and 1918-NS-I226V, 1918-NS-K227R and 1918-NS-I226V+K227R gene segments cloned in the pLLB vectors under the control of the T7 promoter. Briefly, 0.5 µg of the various NS plasmids as well as FLAG-tagged CPSF-F2F3 (FLAG-F2F3) fragment were expressed *in vitro* using TnT T7 Quick-Coupled Transcription/Translation System (Promega) in the presence of 10 µCi ³⁵S-methionine and cysteine (Express ³⁵S Protein Labeling Mix, Perkin Elmer). The mix was incubated with 40 µl TnT master mix in a 50 µl total volume for 90 minutes in a 30 °C water bath then stored at -20°C.

Equal expression of each NS protein as well as FLAG-F2F3 was verified by SDS-PAGE electrophoresis followed by autoradiography. In an α-FLAG pulldown assay, equal volume (10 µl) of the *in vitro* translated and ³⁵S-labeled H5N1 and 1918-NS and the six mutant NS proteins were mixed with FLAG-F2F3 or H₂O as a control and precipitated using Protein G Dynabeads (Invitrogen, Burlington, Ontario) pre-incubated with α-FLAG M2 antibody (Sigma).

The pulldown assay was performed according to the manufacturer's protocol of Protein G Dynabeads (Invitrogen, Burlington, Ontario). Briefly, the proteins were incubated with the antibody-conjugated beads overnight at 4°C while rocking, then were washed with NP-40 lysis buffer four times using a magnetic rack. Following wash, the beads were resuspended in 50 µL 1xSDS sample buffer and heated at 100°C for 1 minute before analysis by SDS-PAGE electrophoresis and autoradiography.

2.27 Statistical analysis

Statistical significance was assessed using the student t-test (Numbers, 09 v.2.0.4) using the parameters of paired or equal variance and two-tailed significance (unless otherwise

indicated) with a probability of ≤ 0.05 considered as statistically significant. Values were calculated as means $\pm$ standard deviation for sample size > 2 and $\pm$ standard error for sample sizes of 2 (for some of the values as indicated in the text and figure legends).

3. Effects of the NS1 mouse adaptive (NS1 MA) mutations in the A/Hong Kong/1/68 (H3N2) NS gene segment rescued onto the A/Puerto Rico/8/34 genetic backbone

3.1 Cloning of the NS1 MA mutations into bidirectional plasmid

The A/HK/1/68 (H3N2) NS gene segment (HK-NS wt) was cloned into the bidirectional pLLB plasmid by ligation independent cloning as described [247]. Thereafter the NS1 mouse adaptive (NS1 MA) mutations were introduced into the HK-NS wt plasmid by site-directed mutagenesis as described [254]. The list of NS1 plasmids cloned is summarized in Table 2. The NS plasmids express the same A/HK/1/68 wildtype NEP gene product and the A/HK/1/68 NS1 gene product that differs solely due to different mutations expressed in the NS1 gene segment.

Table 2 Cloning of MA20 and MA21 NS1 genes into pLLB or pHH21 vectors

VIRUS	NS1 MUTATION	CLONING METHOD	VECTOR
HK-wt	--	Ligation independent	pLLB
MA20a	V23A	Ligation independent	pLLB
MA20b-MA20c-MA20d	F103L	Ligation independent	pLLB
MA41-MA2	M106V	Ligation independent	pLLB
MA43	M106V+M124I	Site directed mutagenesis	pLLB
MA51	M106I	Ligation independent ^{Y, LI}	pHH21
MA52-MA53	L98S+M106I	Ligation independent ^{Y, LI}	pHH21
MA62-MA63	V180A	Ligation independent ^{Y, LI}	pHH21
MA93	D2N	Site directed mutagenesis	pLLB
MA102-MA111	D125G	Site directed mutagenesis	pLLB
MA103	V226I	Ligation independent ^{Y, LI}	pHH21
MA112	R227K	Site directed mutagenesis	pLLB
MA52-MA53	L98S ^Ψ	Site directed mutagenesis	pLLB
MA43	M124I ^Ψ	Site directed mutagenesis	pLLB
MA51 + MA20b-c-d	F103L+M106I ^Φ	Site directed mutagenesis	pLLB
MA43+ MA102-MA111	M124I+D125G ^Φ	Site directed mutagenesis	pLLB
MA103+ MA112	V226I+R227K ^Φ	Site directed mutagenesis	pLLB

^{Y, LI} Cloned by Yishan Li in Earl G. Brown Laboratory

^Ψ Mutations selected in pairs in the MA experiments are cloned separately to determine the effect of each mutation alone

^Φ Mutations cloned together as they map to the same binding site in the NS1 gene segment

3.2 Generation of recombinant viruses possessing the NS1 MA variants

The role of the individual NS1 MA mutations was determined on a defined genetic background by generating synthetic viruses as described previously [255]. The rescue system adopted was the 8-plasmid-system [247]. The system employs eight plasmids: seven of which containing the structural A/PR/8/34 (H1N1) genes in the bidirectional pHW2000 plasmid (provided by R. Webby (St. Jude's Children's Research Hospital, Memphis)) as well as the NS A/HK/1/68 (H3N2) genome segment or its MA variants inserted into either the pHH21 or pLLB vectors (Table 2). The plasmids expressing the vRNA and the mRNA of the IAV genes (with the exception of NS1 when cloned into pHH21 plasmid which only expresses the vRNA of the insert) were transfected into a confluent monolayer of 293T cells to produce the virus of interest. Table 3 summarizes the list of viruses rescued onto the A/PR/8/34 (H1N1) backbone using the 8-plasmid system. These rescued viruses expressed the wildtype A/PR/8/34 (H1N1) NS gene, the wildtype A/HK/1/68 (H3N2) NS gene or the NS1-MA mutations as selected in the mouse adaptation experiments on the A/PR/8/34 (H1N1) backbone (Table 3). Two more viruses have been generated expressing the single mutations L98S and M124I, which were present as a pair, with M106I and M106V respectively in the parental MA53 and MA43 viruses (Table 1). In addition, three viruses were engineered that express combinations of 2 NS1 mutations that map to one binding site such as M124I plus D125G, F103L plus M106I as well as V226I plus R227K. In total 18 rPR8 viruses were generated that differed solely in mutations in their NS genome segment (Table 3).

Table 3 List of rPR8 viruses rescued and their LD₅₀ in BALB/c mice

LIST OF rPR8 RESCUED	NS1 MUTATIONS	LD ₅₀ (log ₁₀ PFU)
rPR8 wt	Wildtype	3.5
rPR8-HK-NS wt	Wildtype	>7.2
rPR8-HK-NS-D2N	D2N	>7.2
rPR8-HK-NS-V23A	V23A	>7.2
rPR8-HK-NS-F103L	F103L	6.8
rPR8-HK-NS-M106V	M106V	6.2
rPR8-HK-NS-M106I	M106I	7.0
rPR8-HK-NS-L98S+M106I	L98S+M106I	6.4
rPR8-HK-NS-M06V+M124I	M06V+M124I	>7.2
rPR8-HK-NS-D125G	D125G	6.8
rPR8-HK-NS-V180A	V180A	6.6
rPR8-HK-NS-V226I	V226I	>7.2
rPR8-HK-NS-R227K	R227K	7.0
rPR8-HK-NS-L98S ^Ψ	L98S	6.8
rPR8-HK-NS-M124I ^Ψ	M124I	>7.2
rPR8-HK-NS-F103L+M106I ^Φ	F103L+M106I	7.0
rPR8-HK-NS-M124I+D125G ^Φ	M124I+D125G	>7.2
rPR8-HK-NS-V226I+R227K ^Φ	V226I+R227K	>7.2

^Ψ Mutations selected in pairs in the MA experiments are cloned separately to determine the effect of each mutation alone

^Φ Mutations cloned together as they map to the same binding site in the NS1 gene segment

3.3 Optimizing the rescue system

Transfection of 4µg of the eight IAV genes (0.5µg/IAV DNA) cloned into bidirectional plasmids was first attempted using transfection reagent (Lipofectamine 2000, Invitrogen) for 5 hours in a co-culture of 293T (90%) and MDCK (10%) as these conditions have been successfully used in the laboratory [196]. Following transfection, the cell supernatant was used to infect a confluent monolayer of MDCK cells to increase the viral titer. However, viral titers were below the levels of detection by HA assay. Upon examination of cell conditions over the course of the experiment, we speculated that the 293T cells were a major factor affecting the failure of virus rescue following DNA transfection. Therefore, the rescue system was compared with a monoculture of 293T cells or in co-culture with MDCK cells at different ratios (90 to 10% and 50 to 50% respectively). Viral titers were not detectable in any of the stated cultures thus indicating that the 293T or growth/culture conditions may have affected DNA transfection efficiency. Therefore, monocultures of 293T cells from three different sources were tested for their transfection efficiency using various amounts (8, 4 and 2 µg) of green fluorescent protein (GFP) expression plasmid (obtained from E. G. Brown Laboratory, University of Ottawa, Ontario). The 293T cells with the best combination of the high transfection efficiency (determined by detection of GFP expressing cells) and cell survival were used subsequently for virus rescue. Additionally, the 293T cells were grown on plates pre-coated with poly -D- lysine to improve cell adherence. Moreover, the DNA transfection incubation was increased from 5 to 16 hours at 37°C in the presence of 5% CO₂. Various amounts of different trypsin sources were subsequently tested to determine the optimal trypsin concentration (1 µg/ml) that results in minimal detrimental effects on

the cells. As a final step, the cell supernatants as well as the 293T cells were collected and grown in 10-day old embryonated chicken embryos. Seventy-two hours following incubation, the HA positive allantoic fluid were collected and the viruses were then sequenced to make sure no unwanted mutations were selected during viral growth. Improving the 293T cell conditions, transfection efficiency and viral growth in embryonated eggs were major factors in improving the rescue system.

3.4 Comparison of mouse adapted A/PR/8/34 (H1N1) versus A/HK/1/68 (H3N2) NS genes on A/PR/8/34 (H1N1) backbone

The viral fitness and lethality were first compared between the two viruses rPR8-wt and the rPR8-HK-NS-wt that possess the same seven structural A/PR/8/34 (H1N1) genes and only differed in their NS genes, which belong to A/PR/8/34 (H1N1) and A/HK/1/68 (H3N2) respectively. To compare viral growth *in vitro*, monolayers of MDCK cells were infected with a multiplicity of infection (MOI) of 0.001 in the presence of trypsin (1µg/ml). The supernatant was collected at 12, 24, 48 and 72 hpi and the virus titer was assessed by plaque assay. Viral growth was enhanced significantly for the rPR8-wt reassortant compared to the rPR8-HK-NS-wt throughout the course of the experiment ($P < 0.001$) with a 10-fold increase in peak titre 48 hpi (Figure 9a). In addition, viral growth was also compared *in vivo* between the two viruses. Groups of 11 BALB/c mice were infected with 5×10^3 pfu with each of two viruses via the intranasal route. Viral growth in BALB/c lungs was also enhanced significantly by >3 logs by the rPR8-wt compared to the rPR8-HK-NS-wt at days 2 and 3 pi ($P < 0.05$) (Figure 9b). Another mouse model was also used; groups of 12 CD-1 mice were infected with 5×10^3 pfu with each of the two viruses differing in the NS gene via the intranasal route and viral growth in the lungs were also compared. The A/PR/8/34 (H1N1)

NS gene also enhanced viral growth in CD-1 mouse lungs significantly ($P < 0.05$) increasing yields by 2 and 3 logs at days 5 and 7 respectively compared to the rPR8-HK-NS-wt virus that possessed the NS gene of A/HK/1/68 (H3N2) (Figure 9c). The attenuation of the viral growth induced by the A/HK/1/68 (H3N2) NS gene relative to the enhanced growth to the virus possessing the PR8-NS gene was also observed at the level of lung tissue tropism. The extent of virus spread and lung tissue tropism was observed by immunofluorescent staining at 2 days following intranasal infection with 10^5 pfu in BALB/c mice. The A/PR/8/34 (H1N1) NS gene resulted in dramatic increase in viral spread in the mouse lungs that was observed to involve extensive infection in the bronchi as well as the alveoli. This was in contrast to the rPR8-HK-NS-wt possessing an NS gene from A/HK/1/68 (H3N2) that dramatically attenuated the viral tissue tropism in the BALB/c lungs to result in very limited areas of infection in the alveoli (Figure 9d). Moreover, the LD_{50} of the rPR8-wt virus determined in both BALB/c and CD-1 mice was $10^{3.5}$ PFU, while the substitution of the NS gene by A/HK/1/68 (H3N2) resulted in avirulent virus even at the highest dose of infection 5×10^6 PFU and therefore the LD_{50} was $>10^{7.2}$ that was thus increased by greater than 3.5 logs due to the rPR8-NS-wt genome segment. Consequently, the substitution of the A/PR/8/34 (H1N1) NS gene by the A/HK/1/68 (H3N2) NS segment resulted in strong attenuation of viral growth of the highly virulent rPR8wt virus *in vitro* and in both mouse models used in our studies (BALB/c and CD-1), indicating that the A/PR/8/34 (H1N1) NS genome segment contributes importantly to the virulence of A/PR/8/34 (H1N1). Thus the low virulence of rPR8-HK-NS-wt makes it a suitable model that permits the assessment of potential increases in fitness induced by the NS-MA mutations.

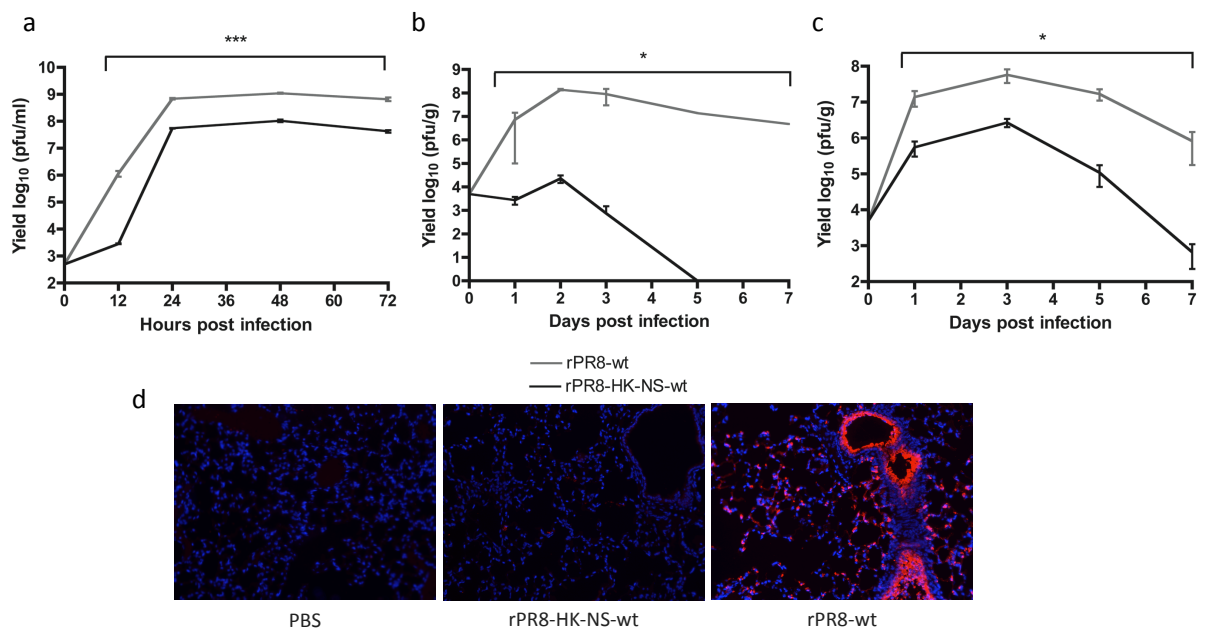


Figure 9 Replacing the A/PR/8/34 (H1N1) by the A/HK/1/68 (H3N2) NS gene segment results in reduced lung tissue tropism of the A/PR/8/34 (H1N1) virus as well as decreased viral growth in MDCK cells, BALB/c and CD-1 mouse lungs **a. Viral growth of rPR8-wt and rPR8-HK-NS-wt in MDCK cells.** Monolayers of MDCK cells were infected with rPR8-wt and rPR8-HK-NS-wt at MOI=0.001 in presence of trypsin. Supernatants were collected at 12, 24, 48 and 72 hpi and viral titer was assessed by plaque assay (*P<0.05, ** P<0.01, ***P<0.001 Student's paired t-test) **b. Viral growth of rPR8-wt and rPR8-HK-NS-wt in BALB/c lungs.** Groups of 11 BALB/c mice were infected intranasally with 5×10^3 pfu. Lungs were collected at days 1, 2, 3, 5 and 7 post infection. Collected lungs were homogenized and viral titer was assessed by plaque assay (*P<0.05, ** P<0.01, ***P<0.001 Student's paired t-test) **c. Viral growth of rPR8-wt and rPR8-HK-NS-wt in CD-1 lungs.** Groups of 12 CD-1 mice were infected intranasally with 5×10^3 pfu. Lungs were collected at days 1, 3, 5 and 7 post infection. Collected lungs were homogenized and viral titer was assessed by plaque assay (*P<0.05, ** P<0.01, ***P<0.001 Student's paired t-test) **d. Lung tissue tropism by rPR8-wt and rPR8-HK-NS-wt in BALB/c lungs.** BALB/c mice were infected intranasally with 10^5 pfu and lungs were collected 2 dpi. The lungs were inflated and fixed with 3.7% formaldehyde and lung sections were stained with anti- PR8 antibody.

3.5 MDCK cell plaque morphology of rPR8 NS1 mutants

The rescued rPR8 viruses with various NS1 mutations were grown in the allantoic cavity of embryonated chicken eggs and the virus titers as well as the plaque morphology were determined by plaque assay in MDCK cells and compared to the rPR8-HK-NS-wt virus. All viruses grew to high titers in the allantoic fluids of embryonated chicken eggs ($\geq 10^7$ pfu/ml) as determined by plaque assay in MDCK cells. The rPR8-HK-NS-wt average plaque diameter is 1.25 ± 0.35 mm. Although all 16 mutants demonstrated similar or increased numerical plaque size in MDCK cells compared to rPR8-HK-NS-wt, 12 of these mutants increased plaque size to a statistically significant level compared to the rPR8-HK-NS-wt ($P < 0.05$ for the rPR8-HK-NS-L98S+M106I, rPR8-HK-NS-M106V and rPR8-HK-NS-D125G; $P < 0.01$ for rPR8-HK-NS-V23A, rPR8-HK-NS-M124I+D125G and rPR8-HK-NS-V226I+R227K; $P < 0.001$ for rPR8-HK-NS-D2N, rPR8-HK-NS-M106I, rPR8-HK-NS-L98S, rPR8-HK-NS-F103L+M106I, rPR8-HK-NS-V180A and rPR8-HK-NS-R227K) (Figure 10). The mutants rPR8-HK-F103L and rPR8-HK-M106V+M124I (1.55 ± 0.5 and 1.48 ± 0.41 respectively) increased plaque size numerically compared to rPR8-HK-NS-wt (1.25 ± 0.35 mm) but the difference was not statistically significant, indicating a trend to greater plaque size. In contrast, the rPR8-HK-NS-M124I and rPR8-HK-NS-V226I mutants showed similar plaque diameter (1.29 ± 0.43 and 1.32 ± 0.46 respectively) compared to the rPR8-HK-NS-wt. The plaque diameter of the rPR8-wt virus 4.34 ± 0.67 mm exceeded all the rPR8-HK-NS wildtype and mutants (Figure 10). The increase in plaque size for most of the mutants indicates an enhanced fitness in MDCK cells.

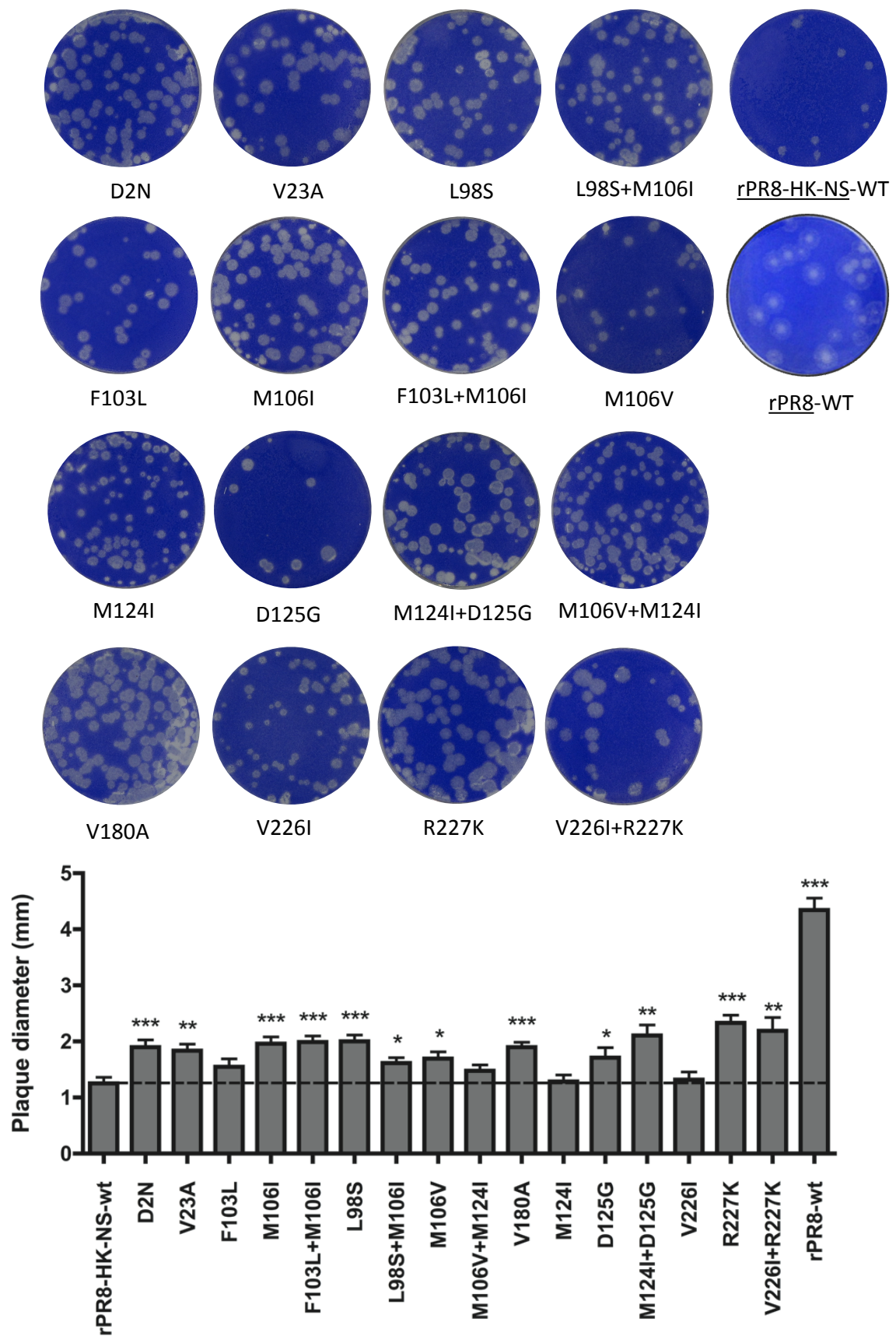
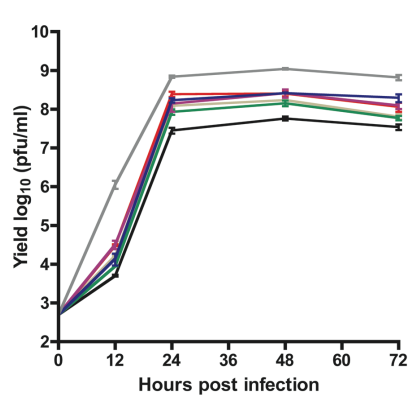


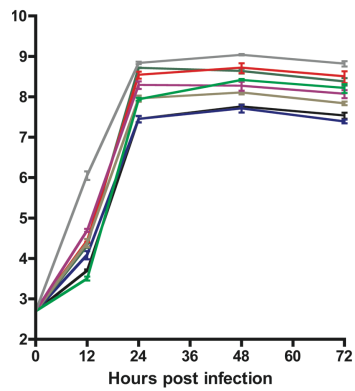
Figure 10 **Plaque morphology of rPR8-HK-NS-WT and the recombinant viruses carrying the NS1-MA mutations selected upon mouse adaptation observed on MDCK cells.** Average plaque size of recombinant rPR8 viruses expressing NS1 mouse-adaptive mutations. Average plaque size $\pm$ SD is noted (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; two-tailed student's t-test)

3.6 Growth of rPR8 NS1 mutants *in vitro*

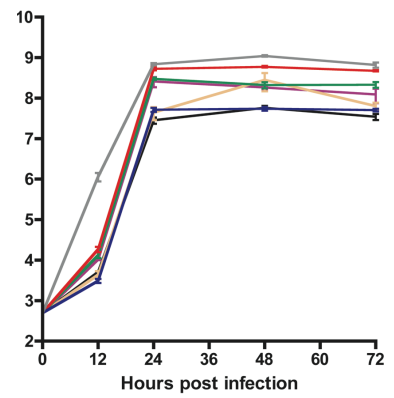
To compare the growth kinetics of the different viruses that differ due to specific changes in their NS1 genes (that in some cases included NEP mutations), MDCK cells were infected in triplicate with a MOI of 0.001 (equivalent to 1.5×10^3 pfu per 35 mm dish) in the presence of trypsin (1 μ g/ml). The supernatant was collected at 12, 24, 48 and 72 hpi and the virus titer was assessed by plaque assay in duplicate. The rPR8-HK-NS-wt reached a peak of 1.03×10^8 PFU at 48 hpi (Figure 11). Growth was enhanced significantly in 13 of the mutant viruses compared to rPR8-HK-NS-wt ($P < 0.05$). Replication was enhanced significantly by the double-mutants rPR8-HK-NS-M124I+D125G, rPR8-HK-NS-F103L+M106I and rPR8-HK-NS-V226I+R227K ($P < 0.001$), and also by rPR8-HK-NS-M106I, rPR8-HK-NS-M106V, rPR8-HK-NS-L98S, rPR8-HK-NS-V180A, rPR8-HK-NS-L98S+M106I and rPR8-HK-NS-D125G ($P < 0.01$) (Figure 11). The following NS1 variants also enhanced viral growth significantly throughout the course of the experiment rPR8-HK-NS-V23A, rPR8-HK-NS-F103L, rPR8-HK-NS-R227K and rPR8-HK-NS-D2N ($P < 0.05$). However, the growth of three mutants rPR8-HK-NS-M124I, rPR8-HK-NS-V226I and rPR8-HK-NS-M106V+M124I was not significantly different from rPR8-HK-NS-wt. Viral growth in MDCK cells correlated with the plaque diameter assayed in the same cell line. Therefore, the NS1 mutations significantly increase the viral replication and growth *in vitro* compared to the rPR8-HK-NS-wt and thus demonstrates their adaptive nature. On the other hand, the rPR8-wt exceeded the titers reached by any of the other synthetic viruses (Figure 11) consistent with the presence of multiple adaptive mutations that had been selected on mouse adaptation.



— rPR8-HK-NS-wt
— rPR8-HK-NS-V23A*
— rPR8-HK-NS-F103L*
— rPR8-HK-NS-M106I**
— rPR8-HK-NS-M106V**
— rPR8-HK-NS-103L+106I***
— rPR8-wt



— rPR8-HK-NS-wt
— rPR8-HK-NS-V226I
— rPR8-HK-NS-R227K*
— rPR8-HK-NS-V226I+R227K**
— rPR8-NS-M106I L98S**
— rPR8-HK-NS-V180A**
— rPR8-HK-NS-L98S**
— rPR8-wt



— rPR8-HK-NS-wt
— rPR8-HK-NS-D2N*
— rPR8-HK-NS-M124I
— rPR8-HK-NS-D125G**
— rPR8-HK-NS-M124I+D125G***
— rPR8-HK-NS-M106V+M124I
— rPR8-wt

Figure 11 **Mouse-adapted NS1 mutations increased viral replication efficiency *in vitro*.** Monolayers of MDCK cells were infected with the rPR8-HK-NS-wt, rPR8-wt and the recombinant viruses expressing the NS1 mouse-adaptive mutations at an MOI=0.001 in presence of trypsin. Supernatants were collected at 12, 24, 48 and 72 hpi and viral titer was assessed by plaque assay (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; two-tailed student's paired t-test with respect to rPR8-HK-NS-wt).

3.7 Lethality and body weight loss in BALB/c mice

To compare the virulence of the different rescued NS mutant viruses, BALB/c mice were infected with 5×10^6 PFU/ml and mortality was monitored for 10 days post-infection. Body weight was also measured daily. The rPR8-HK-NS-wt did not kill mice throughout the whole study time period. However, nine of the sixteen mutants were more virulent when compared to rPR8-HK-NS-wt as they resulted in decreases of LD₅₀ as shown in Table 3 and Figure 12. Although the rPR8-HK-NS-wt virus was avirulent in BALB/c mice on the basis of survival (Figure 12) viral infection resulted in a body weight loss of approximately 15 % of their original weight at day 3 pi, whereafter, the mice started to recover and the body weight increased gradually (Figure 13). Most of the mutants exhibited an increase in virulence when compared to rPR8-HK-NS-wt (Figure 12). The greatest effect was seen with rPR8-HK-NS-M106V, rPR8-HK-NS-V180A and the double mutant rPR8-HK-NS-L98S+M106I, which caused 100, 80, and 60% mortality respectively (Figure 12). This was followed by rPR8-HK-NS-L98S, rPR8-HK-NS-F103L and rPR8-HK-NS-D125G that resulted in 40% mortality (Figure 12). The rPR8-HK-NS-M106I, rPR8-HK-NS-F103L+M106I and rPR8-HK-NS-R227K killed 1 of 5 mice (20%) following infection (Figure 12). Most of the NS1-MA variants also resulted in severe disease in BALB/c mice measured by body weight loss, which was significant when compared to the rPR8-HK-NS-wt for the rPR8-HK-NS-F103L and rPR8-HK-NS-M106V ($P < 0.05$) as well as rPR8-HK-NS-L98S, rPR8-HK-NS-V180A and rPR8-HK-NS-L98S+M106I ($P < 0.001$) (Figure 13). Out of the seven remaining avirulent viruses, mice infected with either rPR8-HK-NS-V23A ($P < 0.001$), rPR8-HK-NS-D2N, or rPR8-HK-NS-M124I+D125G ($P < 0.01$) lost more than 20% of their body weight. Consistent with their roles

in increasing replicative fitness the NS1 adaptive mutations increased virulence *in vivo* as observed by body weight loss and mortality in BALB/c when compared to the rPR8-HK-NS-wt.

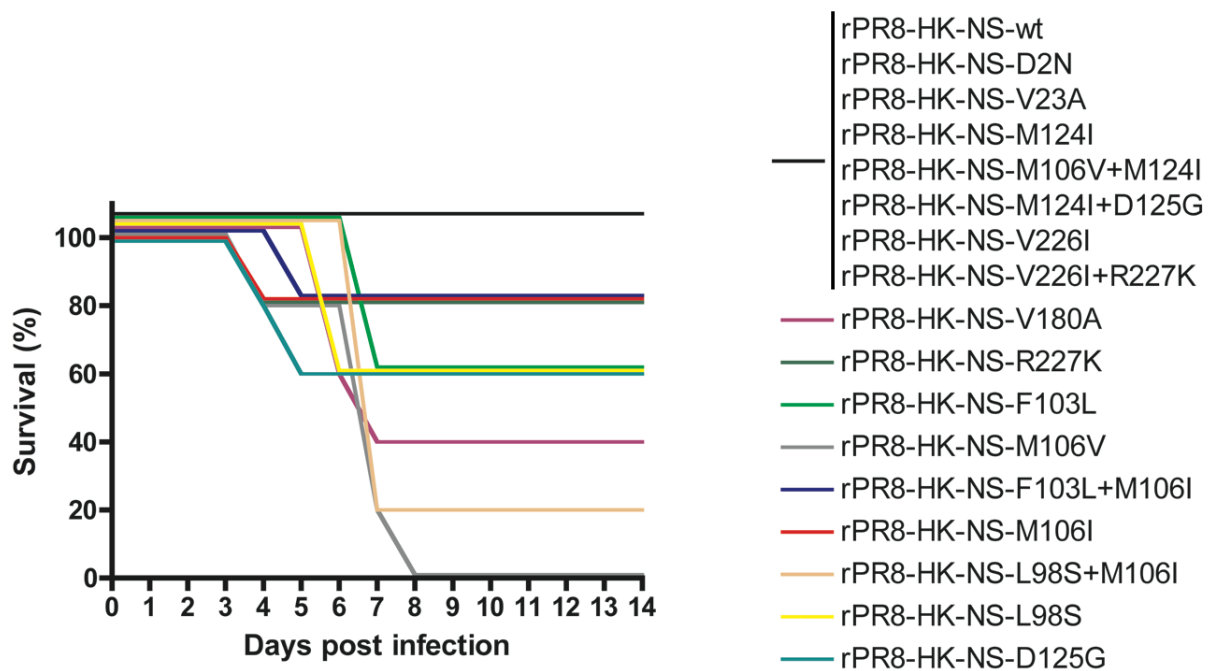
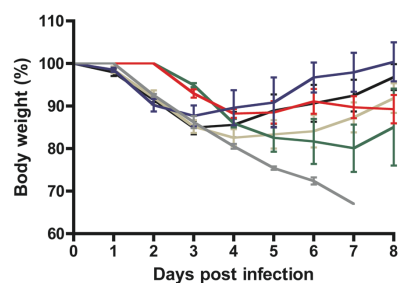
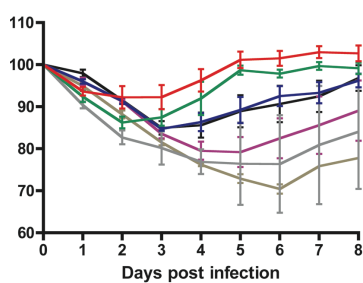


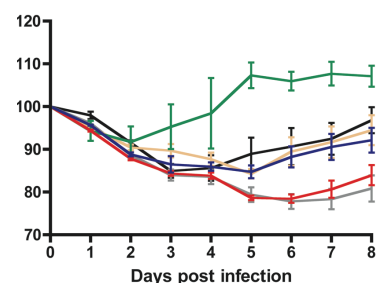
Figure 12 **Mouse-adapted NS1 mutations induce virulence in BALB/c mice.** Groups of 5 BALB/c mice were infected intranasally with 5×10^6 pfu in a 50 μ l volume with the rPR8-HK-NS-wt virus and the recombinant viruses expressing the NS1 mouse-adaptive mutations. Survival was monitored for 14 days following infection.



— rPR8-HK-NS-wt
— rPR8-HK-NS-V23A ***
— rPR8-HK-NS-F103L *
— rPR8-HK-NS-M106I
— rPR8-HK-NS-103L+106I
— rPR8-HK-NS-M106V *



— rPR8-HK-NS-wt
— rPR8-HK-NS-L98S+M106I ***
— rPR8-HK-NS-V180A ***
— rPR8-HK-NS-L98S ***
— rPR8-HK-NS-V226I
— rPR8-HK-NS-R227K
— rPR8-HK-NS-V226I+R227K



— rPR8-HK-NS-wt
— rPR8-HK-NS- D2N
— rPR8-HK-NS-M124I
— rPR8-HK-NS-D125G
— rPR8-HK-NS-M124I+D125G**
— rPR8-HK-NS-M106V+M124I

Figure 13 **Mouse-adapted NS1 mutations increase disease severity expressed by body weight loss in BALB/c mice.** Groups of 5 BALB/c mice were infected intranasally with 5×10^6 pfu in a 50 μ l volume with the rPR8-HK-NS-wt virus and the recombinant viruses expressing the NS1 mouse adaptive mutations. Body weight loss was recorded daily (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; two-tailed student's paired t-test between days 1 and 8).

3.8 Interferon- β transcription inhibition by the recombinant NS1 MA proteins

So far we have demonstrated that the majority of NS1 mutations selected upon mouse adaptation increased virulence and disease severity *in vivo* as well as viral growth *in vitro*. We next asked whether the NS1 MA enhanced fitness is caused by increased IFN inhibition, therefore the effect of NS1 MA recombinant proteins on IFN- β promoter transcriptional activation was determined *in vitro* using luciferase reporter gene expressing plasmid construct driven by the human IFN- β promoter. Monolayers of 293-T cells were transfected with the bidirectional plasmids expressing mutant or HK-wt NS gene segments with a firefly luciferase reporter plasmid driven by the human IFN- β promoter (p125), then activated the IFN- β promoter by transfection with synthetic dsRNA (poly-I:C). The HK-wt NS1 protein was effective at inhibition of poly-I:C transcriptional activation of the human IFN- β promoter (14 % activity) compared to the empty vector control (pLLB). Surprisingly, most of the NS1 MA mutants were not significantly different in inhibiting IFN- β transcriptional activation compared to the HK-wt NS1 plasmid except for NS1 mutants M106I, M106V, M106V+M124I, F103L+M106I and V180A that demonstrated significantly decreased ability to inhibit human IFN- β promoter activation ($p < 0.01$, $p < 0.01$, $p < 0.001$, $p < 0.01$, $p < 0.05$ respectively) (Figure 14). Therefore we can conclude that the NS1 mediated increase in virulence and replication does not correlate with an enhanced ability to antagonize IFN- β transcriptional activation as measured in the standard human IFN- β promoter in the 293T cell assay.

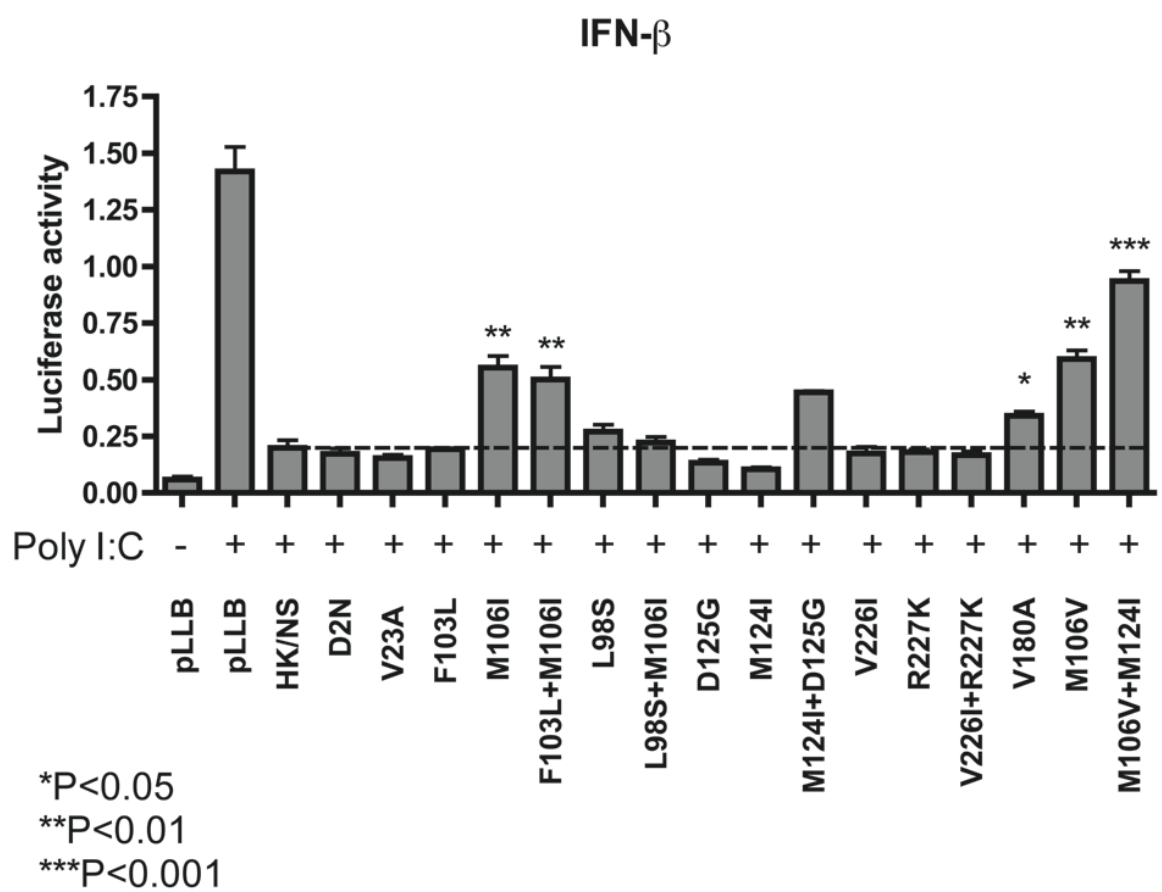


Figure 14 **Mouse-adapted NS1 mutations affect poly I:C induced IFN- β promoter activation *in vitro*.** IFN- β promoter activation was measured in human 293-T cells in triplicate for NS mutants and HK-NS-wt expression plasmids using a dual-luciferase reporter assay. Each of the NS expression plasmids along with the firefly luciferase p125 under the control of the human IFN- β promoter as well as the renilla luciferase control pRL-SV40 reporter plasmids were transfected in 293T cells. Sixteen hours following transfection, IFN- β promoter activation was stimulated by poly I:C transfection. The luciferase activities were measured following a 24h incubation period. Data represent the means $\pm$ SD (* p <0.05, ** p <0.01, *** p <0.001; two-tailed student's t-test) of firefly luciferase activity relative to renilla.

4. Properties of the NS1 MA F103L and M106I mutant combination

4.1 Enhanced growth mediated by F103L and M106I mutations in the rPR8-HK-NS mutant viruses *in vitro*

Mouse adaption had resulted in the individual selection of F103L and M106I mutations however both mutations are present in the A/HK/156/1997 (H5N1) virus, suggesting that these mutations in combination may mediate enhanced NS1 gene functions. To test the interactive roles of F103L and M106I mutations on growth, we first generated viruses possessing the HK-wt (103F+106M), or F103L, M106I and F103L+M106I mutations on the A/PR/8/34 backbone. To compare the growth kinetics of viruses that differed due to specific change in their NS1 genes, rPR8-HK-NS-wt, rPR8-HK-NS-F103L, rPR8-HK-NS-M106I, and rPR8-HK-NS-F103L+M106I were used to infect MDCK and A549 epithelial cells, in triplicate, at a low MOI (0.001) to initiate multicycle replication. Supernatants were collected at 12, 24, 48 and 72 hpi and were assayed for yield of infectious progeny virus as measured by plaque assay. All the mutants had a significantly increased ability to grow in MDCK cells compared to rPR8-HK-NS-wt with the rPR8-HK-NS-M106I and rPR8-HK-NS-F103L+M106I mutants growing to higher titers than the rPR8-HK-NS-F103L mutant (Figure 15a). Growth was particularly enhanced at early times (12 and 24 hpi) ($P < 0.05$) in the three mutant viruses compared to rPR8-HK-NS-wt, where the F103L mutation enhanced yields by 6 and 2 fold respectively; relative to the M106I containing, single and double, mutants that showed 20 and 9, as well as 7 and 5 fold enhanced yields for these respective viruses at 12 and 24 hpi. However, at the 48 hpi time point, only rPR8-HK-NS-M106I, and rPR8-HK-NS-F103L+M106I viruses grew significantly better than rPR8-HK-NS-wt ($P < 0.05$) and at 72 hpi,

the double mutant was the only virus that grew to a significantly higher titer than the rPR8-HK-NS-wt virus ($P<0.05$) (Figure 15a).

In contrast, growth of the different mutants in A549 cells was comparable to rPR8-HK-NS-wt at early time points (i.e. 12 and 24 hpi), whereas at later time points (48 and 72 hpi) there was a trend to increased yield for the NS1 mutants where the rPR8-HK-NS-M106I and rPR8-HK-NS-F103L+M106I mutants demonstrate statistically significant enhanced growth compared to rPR8-HK-NS-wt at 48 hpi ($P<0.05$) (Figure 15b). Therefore, each of the individual as well as combination of the F103L+M106I NS1 mutations increased viral replication and growth *in vitro* compared to the rPR8-HK-NS-wt in MDCK and, in the case of rPR8-HK-NS-M106I and rPR8-HK-NS-F103L+M106I mutants, in A549 cells and thus demonstrates their adaptive nature in both human and canine cells that included host specific enhancement for the F103L mutation.

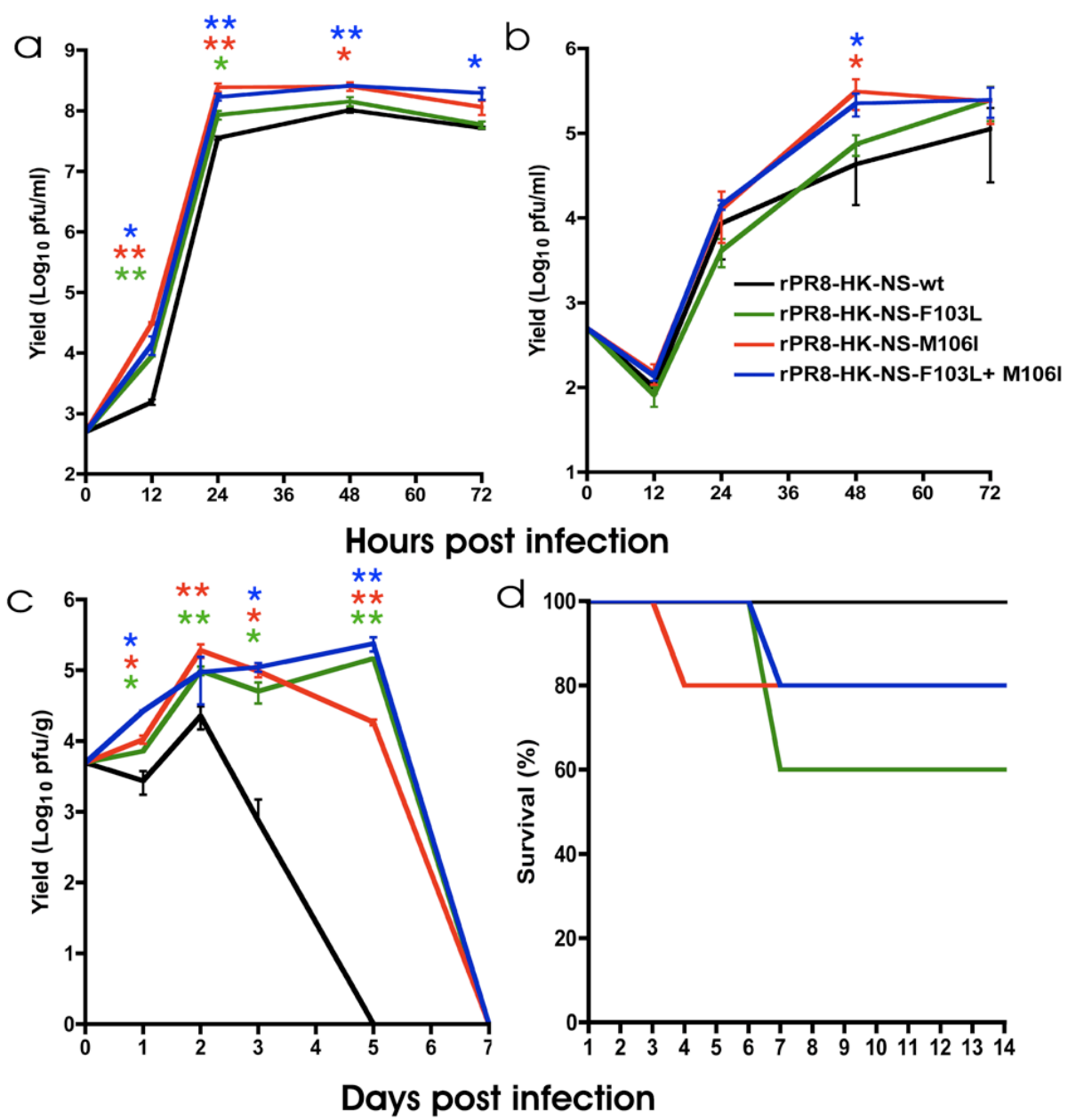


Figure 15 Increased virulence and viral growth by the NS1 mutants in *in vitro* and *in vivo* systems. **a. Growth of rPR8 mutant viruses in MDCK cells.** **b. Growth of rPR8 mutant viruses in A549 human epithelial cells.** Monolayers of MDCK or A549 cells were infected with each of the recombinant viruses at MOI= 0.001 in presence of trypsin. Supernatants were collected at 12, 24, 48 and 72 hpi and viral titer was assessed by plaque assay. Values are shown as averages +/- standard deviation (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; two-tailed equal variance student's t-test). **c. Growth of rPR8 mutant viruses in BALB/c lungs.** Groups of 11 BALB/c mice were infected intranasally with 5×10^3 pfu. Lungs were collected at days 1, 2, 3, 5 and 7 post infection. Organs were sonicated on ice and viral titer was assessed by plaque assay. Values are shown as averages +/- standard deviation at 3 dpi and +/- standard error for the rest of the time points (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; two-tailed equal variance student's t-test). **d. Virulence of rPR8 mutant viruses in BALB/c mice.** Groups of 5 BALB/c mice were infected intranasally with 5×10^6 pfu of the rPR8 viruses. Survival was monitored for 14 dpi.

4.2 Enhanced growth mediated by F103L and M106I mutations in the rPR8-HK-NS mutant viruses *in vivo*

The three mutant rPR8 viruses along with the rPR8-HK-NS-wt were used to infect groups of 11 BALB/c mice with 5×10^3 pfu each via the intranasal route, and their lung growth properties in lung tissue were compared. The rPR8-HK-NS-wt virus reached a peak titer of 3.7×10^4 pfu/g in mouse lungs at 2 dpi and was cleared from the lungs by 5 dpi (Figure 15c). All the NS1 adaptive mutations significantly increased replication in mouse lung compared to rPR8-HK-NS-wt at 1 dpi ($P < 0.05$) and for each of the single mutants 2 days after infection ($P < 0.01$). Thereafter, viral growth was increasingly enhanced (100 fold at 3 dpi ($P < 0.05$), and 5 logs by 5 dpi ($P < 0.01$)) (Figure 15c). Thus all the NS1 mutants delayed viral clearance by 2 days in mouse lungs compared to rPR8-HK-NS-wt. In addition, rPR8-HK-NS-F103L and the double mutant rPR8-HK-NS-F103L+M106I demonstrated enhanced viral growth relative to the rPR8-HK-NS-M106I mutant at 5 dpi (Figure 15c). This indicated that both of the mouse adaptive mutations, F103L and M106I, were associated with increased replication in mouse lungs such that they grew faster and to a higher yield as well as persisted longer in the mouse lung compared to rPR8-HK-NS-wt. The level of IFN- β was undetectable by ELISA in these infected mouse lung extracts. No virus was detected in the liver of any of the mice indicating that the viruses are incapable of growing systemically in BALB/c mice following infection at 5×10^3 pfu (data not shown).

4.3 F103L and M106I mutations are associated with large increases in virulence in the rPR8-HK-NS mutant viruses in BALB/c mice

To compare the virulence of the different recombinant viruses, groups of 5 BALB/c mice were infected intranasally with 5×10^6 pfu of the different recombinants and mortality

was monitored for 14 days pi. Although the LD₅₀ of the rPR8 virus was 10^{3.5} pfu (Table 3), introduction of the HK-wt NS1 gene onto this backbone resulted in rPR8-HK-NS-wt that was avirulent in mice and did not result in mortality (LD₅₀>10⁷ pfu) (Figure 15d) indicating an important role for PR8 NS1 in virulence. Similar infection of mice with 5 x 10⁶ pfu of each of the three mutants resulted in mortality within each group (Figure 15d). A single mutation (F103L) introduced to the HK-wt NS gene resulted in 40% mortality in BALB/c, while the infection with the viruses possessing M106I and F103L+M106I mutations resulted in a 20% mortality rate by 4 and 7 dpi respectively. Body weight loss was also recorded daily throughout the course of the experiment. The rPR8-HK-NS-wt virus resulted in 15% body weight loss 3 dpi (Figure 13). The F103L NS1 mutation not only enhanced virulence but also resulted in 20% body weight loss by day 6pi (P<0.05) (Figure 13). However although both M106I and F103L+M106I NS mutants resulted in 20% mortality in BALB/c mice both mutants only caused 12% body weight loss by day 3 pi. Consistent with their increased growth in mouse lungs, the NS1 adaptive mutations increased virulence *in vivo* as observed by mortality when compared to the rPR8-HK-NS-wt.

4.4 The F103L and M106I HK-NS1 mutations increase tropism in BALB/c lungs

The recombinant PR8 viruses were used to assess the extent of infection and tropism in BALB/c mouse lungs infected with 10⁵ pfu at 2 dpi by immunofluorescent staining. The lungs infected with the rPR8-HK-NS-wt showed a minimal pattern of detectable infection that was mainly in the bronchioles with scattered alveolar staining (Figure 16b). The NS1 mutants showed an increased extent of alveolar tissue infection with larger foci in the lung parenchyma that was more pronounced in the single mutants rPR8-

HK-NS-F103L and rPR8-HK-NS-M106I compared to rPR8-HK-NS-wt (Figure 16b, c, d, e), whereas very little bronchiolar infection was observed for these mutants.

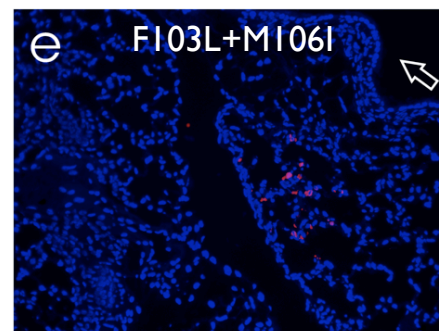
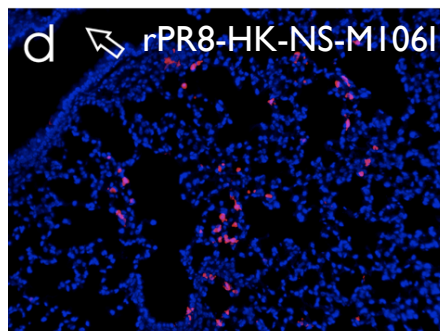
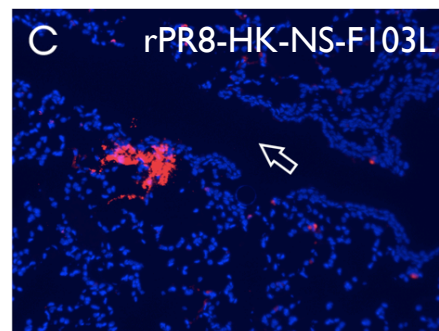
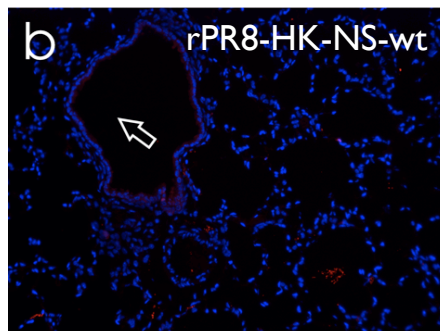
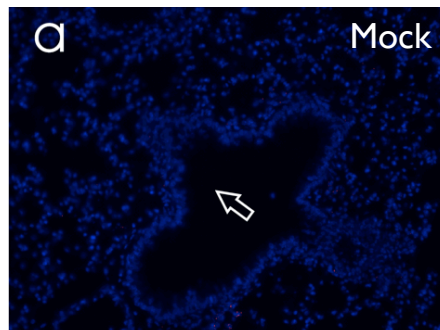


Figure 16 **103L, 106I and 103L+106I mutations in the A/HK/1/68-NS enhance viral tropism in BALB/c lungs.** BALB/c mice were infected intranasally with 10^5 pfu and lungs were collected 2 dpi. The lungs were inflated and fixed with 3.7% formaldehyde and lung sections were stained with anti- PR8 antibody. The bronchiolar regions are indicated by an arrow. **a.** Mock uninfected lungs **b.** rPR8-HK-NS-WT infected lungs **c.** rPR8-HK-NS-F103L infected lungs **d.** rPR8-HK-NS-M106I infected lungs **e.** rPR8-HK-NS-F103L+M106I infected lungs

4.5 Rate of viral protein synthesis

The mouse adapted NS1 mutations F103L and M106I map to binding sites for cellular translational (eIF4G-I and PABPI) and post-transcriptional factors (CPSF-30). To explore whether the enhanced growth and pathogenicity of the mutant viruses correlated with enhanced viral protein synthesis, the rate of viral protein synthesis was determined by ³⁵S-met+cys labeling of infected MDCK cells. MDCK cells were infected with the different rPR8 viruses at MOI=3 and cells were pulse-labeled with ³⁵S met and ³⁵S cys for 1 h at 2, 4, 6 and 8 hpi before SDS PAGE and autoradiography. A representative gel of 1 of 2 independent experiments that demonstrated the same patterns is shown in Figure 17a. The rate of viral protein synthesis was enhanced by the F103L mutation at all time points as demonstrated for the M1 and NS1 bands with the most dramatic increase shown early after infection (at 2 hpi). M106I also enhanced viral protein synthesis at 2 hpi but not at later time points (Figure 17a). The double mutant produced similar or reduced levels of protein synthesis compared to rPR8-HK-NS-wt at all times (Figure 17a). These data indicated that each of the F103L and M106I mutations resulted in more rapid gene expression seen at the level of protein synthesis, but that these mutations in combination were not as effective at enhancing protein synthesis.

The same experiment was also performed in mouse M1 kidney cells. Overall the rate of viral protein expression was less pronounced in M1 compared to MDCK cells indicating host restriction of replication in the mouse cell line. Viral protein synthesis was first detected at 6 hpi by the rPR8-HK-NS-wt as observed for the M1 and NS1 bands (Figure 17b). The F103L NS1 mutation also enhanced the rate of viral protein synthesis in the mouse cell line at 6

and 8 hpi with a remarkable increase observed at 8 hpi compared to the rPR8-HK-NS-wt virus (Figure 17b). However, the M106I containing mutants did not enhance viral protein synthesis in M1 cells as viral protein expression was only detected at 6 hpi by the M106I mutation followed by viral protein degradation as observed for the M1 and NS1 at 8hpi. The double mutant rPR8-HK-NS-F103L+M106I mutant expressed similar to decreased protein levels compared to the rPR8-HK-NS-wt (Figure 17b), a phenotype that was also observed in MDCK cells. Therefore, the F103L mutation results in a large increase in viral protein synthesis compared to rPR8-HK-NS-wt in both canine and mouse cell lines. The rPR8-HK-NS-M106I mutant that also enhanced viral protein synthesis in MDCK cell line showed an opposite effect in M1 cells indicating a specific host-virus relationship. However both mutations in combination resulted in an antagonistic effect such that they were not effective at improving viral protein synthesis in canine or mouse cells.

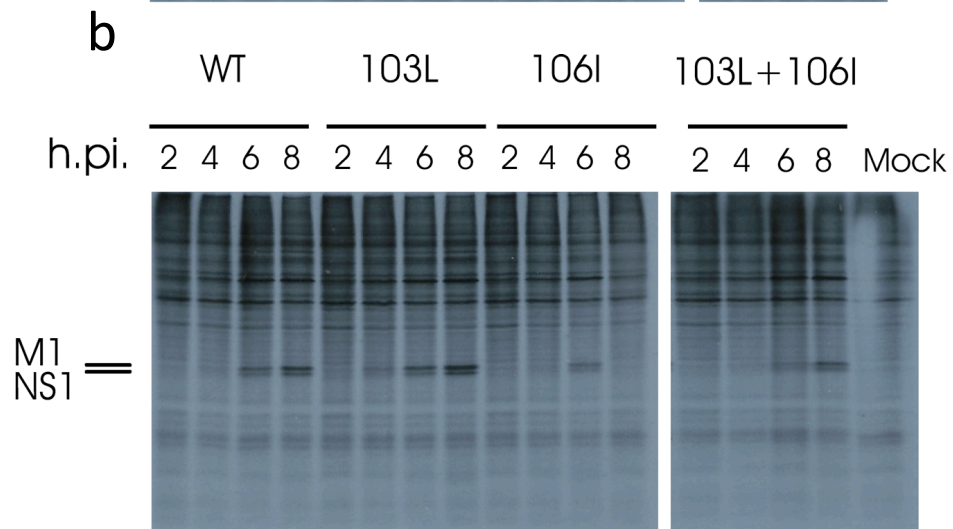
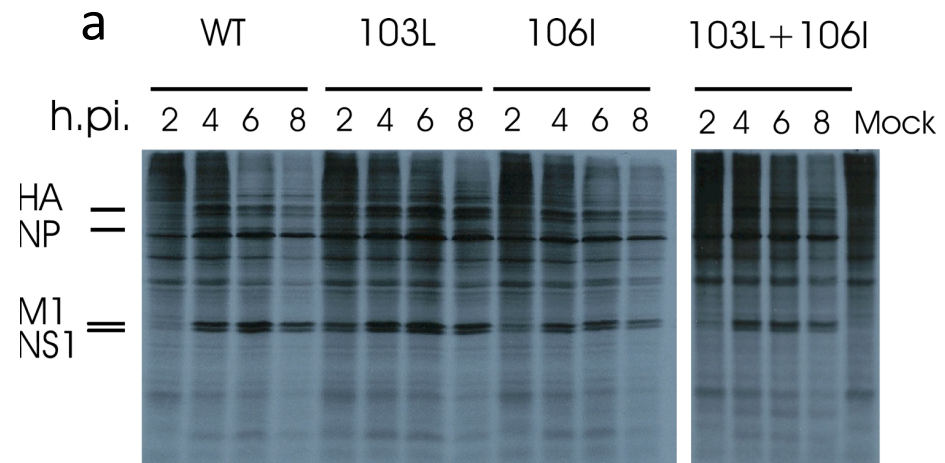


Figure 17 **Effect of F103L, M106I, and F103L + M106I mutations in the A/HK/1/68 NS1 on the rate of viral protein synthesis in MDCK and M1 cells** **a.** MDCK cells were infected with each of the rPR8 viruses at a MOI=3 and pulsed for 1 hour with ³⁵S at 2, 4, 6, and 8 hpi. Cell lysate was collected after pulse and used for SDS page and autoradiography. The experiment was performed twice and a representative SDS-page analysis is shown **b.** The same experiment was repeated in M1 cells.

4.6 NS1 mouse adaptive mutations F103L and M106I increase replication in IFN- β treated and untreated mouse cells

NS1 is involved in IFN antagonism as the NS1 deleted IAV A/PR/8/34 mutants are only capable of replicating in IFN unresponsive cells or mice such as Vero cells or in STAT-1 knockout mice [149]. Therefore, to determine whether the difference in virulence and replication between the mutant viruses and the rPR8-HK-NS-wt was due to NS1 IFN antagonistic effect, the viral growth in murine M1 cells was assessed in the presence and absence of IFN- β pre-treatment. Monolayers of M1 cells were either untreated or pre-treated with 200 U/ml of murine IFN- β for 24 hours. Twenty-four hours post treatment, cells were washed with PBS and infected in triplicate with the different rPR8 viruses at MOI=1 in the presence of trypsin (1 μ g/ml) and the cell supernatants were collected at 0, 8, 24 and 48 hpi. Without prior IFN- β treatment, the rPR8-HK-NS-wt reaches a peak of 3.8×10^4 pfu/ml at 24 hpi that was lower than each of the single mutants at that time, where each of the mutations (F103L and M106I) enhanced growth at the earliest (8 hpi) (by > 6 fold and >4 fold for the F103L and M106I mutants respectively) and at the latest time point (48 hpi) compared to rPR8-HK-NS-wt ($P < 0.01$) (Figure 18a). The double mutant grew better than the rPR8-HK-NS-wt at 8 hpi but to lower yields compared to each of the single mutants, and was reduced to lower than the rPR8-HK-NS-wt levels thereafter. Following IFN- β pretreatment, the rPR8-HK-NS-wt reached a peak of 3×10^3 pfu/ml at 8 hpi and all the viral mutants grew better than the rPR8-HK-NS-wt at this time ($P < 0.01$ for the single mutants and $P < 0.05$ for the double mutant) (>7 fold increase by F103L mutant). At later time points (24 and 48 hpi) only the single mutants 103L and 106I were able to enhance

growth in the presence of IFN- β relative to rPR8-HK-NS-wt ($P \leq 0.01$) (Figure 18b). Thus, following IFN- β treatment, the rPR8-HK-NS-F103L and rPR8-HK-NS-M106I mutants had demonstrated enhanced growth throughout infection relative to rPR8-HK-NS-wt, but enhanced growth was only seen at early times for the double mutant.

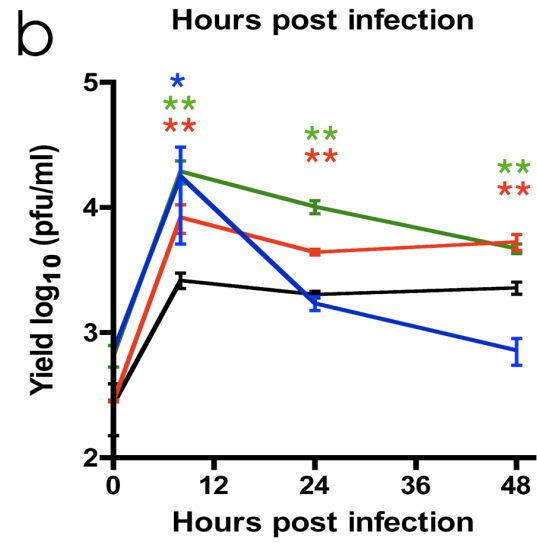
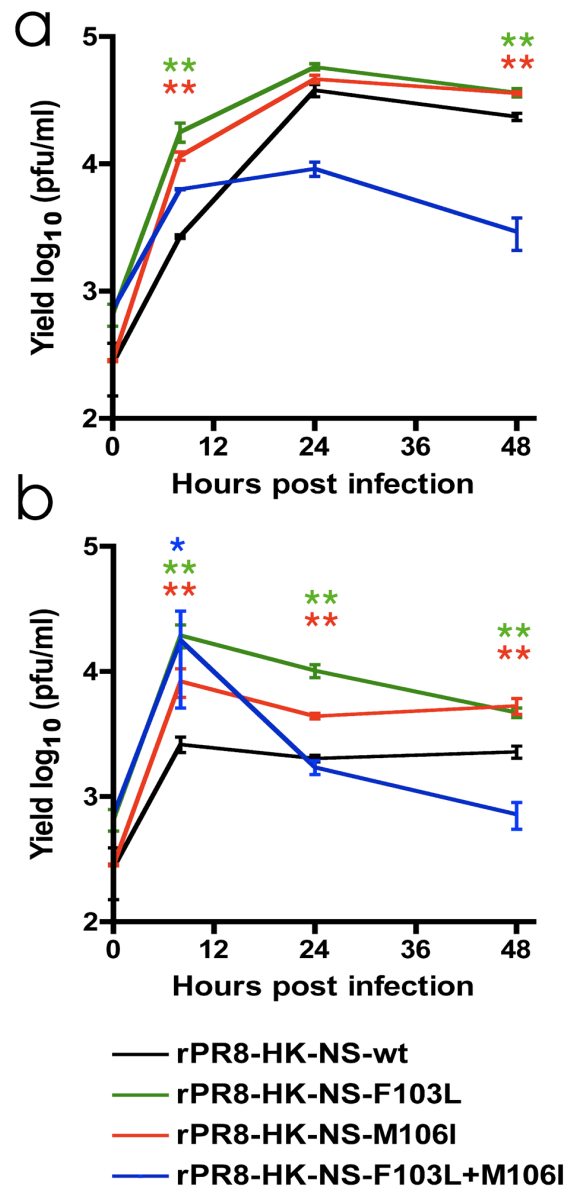


Figure 18 Interferon sensitivity of the rPR8 viruses in mouse epithelial cell lines. a. Growth curve of the rPR8 mutant viruses in M1 cells. Monolayers of M1 cells were infected with each of the rPR8 viruses at MOI=1. Cell supernatants were collected at 0, 8, 24 and 48 hpi. Viral titer was assessed by plaque assay. **b. Growth curve of the rPR8 mutant viruses in M1 cells in the presence of IFN- β .** Monolayers of M1 cells were treated with 200U/ml of murine IFN- β . Twenty-four hours post treatment; monolayers were infected with each of the rPR8 viruses at MOI 1. Cell supernatants were collected at 0, 8, 24 and 48 hpi. Viral titer was assessed by plaque assay. Values are shown as average +/- standard deviation (*p<0.05, **p<0.01, *** p<0.001; two-tailed equal variance student's t-test).

4.7 Measurement of the extent of Interferon- β , NF- κ B, ISRE and IRF-3 transcription inhibition by the recombinant NS1 F103L and M106I proteins

Following infection, the viral RNA products are recognized by IAV PRR (i.e. RIG-I, TLR-3 and TLR-7) thus initiating a cascade of events including the phosphorylation and dimerization of IFN- β transcription factors IRF-3 and NF- κ B which then translocate to the nucleus and drive the transcription of ISRE promoters resulting in the induction of type 1 IFN. The effect of NS1 protein at inhibiting IFN induction have been demonstrated both at the pre-transcriptional level and post transcriptional level as well as IFN signaling (reviewed in [88]). To determine whether the enhanced viral fitness of the NS1 F103L and M106I mutations would also correlate with increased NS1 ability to limit IFN- β production at the pre-transcriptional level, the inhibitory effect of NS1 recombinant proteins on IFN- β , NF- κ B, ISRE and IRF-3 promoter transcriptional activation was determined *in vitro*. Monolayers of 293-T cells were transfected with the bidirectional plasmids expressing mutant (F103L, M106I and F103L+M106I) or HK-wt NS gene segments with a firefly luciferase reporter plasmid driven by the human IFN- β , NF- κ B, ISRE and IRF-3 promoters, then triggered promoter activation upon transfection with synthetic dsRNA (poly-I:C). The HK-wt NS1 protein was effective at inhibiting poly I:C induced transcriptional activation of the human IFN- β , NF- κ B, ISRE and IRF-3 promoter compared to the empty vector control (pLLB) (Figure 19). Surprisingly, the NS1 mutant F103L was comparable to the HK-wt at inhibiting IFN- β , NF- κ B, ISRE and IRF-3 transcriptional activation (Figure 19). However the NS1 mutants M106I and F103L+M106I demonstrated significantly decreased ability to inhibit human IFN- β ($p<0.01$), NF- κ B ($p<0.001$), ISRE ($p<0.001$) and IRF-3 ($p<0.001$) promoter activation (Figure

19). Therefore the HK-wt NS1 protein is capable of preventing IFN- β transcription as well as promoter activation of IFN- β transcription factors NF- κ B and IRF-3 compared to the empty bidirectional vector. However the NS1 F103L and M106I mutations did not improve the ability of the HK-wt NS1 recombinant protein to inhibit IFN- β , NF- κ B, ISRE and IRF-3 transcription in human 293T cells. Therefore the enhanced replicative abilities of HK-NS possessing F103L and M106I can not be attributed to a better ability to inhibit IFN- β transcriptional activation in human embryonic cells *in vitro*.

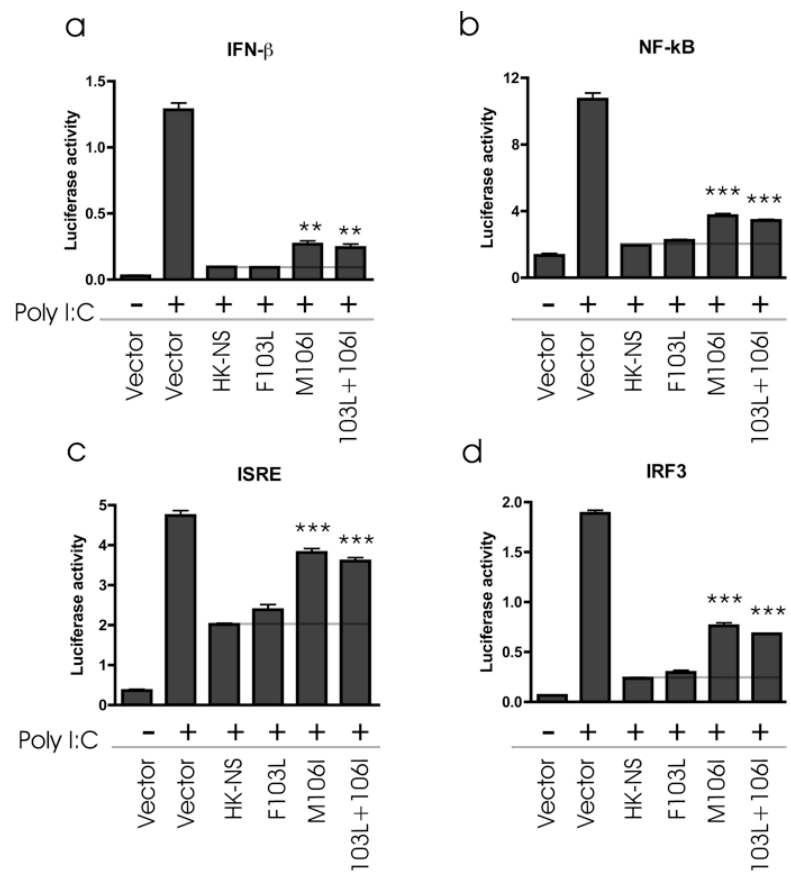


Figure 19 Mouse-adapted NS1 M106I and F103L+M106I mutations affect poly I:C induced IFN- β , IRF-3, NF- κ B and ISRE promoter activation *in vitro*.

a. IFN- β promoter activation was measured in human 293-T cells in triplicate for the HK-NS-wt and the three NS mutant (F103L, M106I and F103L+M106I) expression plasmids using a dual-luciferase reporter assay. Each of the NS expression plasmids along with the firefly luciferase p125 under the control of the human IFN- β promoter as well as the renilla luciferase control pRL-SV40 reporter plasmids were transfected in 293T cells. Sixteen hours following transfection, IFN- β promoter activation was stimulated by poly I:C transfection. The luciferase activities were measured following a 24h incubation period. **b.** The same experiment was repeated using the firefly reporter plasmid under the control of the human NF- κ B promoter **c.** The same experiment was repeated using the firefly reporter plasmid under the control of the human ISRE promoter **d.** The same experiment was repeated using the firefly reporter plasmid under the control of the human IRF-3 promoter. Data represent the means $\pm$ SD (* p <0.05, ** p <0.01, *** p <0.001; two-tailed student's t-test) of firefly luciferase activity relative to renilla.

4.8 Interferon induction by NS1 mutants in M1 cells

As both the F103L and M106I mutations were associated with greater levels of growth than the double mutant at later times following IFN- β pretreatment, we assessed the amount of IFN- β production during infection in the same mouse cell line, which could explain the differences in mutant gene functions among these mutants. To assess the roles of our NS mutants in controlling IFN- β production, monolayers of M1 cells were infected with each of the rPR8 viruses at MOI=2. Twenty-four hours post infection, cell supernatants were collected and IFN- β production was assessed by ELISA. Infection with the rHK-wt and the rPR8-wt viruses resulted in low or undetectable levels of IFN- β respectively indicating that both NS1 (and possible roles of the NEP) proteins can suppress IFN- β induction on their respective genetic backbones. The rPR8-HK-NS-wt recombinant also induced low levels of IFN- β (22 pg/ml) whereas each of the recombinants rPR8-HK-NS-F103L or rPR8-HK-NS-M106I induced 2 and 3 fold more IFN- β respectively compared to rPR8-HK-NS-wt (40 and 68 pg/ml respectively) while the double mutations together had a cumulative effect on increasing IFN- β induction to 113 pg/ml ($P<0.001$) (Figure 20). The increased IFN- β induction levels produced by the different mutants is consistent with the decreased ability of the respective NS1 recombinant proteins to antagonize IFN- β transcriptional activation in 293T cells. The reduced viral growth by the rPR8-HK-NS-F103L+M106I virus at later times in M1 cells relative to rPR8-HK-NS-wt also to the increasing amounts of IFN- β that have accumulated to exert an antiviral effect (as shown by the effect of IFN- β pretreatment in Figure 18b). The extent of viral growth was thus inversely correlated with IFN- β induction where the rPR8-HK-NS-F103L virus grew to the highest yield in M1 cells in the presence and

absence of IFN- β and induced the least amount of IFN- β , while the double mutant grew the least with and without IFN- β pre-treatment and induced the highest levels of IFN- β compared to the rest of the mutants. The increased IFN- β induction by the double mutant explains the reduced replication of this virus relative to the single mutants.

We also inserted the A/HK/156/97 (H5N1) NS and A/Ck/Bj/1/95 (H9N2) NS genome segments possessing both the 103L and 106I NS1 residues into the mouse adapted PR8 and WSN backbones respectively. The levels of IFN- β induction in M1 cells following infection with the recombinants that possess the H5N1 NS and H9N2 NS genes were also measured and showed minimal levels of IFN- β induction at 24 hpi (Figure 20). Unlike the rPR8-HK-NS virus that increased IFN- β levels when the HK-NS substituted the PR8-NS (20 pg/ml vs. undetected levels respectively (Figure 20)), the substitution of the PR8-NS by H5N1-NS on the rPR8 backbone resulted in the same IFN- β levels compared to the rPR8-wt virus (undetected IFN- β levels for both rPR8-wt and rPR8-H5N1-NS (Figure 20)). Similarly, the rWSN-H9N2-NS also produced the same IFN- β levels 24h following infection compared to the rWSN virus (Figure 20). This suggests that the rPR8-H5N1-NS and rWSN-H9N2-NS viruses might provide a better model for understanding the effects of the NS1 mutations in a system that controlled the level of IFN- β induction. Mutating individually the NS1 sites 103L and 106I to L103F and I106M in either of the NS genes (H9N2 and H5N1) resulted in viruses of low and comparable interferon induction ($P>0.05$, t-test unequal variance) indicating a role of other NS1 gene mutations in the regulation of IFN- β induction by these viruses. Interestingly when we mutated both 103L and 106I NS1 sites back to consensus L103F+I106M in the H5N1 NS1 gene, the IFN- β levels were increased *in vitro* ($P<0.001$)

(Figure 20). The effectiveness of the influenza NS1 mutants to inhibit IFN- β induction was shown in comparison with the VSV Δ 51 mutant that has a defect in its ability to block innate immunity and resulted in 1,306 pg/ml IFN- β . We can conclude that IFN- β induction is controlled by multiple genetic factors involving the nature of the NS1 gene as well as interactions with the genetic backbone. Furthermore, the adaptive nature of the 103L and 106I NS1 mutations was next investigated in the H5N1 and H9N2 NS genes onto the mouse adapted backbones A/PR/8/34 (H1N1) and A/WSN/1/33 (H1N1) respectively that do not induce elevated levels of IFN- β *in vitro*.

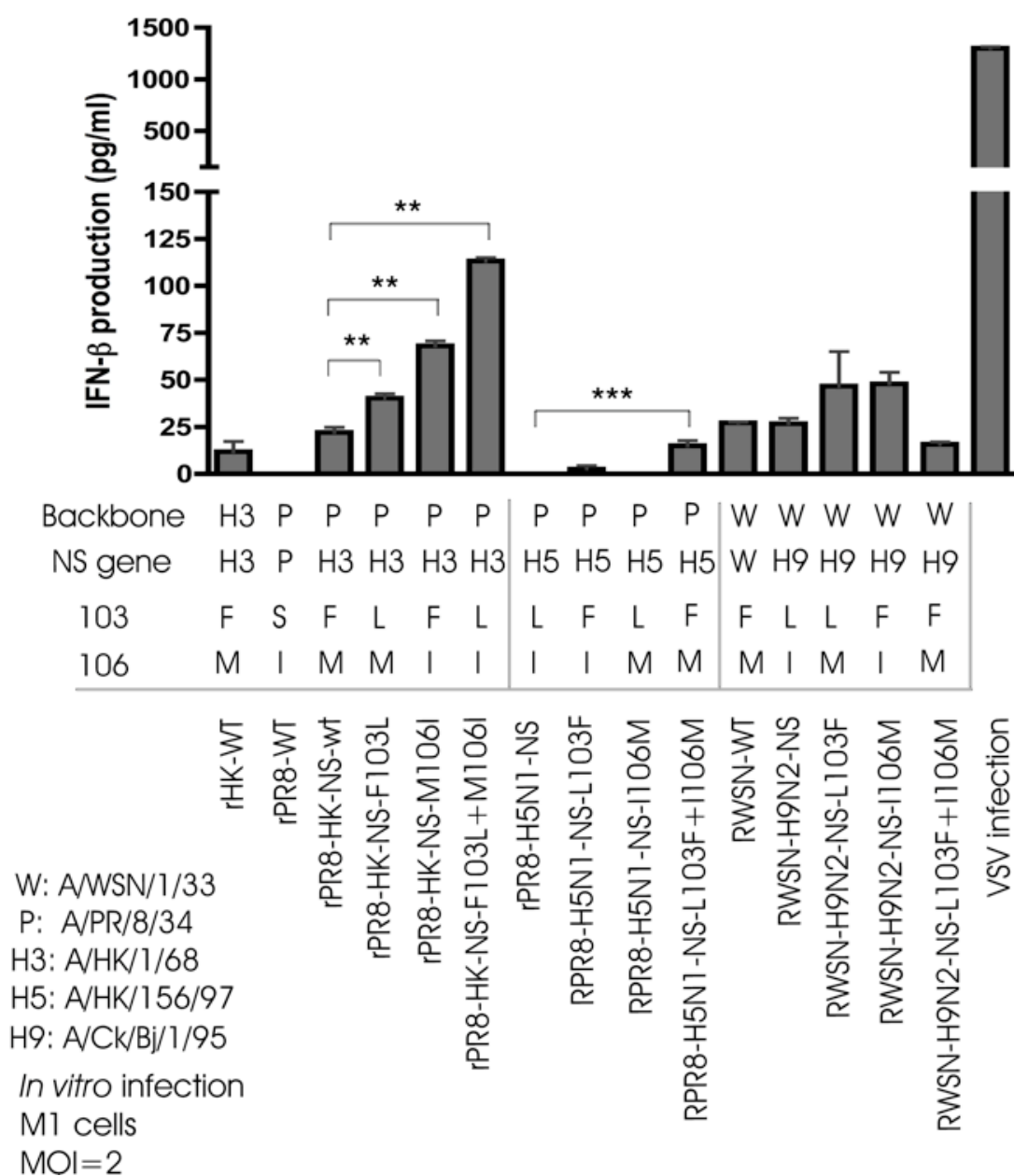
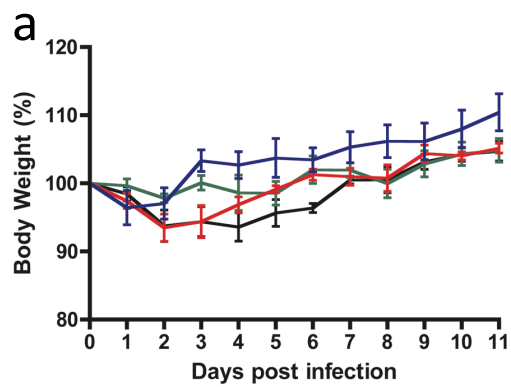


Figure 20 Interferon production in M1 cells. Monolayers of M1 cells were infected with the parental recombinant viruses rHK-wt, rPR8-wt and rWSN-wt as well as the different recombinant viruses: rPR8-HK-NS, rPR8-H5N1-NS and rWSN-H9N2-NS and their respective mutants at the 103 and 106 sites (rPR8-HK-NS-F103L, rPR8-HK-NS-M106I and rPR8-HK-NS-F103L+M106I) (rPR8-H5N1-NS-L103F, rPR8-H5N1-NS-I106M and rPR8-H5N1-NS-L103F+I106M) (rWSN-H9N2-NS-L103F, rWSN-H9N2-NS-I106M and rWSN-H9N2-NS-L103F+I106M) at MOI=2. Twenty-four hours post infection, the supernatants were collected and the level of IFN- β production was assessed by ELISA. The IFN- β levels were compared to a positive control (VSV Δ 51 infection). Values are shown as average +/- standard deviation (*p<0.05, **p<0.01, *** p<0.001; two-tailed student's t-test).

4.9 F103L and 106I in H5N1 NS1 control virulence in CD-1 mice

To further assess the virulence associated with 103L and 106I sites, the H5N1-NS, that possess the L and I amino acids in the 103 and 106 sites respectively, was rescued in A/PR/8/34 (H1N1) and A/HK/1/68 (H3N2) backbones. The virulence of these viruses on the PR8 and the HK backbones were determined in groups of 3 CD-1 mice following intranasal infection with 10^4 pfu and 5×10^6 pfu respectively. The rHK-H5N1-NS and the three mutants (rHK-H5N1-NS-L103F, rHK-H5N1-NS-I106M and rHK-H5N1-NS-L103F+I106M) did not result in any mortality in CD-1 mice ($LD_{50} > 10^{7.2}$) throughout the study. Body weight loss recorded daily showed a slight decrease in body weight loss (~ 7% at day 5 pi) by the rHK-H5N1-NS virus. When mutated back to consensus, the rHK viruses possessing L103F, I106M and L103F+I106M showed a decrease in body weight loss compared to the rHK-H5N1-NS wildtype ($P < 0.01$, $P < 0.05$, $P < 0.001$ respectively) (Figure 21a). The rPR8-H5N1-NS-(103L+106I) resulted in 100% mortality by 8 dpi indicating a LD_{50} of $10^{2.75}$ that was associated with an average 26% body weight loss at the time of death (Figure 21b). We then mutated the 103L and 106L sites back to consensus to generate PR8 recombinants possessing the H5N1-NS-L103F, H5N1-NS-I106M and H5N1-NS-L103F+I106M. The single mutants H5N1-NS-L103F, H5N1-NS-I106M increased the LD_{50} to $10^{3.0}$ and $10^{3.6}$ respectively and resulted in similar body weight loss compared to the virulent wildtype rPR8-H5N1-NS. The rPR8-H5N1-NS-L103F+I106M did not cause any mortality in mice, LD_{50} of $> 10^{6.5}$; in addition, the infected mice only lost 4% of their body weight at day 2 pi which was consistent with a loss of virulence ($P < 0.001$) (Figure 21b). Therefore, each of the 103L and

106I mutations were associated with severe disease, and increased virulence by >1000 fold in the H5N1-NS gene when assessed on the mouse adapted A/PR/8/34 backbone.



— rHK-H5N1-NS
 — rHK-H5N1-NS-L103F **
 — rHK-H5N1-NS-I106M *
 — rHK-H5N1-NS-L103F+I106M ***

Dose of infection: 5×10^6 pfu/ml

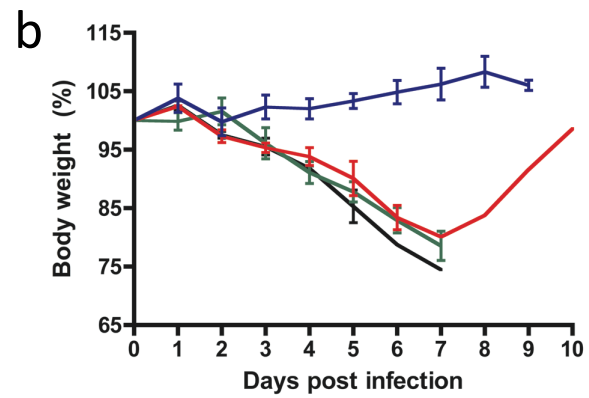
CD-1 mice

Paired t-test

*P<0.05

** P<0.01

***P<0.001



— rPR8-H5N1-NS
 — rPR8-H5N1-NS-L103F
 — rPR8-H5N1-NS-I106M
 — rPR8-H5N1-NS-L103F+I106M ***

Dose of infection: 10^4 pfu/ml

CD-1 mice

Paired t-test

***P<0.001

Figure 21 **Body weight loss is increased by 103L and 106I NS1 mutations in H5N1-NS on both the rPR8 and rHK recombinant viruses.** **a.** Groups of 3 CD-1 mice were infected intranasally with 5×10^6 pfu with the different rHK viruses. **b.** Groups of 3 CD-1 mice were infected intranasally with 10^4 pfu with the different rPR8 viruses. The percent of body weight loss was calculated as the mice body weight loss was recorded daily throughout the whole course of the experiment. Values are shown as average $\pm$ standard deviation (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; two-tailed paired student's t-test).

4.10 F103L and M106I mutations in the H5N1-NS gene control the extent of viral spread in the alveoli and bronchi

Although the reduced disease severity by L103F and I106M NS1 mutations was observed on both the HK and PR8 backbones, the effect was much more pronounced on the PR8 backbone. Therefore to further assess the effect of the F103L and M106I mutations on the H5N1-NS gene, the rPR8 backbone viruses were used. The extent of CD-1 mouse lung infection and tropism was determined by immunofluorescent staining on frozen lung tissues at 3 days post intranasal infection with 10^5 pfu. The lungs infected with the rPR8-H5N1-NS showed extensive virus infection and spread that encompassed large regions of the bronchi as well as the alveolar regions (Figure 22b). When mutated back to consensus L103F and I106M NS1 single mutants showed a decrease in the extent of alveolar tissue infection as well as a decreased bronchiolar infection that was more pronounced for the rPR8-H5N1-NS-I106M mutant compared to rPR8-H5N1-NS (Figure 22b, c, d). Small regions of bronchiolar and alveolar infection were observed for the rPR8-H5N1-NS-L103F+I106M mutant (Figure 22e). The enhanced tropism and spread observed by increased bronchiolar and alveolar infection in the lung further proves the increased ability of 103L and 106I NS1 mutations to dramatically enhance viral replication and disease severity.

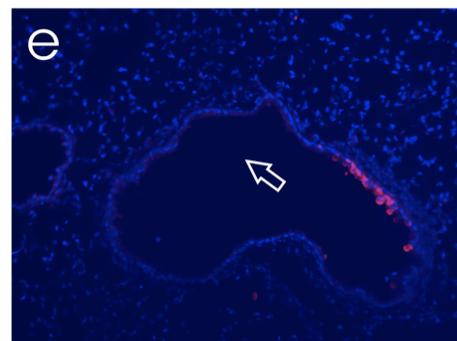
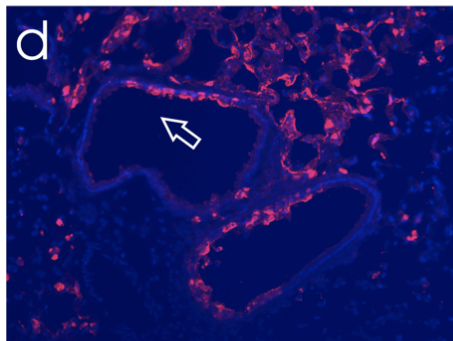
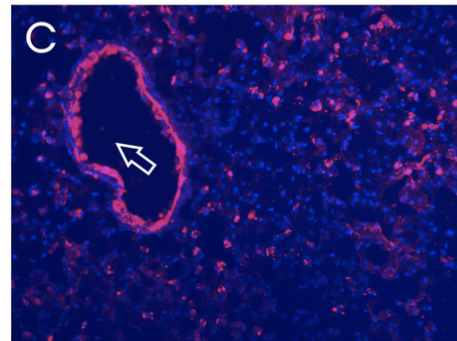
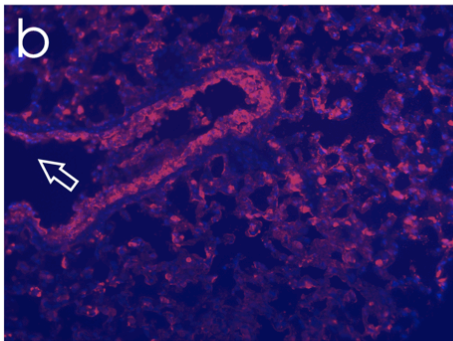
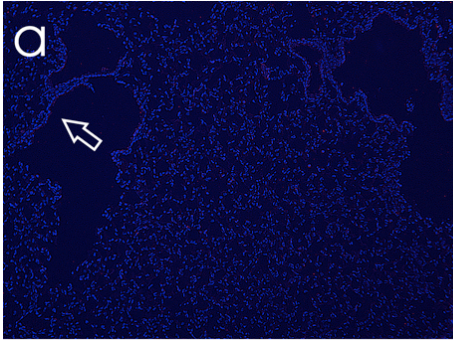


Figure 22 **103L, 106I and 103L+106I mutations in the H5N1-NS gene enhance viral tropism in CD-1 lungs.** CD-1 mice were infected intranasally with 10^5 pfu and lungs were collected 3 dpi. The lungs were inflated and fixed with 3.7% formaldehyde and frozen lung sections were stained with anti-PR8 antibody. The bronchi regions are indicated by an arrow. **A.** Mock uninfected lungs **B.** rPR8-H5N1-NS infected lungs **C.** rPR8-H5N1-NS-L103F infected lungs **D.** rPR8-H5N1-NS-I106M infected lungs **E.** rPR8-H5N1-NS-L103F+I106M infected lungs

4.11 103L and 106I in H5N1-NS increase viral growth in CD-1 lungs but do not affect IFN- β lung levels

Given that the H5N1 NS gene possessing the 103L and 106I residues increased virulence, disease severity and lung tropism *in vivo* another aspect of virulence, viral growth in lung tissues, was also assessed in CD-1 lungs. Twelve CD-1 mice were infected intranasally with 5×10^3 pfu in a 50 μ l volume of the rPR8-H5N1-NS virus possessing the NS1 103L+106I residues as well as the three mutants (rPR8-H5N1-NS-L103F, rPR8-H5N1-NS-I106M and rPR8-H5N1-NS-L103F+I106M). Lungs from 3 mice were collected at days 1, 3, 5 and 7 pi and the lung titers were determined by plaque assay. The rPR8-H5N1-NS virus possessing the L+I residues on the 103 and 106 NS1 sites respectively, grew to a high titer in CD-1 mouse lungs and reached a peak titer of 1.05×10^8 pfu/g at day 3 pi (Figure 23a). When each of the 103L and 106I sites in the H5N1-NS gene was mutated back to consensus, virus growth of rPR8-H5N1-NS-L103F and rPR8-H5N1-NS-I106M were similar to the rPR8-H5N1-NS virus that possessed both 103L+106I sites ($P > 0.05$ paired t-test) (Figure 23a). However when both sites were mutated in combination, the H5N1-NS-L103F+I106M virus decreased viral growth dramatically (>100 fold at day 5 pi) ($P < 0.01$ paired t-test) compared to the rPR8-H5N1-NS and the virus was cleared from the lungs by day 7 pi (Figure 23a). Therefore the presence of either one of the 103L and/or 106I mutations in the H5N1 NS gene resulted in a dramatic increase in virus growth and a delay in viral clearance in lungs. To further explore whether the >100 fold increase in viral growth associated with the combination of 103L+106I mutations correlated with an increase in IFN- β production eighteen CD-1 mice (6 mice/time point) were infected intranasally with 5×10^3 pfu of rPR8-

H5N1-NS and rPR8-H5N1-NS-L103F+I106M viruses. Lungs were collected at 1, 3 and 5 dpi and homogenized before determining IFN- β levels by ELISA. Both rPR8-H5N1-NS and rPR8-H5N1-NS-L103F+I106M viruses produced similar lung IFN- β levels at days 1 and 3 pi ($P>0.05$, equal variance t-test) (Figure 23b) even though the rPR8-H5N1-NS-L103F+I106M and rPR8-H5N1-NS viruses different in yield by >100 fold indicating a reduced IFN- β production due to the F103L+M106I mutations when standardized for amount of growth. The 103L+106I residues associated with >100 fold increase in viral growth (day 5 pi) also increased lung IFN- β levels at day 5 pi although not to a statistically significant level compared to the virus possessing L103F+I106M mutations (Figure 23b). Therefore the increase in viral growth, virulence, and lung tissue tropism associated with the 103L+106I residues may have been, at least in part, due to a better ability of the H5N1 NS protein to inhibit lung IFN- β levels at early times post infection. The data are consistent with increased replication associated with decreased IFN- β induction at early times but that the increased infection of lung tissues eventually induced high levels of IFN- β that are associated with toxic levels which have been associated with cytokinemia as has been described in human H5N1 infections [12].

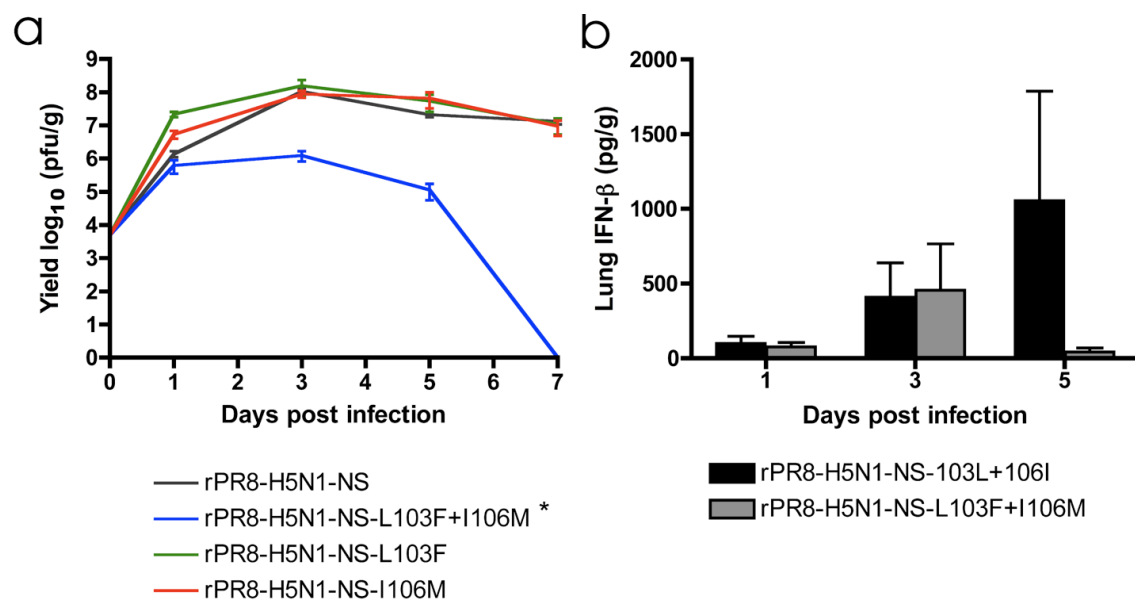


Figure 23 **103L, 106I and 103L+106I mutations in the H5N1-NS gene enhance viral growth in CD-1 lungs but do not affect IFN- β of the lung homogenates.** **a.** Groups of 12 CD-1 mice were infected intranasally with 5×10^3 pfu of the rPR8-H5N1-NS and the three mutants (rPR8-H5N1-NS-L103F, rPR8-H5N1-NS-I106M and rPR8-H5N1-NS-L103F+I106M). Lungs were collected at days 1, 3, 5 and 7 pi and viral titers were assessed by plaque assay of the lung homogenates **b.** Groups of 18 CD-1 mice were infected intranasally with 5×10^3 pfu of the rPR8-H5N1-NS and the rPR8-H5N1-NS-L103F+I106M. The levels of IFN- β lung homogenates were determined by ELISA at days 1, 3 and 5 pi. Values are shown as average +/- standard deviation (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; two-tailed paired student's t-test).

4.12 IFN- β transcription inhibition and cleavage and polyadenylation specificity factor (CPSF-F2F3) binding

The rPR8-H5N1-NS that possessed the 103L+106I mutations was associated with a significant decrease in *in vitro* IFN- β production compared to the respective virus possessing L103F+I106M consensus NS1 residues. Although the *in vitro* IFN- β production inhibition was not observed for the *in vivo* lung IFN- β levels, the similar level of IFN- β seen in mouse lungs were associated with >100 fold more virus suggesting that the rPR8-H5N1-NS 103L+106I residues were inducing reduced levels of IFN- β when standardized to the level of infection. To further understand the ability of the 103L+106I NS1 sites to affect IFN- β production, different aspects of NS1 IFN inhibition were examined at the pre-transcriptional level (by assessing IFN- β promotor activation) as well as the post-transcriptional level (by assessing CPSF binding affinity). Since the 103 and 106 sites map to CPSF binding domain (Figure 7), we determined whether the NS1 mutations selected upon mouse adaptation affected NS1 binding to the F2F3 fragment of CPSF30 in the context of H5N1-NS gene. Briefly, H5N1-NS, H5N1-NS-L103F, H5N1-NS-I106M and H5N1-NS-L103F+I106M gene segments as well as FLAG-tagged CPSF-F2F3 (FLAG-F2F3) fragment were expressed *in vitro* in the presence of ^{35}S -methionine and cysteine. Equal expression of each NS protein as well as FLAG-F2F3 was verified by SDS-Page electrophoresis followed by autoradiography (Figure 24a). In an α -FLAG pulldown assay, *in vitro* translated and ^{35}S -labeled H5N1-NS and the three mutant NS proteins were mixed with FLAG-F2F3 or H_2O as a control and precipitated using Protein G Dynabeads (Invitrogen, Burlington, Ontario) pre-incubated with α -FLAG M2 antibody. Following incubation and washing, bound proteins

were eluted in SDS buffer and subject to SDS-PAGE electrophoresis followed by autoradiography (Figure 24b). The H5N1 possessing either the L+I or F+I on the 103 and 106 NS1 sites show undetectable NS1 binding affinity to F2F3 fragment *in vitro*. However, the mutation I106M in the H5N1-NS gene restored binding to F2F3 and the binding was maintained when both 103 and 106 sites possessed both consensus amino acids F+M. Therefore we can conclude that the presence of 106M in the H5N1-NS was sufficient for CPSF-F2F3 binding and more importantly could compensate for the deficit in binding due to the 103L mutation as reported by Forbes *et al.* [160]. However, the F2F3 binding data did not correlate with the IFN- β levels following infections of M1 cells with rPR8-H5N1-NS and the three mutants (Figure 20), therefore a different aspect of IFN- β inhibition at the pre-transcriptional level was assessed. Transfection experiments were performed as described earlier (section 4.7) where monolayers of 293T cells were transfected independently with the H5N1-NS bidirectional plasmid or the H5N1-NS-L103F, H5N1-NS-I106M and H5N1-NS-L103F+I106M along with a firefly reporter plasmid under the control of human IFN- β and IRF-3 promoters and the levels of IFN- β and IRF-3 promoters' transcriptional activation was compared. The H5N1-NS gene possessing the 103L+106I residues inhibited IFN- β transcription compared to the empty control vector however the presence of the consensus amino acids 103F, 106M and 103F+106M all decreased IFN- β promoter activation significantly compared to the H5N1-NS ($P<0.05$) (Figure 24c). Furthermore the greatest IFN- β transcription inhibition was observed by the NS1 gene possessing the F103L+I106M sites. Transcription inhibition of the IRF-3 promoter was also more pronounced by the 103L+I106M ($P<0.05$) (Figure 24d). As a conclusion, the combination of 103L+I106M was

associated with an ability to bind to CPSF-F2F3 and an enhanced IFN- β and IRF-3 inhibition at the level of transcription that translates to undetectable levels of IFN- β production following infection in mouse cells (Figure 20). However, the H5N1-NS gene possessing L+I residues which was not able to bind CPSF (Figure 24b) and possess the least ability of pre-transcriptional inhibition (Figure 24 c,d) was still capable of inhibiting IFN- β induction (Figure 20). This directs us to the possibility that other IAV viral genes are also playing a role in IFN- β inhibition as recently discovered [45, 46].

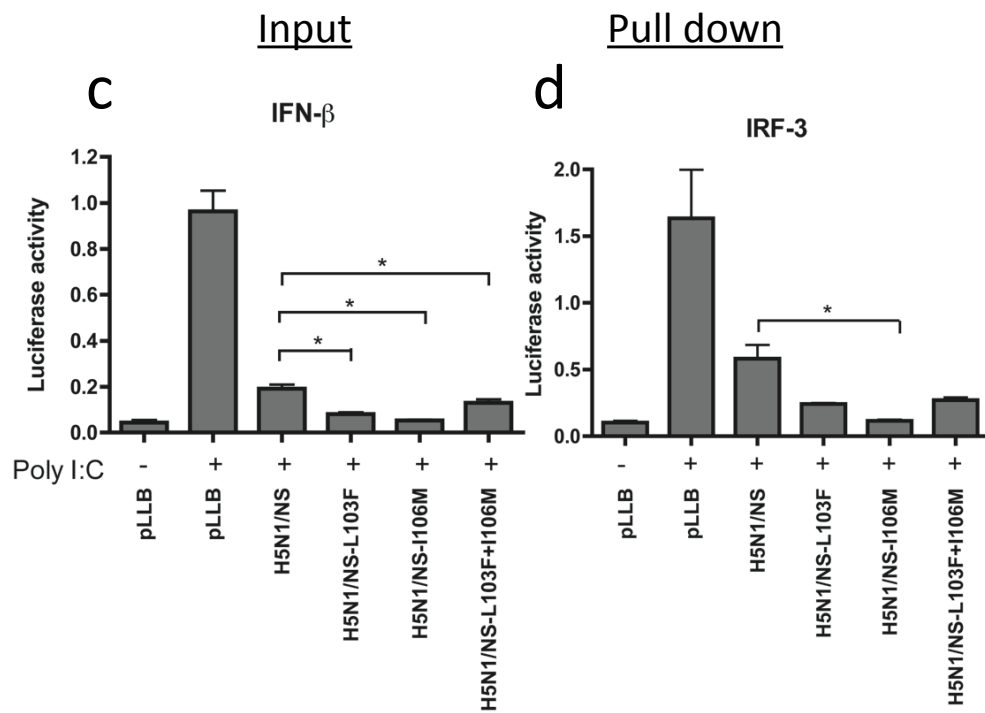
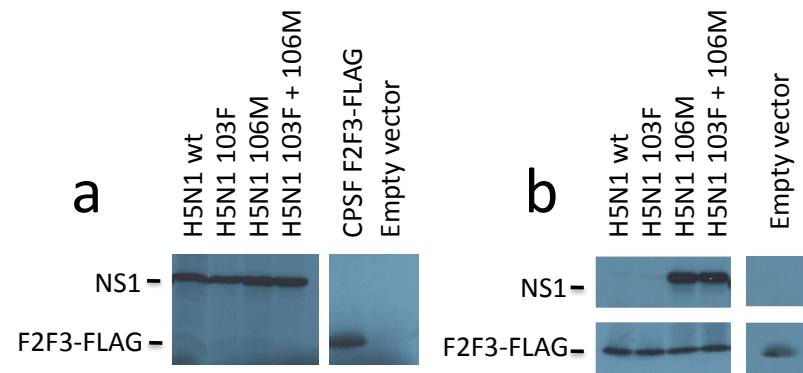


Figure 24 **The 103L and 106I residues in the H5N1-NS gene affect IFN- β production at the pre-transcriptional (IFN- β and IRF-3 promoter transcriptional activation) and post-transcriptional levels (CPSF-30 F2F3 segment binding affinity *in vitro*).** **a.** H5N1-NS1 proteins (wt and mutants) as well as CPSF30-F2F3-FLAG were expressed by *in vitro* coupled transcription and translation of expression plasmids or empty vector in the presence of ^{35}S labeled methionine and cysteine and expression was evaluated by autoradiography. Radiolabeled NS1 proteins or empty vector were mixed with radiolabeled CPSF30- F2F3-FLAG and subject to pull-down using α -FLAG M2 antibody bound to Protein G Dynabeads then SDS-PAGE electrophoresis and autoradiography. **b.** Representative pull-down of 2 independent replicates. **c.** IFN- β promoter activation was measured in human 293-T cells in triplicate for the H5N1-NS-wt and the three NS mutant (L103F, I106M and L103F+I106M) expression plasmids using a dual-luciferase reporter assay. Each of the NS expression plasmids along with the firefly luciferase p125 under the control of the human IFN- β promoter as well as the renilla luciferase control pRL-SV40 reporter plasmids were transfected in 293T cells. Sixteen hours following transfection, IFN- β promoter activation was stimulated by poly I:C transfection. The luciferase activities were measured following a 24h incubation period. **d.** The same experiment was repeated using the firefly reporter plasmid under the control of the human IRF-3 promoter. Data represent the means $\pm$ SD (* p <0.05, ** p <0.01, *** p <0.001; two-tailed student's t-test) of firefly luciferase activity relative to renilla.

4.13 Role of polymerase genes in IFN- β inhibition

As recently revealed, the polymerases PB2, PB1 and PA play important roles in IFN- β antagonism at the level of inhibition of IFN- β transcriptional activation, which is more pronounced by the PB2 gene through an interaction with MAVS (Cardif, IPS-1) protein [45, 46]. To observe whether any of IAV genes is affecting IFN- β transcription, monolayers of 293T cells were transfected independently with each of the 8 recombinant HK plasmids with a firefly luciferase reporter plasmid driven by the human IFN- β promoter, when IFN- β promoter activation is triggered upon transfection with synthetic dsRNA (poly I:C). Interestingly each of the viral polymerase proteins (PB2, PB1, PA and NP) as well as the NS1 and NS2 recombinant proteins significantly decrease poly I:C mediated IFN- β promoter activation compared to the pLLB empty plasmid ($P < 0.001$) with the PA and NP being the most effective at repressing IFN- β transcription (Figure 25a). The HA, NA and M genes did not affect poly I:C induced IFN- β transcription *in vitro* compared to the pLLB control vector. To observe whether combinations of polymerase subunits and their possible heterodimers PB1+PA, PB2+PB1, and PB2+PA, as well as heterotrimer (PB2+PB1+PA) or this POL heterotrimer + NP have a synergistic effect at repressing poly I:C mediated IFN- β expression, the extent of IFN- β transcription inhibition following these various combinations of POL proteins was determined. The results indicated that IFN- β expression was mostly inhibited by the PB1+PA heterodimer as well as the heterotrimer PB2+PB1+PA, however none of these POL combinations enhanced IFN- β transcription inhibition significantly compared to the PA polymerase subunit that is the most effective viral POL subunit at decreasing IFN- β promoter transcriptional activation (Figure 25a). It is important

to note that the IFN- β transcription inhibitory activity mediated by NS1 protein was greater than that of any of the polymerase proteins. To further explore whether the POL subunit functions of inhibiting IFN- β is synergistic with that of NS1, each of the single IAV genes as well as the 5 different POL combinations stated above were transfected along with the HK-NS or the HK-NS-M106I mutant. The results showed that the 3 POL subunits when combined with the HK-NS recombinant protein did not reduce IFN- β promoter activation although compared to that mediated by HK-NS alone (0.124 ± 0.03 vs. 0.129 ± 0.04 , respectively) (Figure 25b).

However when the 3 POL subunits were expressed with the HK-NS-M106I recombinant protein the level of IFN- β promoter transcriptional activation inhibition was more pronounced although still not to a statistically significant point compared to the level of IFN- β inhibition mediated by M106I-NS alone (0.27 ± 0.09 vs. 0.41 ± 0.16 , respectively) (Figure 25b). Therefore we can conclude that the A/HK/1/68 POL as well as both NS1 and NS2 genes are capable of inhibiting Poly I:C mediated IFN- β promoter transcriptional activation. However, the ability of the individual POL genes, 3POL + NP, PB2+PA as well as the heterodimers to inhibit poly I:C induced IFN- β is not additive with that mediated by HK-NS or M106I-NS in 293T cells. Nevertheless, there is a possible trend towards an enhanced IFN- β promoter transcriptional inhibition mediated by the NS1-M106I when combined with the 3 POL proteins.

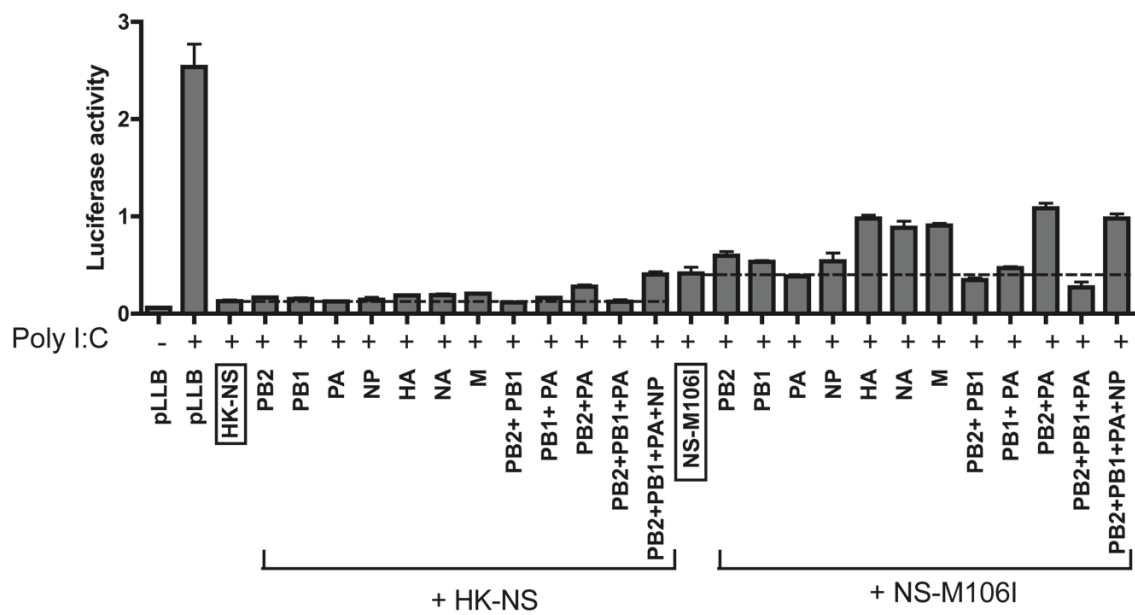
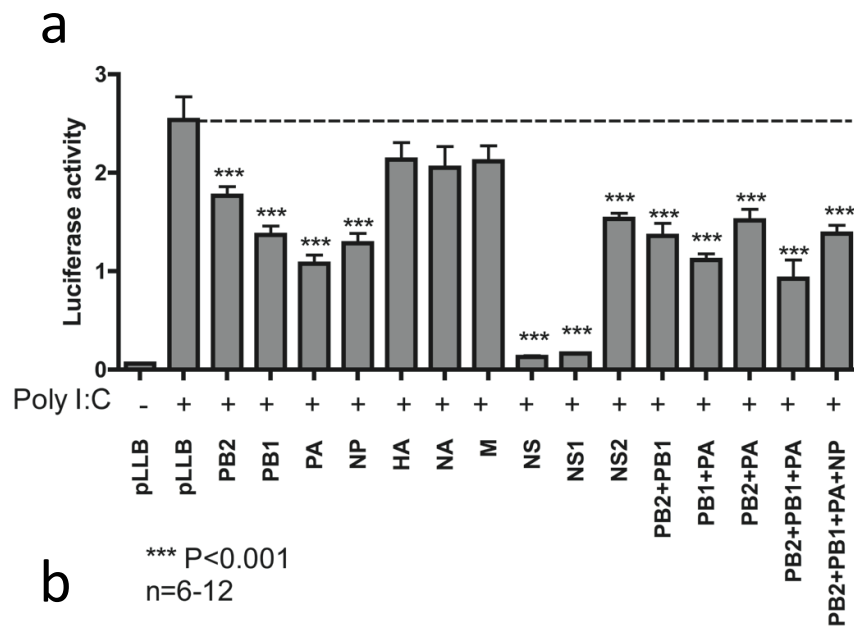


Figure 25 PB2, PB1, PA and NP proteins play a role in inhibiting IFN- β transcriptional activation *in vitro*. **a.** IFN- β promoter activation was measured in human 293-T cells in triplicate for each of the IAV expression plasmids using a dual-luciferase reporter assay. Each of the IAV expression plasmids along with the firefly luciferase p125 under the control of the human IFN- β promoter as well as the renilla luciferase control pRL-SV40 reporter plasmids were transfected in 293T cells. Sixteen hours following transfection, IFN- β promoter activation was stimulated by poly I:C transfection. The luciferase activities were measured following a 24h incubation period. **b.** The same experiment was repeated and the IFN- β promoter activation was measured in the presence of the HK-NS-wt or the NS-M106I mutant expression plasmids. Data represent the means $\pm$ SD (*p<0.05, **p<0.01, ***p<0.001; two-tailed student's t-test) of firefly luciferase activity relative to renilla.

4.14 103L and 106I in the H9N2 NS1 gene on the WSN backbone control virulence in CD-1 mice

The properties of 103L and 106I mutations were also studied in the context of the A/Ck/Bj/1/95 (H9N2) NS gene that was the donor of the A/HK/156/1997 (H5N1) NS1, that possesses both 103L and 106I residues, on another mouse adapted backbone A/WSN/33 backbone. We tested this backbone because WSN NS1 inhibits IFN- β induction by a different mechanism that includes CPSF-30 binding in contrast to PR8 NS1 that does not bind CPSF-30 [256] (reviewed in [88]) and recent findings that multiple polymerase components act to block IFN induction [45, 46] suggest that IFN antagonism is a function of multiple genes that may differ among strains. We then mutated the A/Ck/Bj/1/95 (H9N2) NS gene to possess L103F+I106M to generate the consensus sequence at these sites. The virulence and growth of the recombinant WSN virus having the unmodified H9N2-NS1 gene possessing 103L and 106I mutations was determined in BALB/c mice. The virus was highly virulent in BALB/c mice causing 100% mortality 5 dpi following intranasal infection with 10^6 pfu (Figure 26a). When both sites were mutated to L103F+I106M, the virus lost its ability to cause fatal infection at this dose in the mouse, as the virus did not cause any mortality throughout 14 dpi, thus the LD₅₀ was reduced by $>10^{1.0}$ fold (Figure 26a).

The ability of the two rWSN viruses (rWSN-H9N2-NS-103L+106I and rWSN-H9N2-NS-L103F+I106M) to grow in BALB/c mice was also determined by infecting groups of 8 mice intranasally at a lower infection dose of 5×10^3 pfu. The presence of the H9N2 NS1 103L+106I residues increased growth in BALB/c lungs by 100 fold at 1 dpi and >10 fold at 3 and 5 dpi relative to the L103F+I106M mutant that was significantly different by paired t-

test ($P \leq 0.01$) (Figure 26b). The growth was also more persistent at 7 dpi with >2 logs increased lung titer due to the presence of F103L+M106I mutations ($P < 0.05$) (Figure 26b). Thus the A/Ck/Bj/1/95 NS1 F103L+M106I mutations conferred both increased replication and virulence in mouse lungs.

We also assessed the extent of lung infection using immunofluorescent staining of infected lung sections to show that the presence of L103F and I106M mutations in the A/Ck/Bj/1/95 H9N2-NS1 gene on the WSN backbone resulted in lung infection that was restricted to specific foci in the bronchiolar epithelium (Figure 26c). In contrast, the presence of 103L and 106I in the H9N2-NS1 dramatically enhanced viral spread and tropism in bronchiolar and alveolar tissues to encompass all of the alveoli except for regions around the bronchioles (Figure 26d). The extent of lung infection correlated with viral replication and virulence for both viruses in BALB/c mice. Thus we have identified two mutations in the NS1 gene that mediated increased replicative fitness and viral tropism in the mouse lung. Therefore the presence of Leu at position 103 and an Ile at position 106 are necessary and sufficient for a virus with a human (A/HK/1/68) on the A/PR/8/34 backbone or the avian (A/Ck/Bj/1/95 and A/HK/156/97) NS genes on either the A/PR/8/34 or A/WSN/1933 backbones respectively, to become virulent and enhance growth in BALB/c mice.

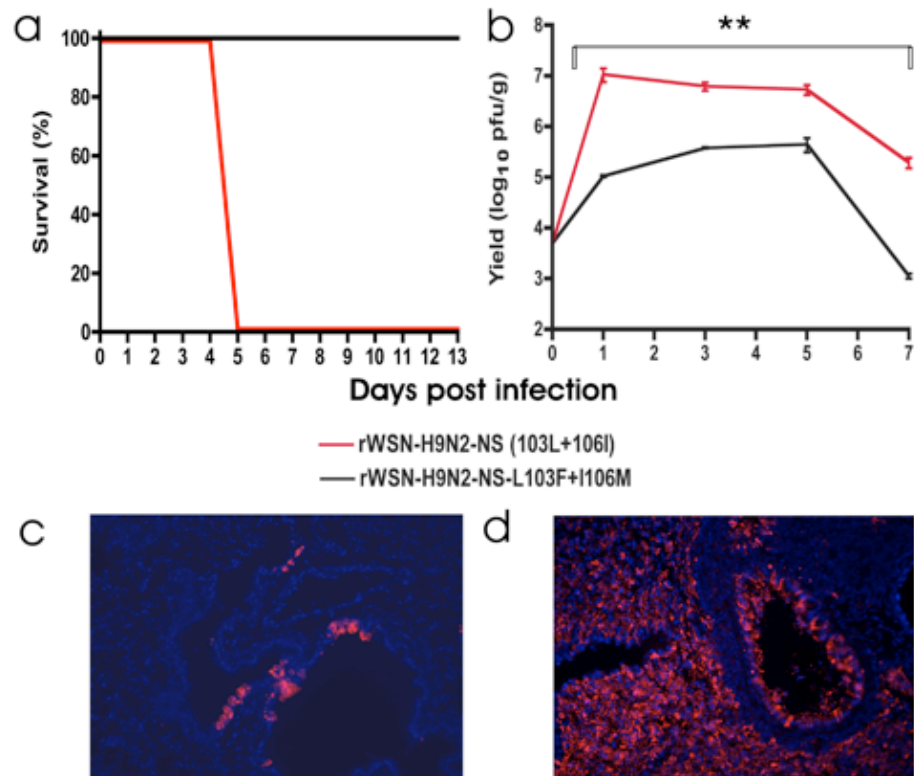


Figure 26 Growth, virulence and viral tropism are increased by 103L and 106I NS1 mutations in rWSN-H9N2-NS. **a.** Groups of 5 BALB/c mice were infected intranasally with 10^6 pfu with the different rWSN viruses. Survival was monitored for 14 dpi. **b.** Groups of 12 BALB/c were infected intranasally with 5×10^3 pfu with the rWSN viruses. Lungs were collected at days 1, 3, 5 and 7 pi and the viral titer was assessed by plaque assay. Values are shown as average of 2 replicates $\pm$ standard error for each value. Statistical analysis were performed using the parameters of paired and two-tailed student's t-test with probability < 0.01 . **c.** BALB/c mice were infected intranasally with 10^5 pfu of rWSN-H9N2-NS-L103F+I106M and lungs were collected 2 dpi. The lungs were inflated and fixed with 3.7% formaldehyde and lung sections were stained with anti-WSN antibody. The bronchiolar regions are indicated by an arrow. **D.** rWSN-H9N2-NS (possessing the 103L+106I residues) infected lungs.

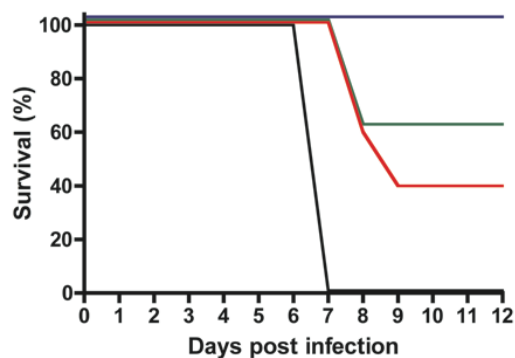
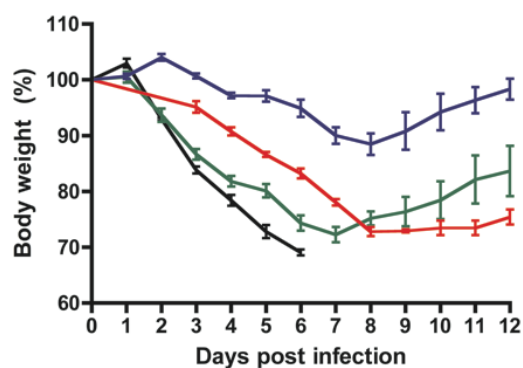
4.15 The properties of the 103S and 106I mutations studied on the A/PR/8/34 (H1N1) NS gene in BALB/c and CD-1 mice

The properties of 103 and 106 residues were also studied in the context of the A/PR/8/34 (H1N1) NS gene, that possesses mutations at both residues relative to the consensus sequence, 103S and 106I, on the same mouse adapted A/PR/8/34 backbone that was used throughout our studies. The PR8-NS1 103S and 106I sites were mutated to S103F, I106M and S103F+I106M. The four rPR8 viruses (rPR8wt, rPR8-NS-S103F, rPR8-NS-I106M, rPR8-NS-S103F+I106M) were used to infect groups of BALB/c and CD-1 mice intranasally with 10^4 pfu in a 50 μ l volume. The rPR8-wt results in a severe disease observed by 31% body weight loss by day 6pi and 100% mortality by day 7 pi in BALB/c mice (Figure 27a, b). In contrast, the infection with rPR8wt at 10^3 pfu resulted in a severe disease but no mortality was reported throughout the study (data not shown) (LD_{50} of the rPR8-wt in BALB/c is $10^{3.5}$ pfu). The NS1 S103F mutation in the PR8-NS1 decreased virulence (LD_{50} = $10^{4.1}$ pfu) and resulted in 40% mortality by day 8 pi. In experiments performed in parallel the I106M NS1 mutation also decreased virulence (60% mortality by day 9 pi LD_{50} = $10^{3.9}$ pfu) and disease severity expressed by body weight loss in BALB/c mice (27% day 8 pi) (Figure 27a, b). However, both NS1 mutations S103F+I106M in combination were avirulent at this dose in BALB/c mice and only resulted in 11% body weight loss by day 8 pi (Figure 27a, b). Again, the 103F and 106M mutations in the PR8-NS completely reversed mortality and thus reduced virulence and disease severity in BALB/c mice and resulted in the least body weight loss among the viruses tested. The same experiment was repeated in CD-1 mice. The rPR8-wt resulted in a severe disease and 100% mortality by day 9 pi (LD_{50} = $10^{3.5}$

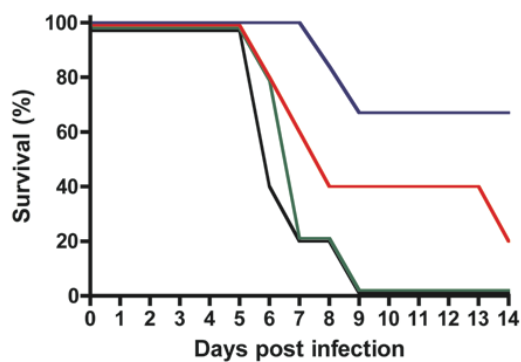
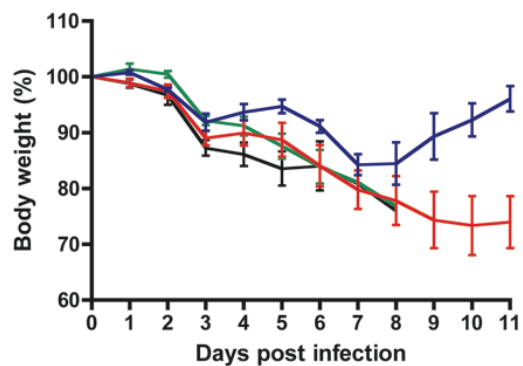
pfu) (Figure 27 c, d). Each of the NS1 S103F and I106M mutations decreased body weight loss compared to rPR8-wt (up to day 6 pi) however, the rPR8-NS-S103F and rPR8-NS-I106M mutants did not result in a detectable decrease in virulence compared to the rPR8-wt virus (100% and 80% mortalities at days 9 and 8 pi and $LD_{50} = 10^{3.5}$ and $10^{3.7}$ pfu respectively) (Figure 27c, d). Whereas the presence of both S103F+I106M NS1 mutations in combination reduced disease severity as well as virulence in CD-1 mice to result in 33% mortality compared to 100% mortality induced by the rPR8-wt virus possessing 103S +106I (Figure 27 c, d).

Again the 103S and 106I NS1 mutations were associated with an increase in virulence with a greater effect seen for the 106I than the 103S mutant and showed a cumulative effect where disease severity was more pronounced when both 103S+106I mutations were expressed in the mouse adapted A/PR/8/34 NS in different mouse models (BALB/c and CD-1), a phenotype that has been observed in the avian H9N2, human H5N1, human H3N2 and mouse H1N1 NS genes for the 103L and 106I mutations. We also observed that the BALB/c mice were more resistant than CD-1 mice to disease upon infection with the A/PR/8/34 (H1N1)-wt and mutants.

BALB/c mice



CD-1mice



— rPR8wt
— rPR8-NS-S103F
— rPR8-NS-I106M
— rPR8-NS-S103F+I106M

Figure 27 Virulence and disease severity are increased by 103S and 106I NS1 mutations in rPR8 virus in BALB/c and CD-1 mice. **a.** Groups of 5 BALB/c mice were infected intranasally with 10^4 pfu with the rPR8-wt and the different mutant viruses (rPR8-NS-S103F, rPR8-NS-I106M and rPR8-NS-S103F+I106M). Body weight loss was calculated as body weight loss was recorded daily. **b.** Survival was monitored for 14 dpi. **c.** Groups of 5 CD-1 mice were infected intranasally with 10^4 pfu with the rPR8-wt and the different mutant viruses (rPR8-NS-S103F, rPR8-NS-I106M and rPR8-NS-S103F+I106M). Body weight loss was calculated as body weight loss was recorded daily. Data represent the means $\pm$ SD (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; two-tailed student's t-test). **d.** Survival was monitored for 14 dpi.

4.16 The effect of the 103L and 106I mutations on the highly virulent MA20 backbone

The F103L and M106I NS1 mutations were selected upon mouse adaptation of the human prototype A/HK/1/68 virus following 20 and 21 serial passages respectively [202, 203]. To determine the effect of the 103L and 106I mutations on virulence on a distinct highly virulent mouse-adapted virus, the effect of the NS1 mutations was assessed on the rMA20 backbone. The rMA20 virus, a mouse-adapted variant of the A/HK/1/68 (H3N2) virus is highly pathogenic in CD-1 mice ($LD_{50} = 10^{2.7}$ pfu) and possesses mutations in all the IAV genes compared to the HK-wt including the NS1 V23A mutation [202]. We therefore generated rMA20 viruses possessing the HK-NS wt, HK-NS-F103L, HK-NS-M106I and HK-NS-F103L+M106I genes. Groups of 5 CD-1 mice were infected intranasally with 10^3 pfu of the 4 MA20 recombinant viruses as well as the rMA20 wildtype. The rMA20 virus possessing the MA20-NS gene caused a severe disease in CD-1 mice with >27% body weight loss at day 10 pi that resulted in 80% mortality (Figure 28 a, b). The substitution of the MA20-NS gene possessing the V23A mutation with the HK-wt NS gene did not affect virulence in CD-1 mice, as the rMA20-HK-NS virus caused also 80% mortality; however the mice that did not die recovered faster than those infected with the rMA20 virus (Figure 28 a, b). Unexpectedly, the rMA20 virus possessing the NS-F103L mutation decreased virulence and body weight loss (40% mortality at day 11 pi) compared to the rMA20 and rMA20-HK-NS viruses. Whereas the addition of each of the M106I containing mutations (M106I and F103L+M106I) increased mortality (100% at days 9 and 10 pi respectively) and also enhanced disease severity as observed by the severe body weight loss at day 8 pi (24 and 25%

respectively) compared to the rMA20-HK-NS virus (18% body weight loss by day 8 pi) (Figure 28 a and b). As a result, the addition of the M106I and F103L+M106I NS1 mutations enhanced the virulence of the rMA20 virus, whereas the F103L did not enhance virulence of the highly virulent rMA20 virus.

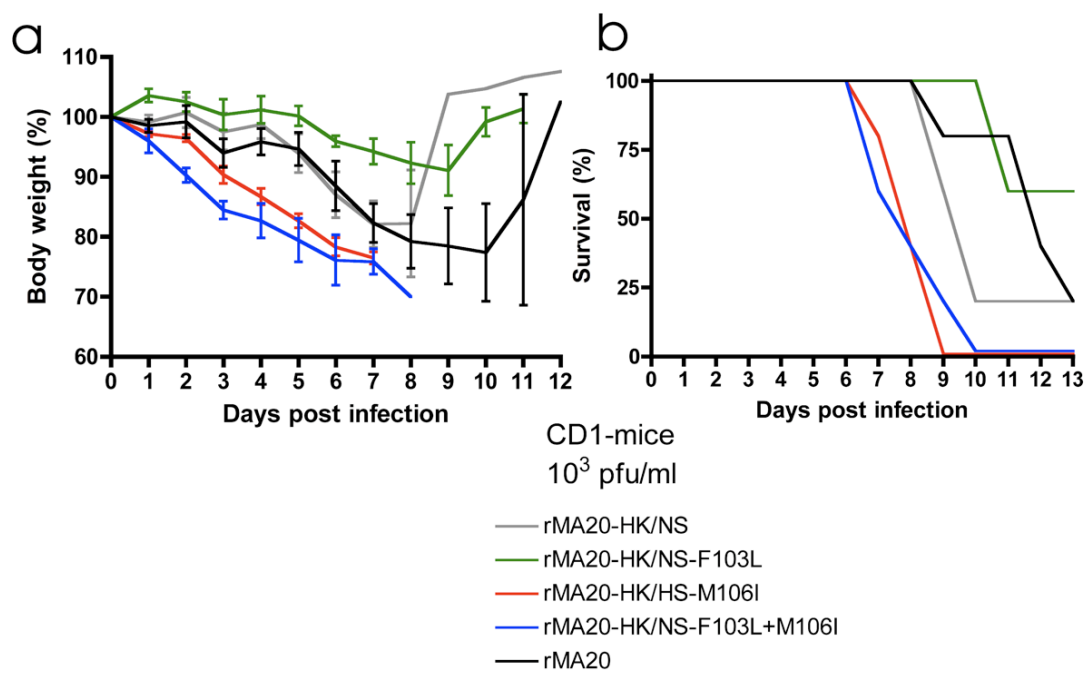


Figure 28 The 103L and 106I NS1 mutations increase the rMA20 virus virulence and disease severity in CD-1 mice. **a.** Groups of 5 CD-1 mice were infected intranasally with 10^3 pfu of the rMA20 and the different mutant viruses (rMA20-HK-NS, rMA20-NS-F103L, rMA20-NS-M106I and rMA20-NS-F103L+M106I). Body weight loss was calculated as body weight loss was recorded daily. **b.** Survival was monitored for 14 dpi.

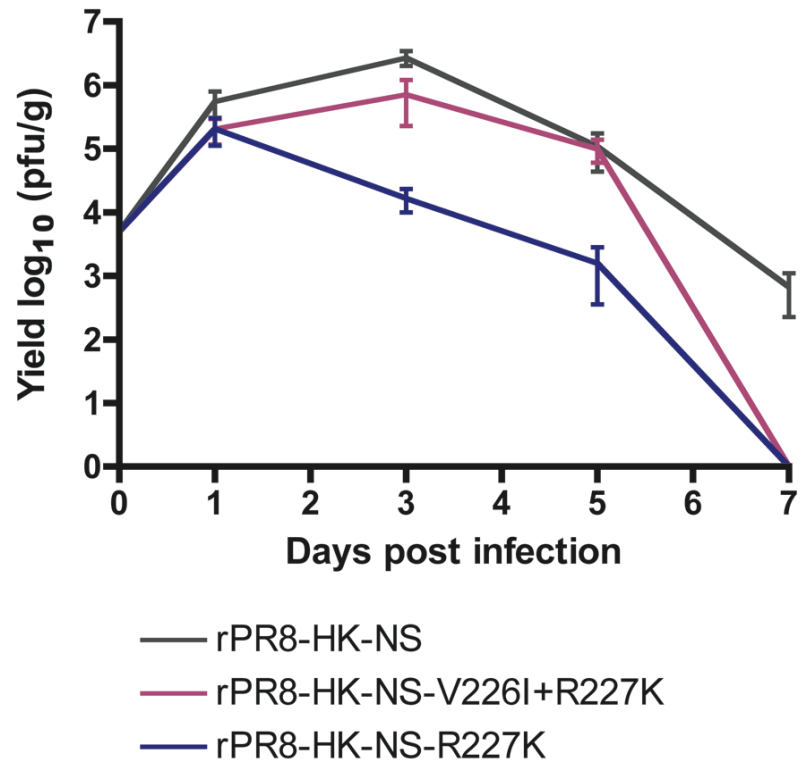
5. Role of mouse adaptive V226I and R227K in both the A/HK/1/68 (H3N2) and A/Brevig Mission/1/18 (H1N1) NS genes on the A/HK/1/68 (H3N2) and A/PR/8/34 (H1N1) backbone

In section 3 we have demonstrated that most of the NS1 mouse adaptive mutations conferred increased virulence and disease severity in BALB/c mice and simultaneously increased viral growth *in vitro* in MDCK cells. Here we will further explore the adaptive properties of the NS1 C-terminal mutations V226I and R227K that had been selected separately on MA 103 and MA112 mouse adaptive strains respectively (Table 1). These NS1 mutations have been independently selected in the pandemic Spanish flu virus A/Brevig Mission/1/18 (H1N1) suggesting a potential role in enhancing virulence and a possible association with pandemic properties.

5.1 Viral growth of NS1 V226I and R227K in CD-1 mouse lungs

In earlier results we showed that the A/HK/1/68 NS1 gene possessing the R227K mutation on the PR8 backbone enhanced virulence in BALB/c mice and resulted in 20% mortality (Figure 12). In addition, the R227K and V226I+R227K NS1 mutations are associated with enhanced plaque diameter in MDCK cells ($P < 0.001$ and $P < 0.01$ respectively) (Figure 12) as well as a significant increase in viral growth in MDCK cells ($P < 0.05$ and $P < 0.01$ respectively) (Figure 13). While the NS1 mutation V226I did not affect plaque diameter, *in vitro* growth and virulence (Figures 10, 12 and 13). Therefore to further explore whether the NS1 C-terminus mutations enhance viral growth in CD-1 mouse lungs, groups of 12 mice were infected intranasally with 5×10^3 pfu in a 50 μ l volume intranasally. Mice infected with the rPR8 viruses possessing the A/HK/1/68 NS1 wildtype gene as well as the rPR8-HK-NS-

R227K and rPR8-HK-NS-V226I+R227K were sacrificed at 1, 3, 5 and 7 dpi and lungs were collected, homogenized and the viral titer was determined from the lung homogenates by plaque assay. The rPR8-HK-NS-wt virus reaches a peak of 2.7×10^6 pfu/g at day 3 pi and drops to 6.7×10^2 pfu/g by day 7 pi (Figure 29). Both the R227K and V226I+R227K mutations resulted in similar virus growth compared to the rPR8-HK-NS-wt early after infection (1 dpi). Surprisingly the R227K and V226I+R227K NS1 mutations that were previously associated with an enhanced growth *in vitro* showed attenuated viral growth in CD-1 mouse lungs compared to the rPR8-HK-NS-wt virus throughout the course of the experiment ($P=0.053$ and $P=0.059$ respectively paired student's t-test) (Figure 29). Both rPR8 viruses possessing NS1 MA mutations were cleared from mice lungs by day 7pi whereas the rPR8-HK-NS-wt infected mouse lungs still contained infectious virus. Therefore the NS1 MA C-terminal mutations R227K and V226I+R227K in the human A/HK/1/68 (H3N2) NS1 gene on the A/PR/8/34 (H1N1) backbone resulted in attenuated growth in CD-1 mouse lungs.



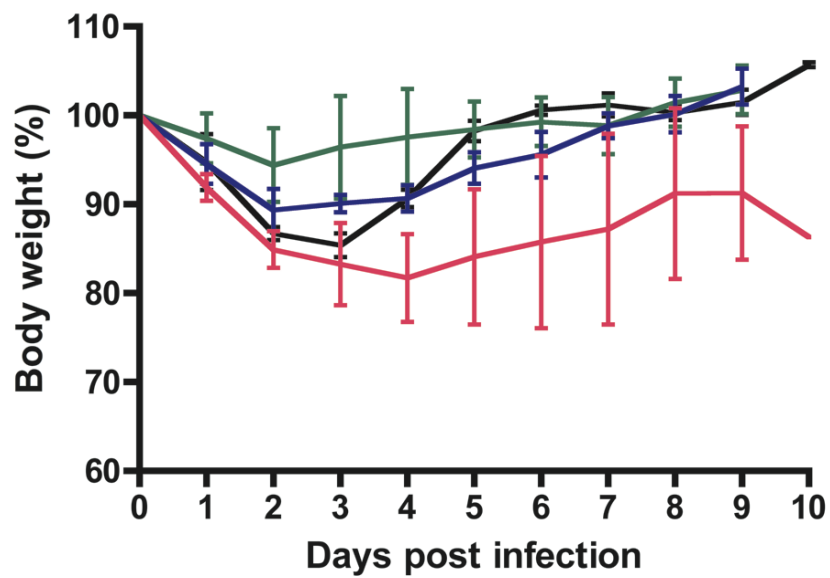
Dose of infection: 5×10^3 pfu/ml
CD-1 mice

Figure 29 The NS1-MA C-terminal mutations V226I and R227K mutations decrease viral growth in CD-1 mice. Groups of 12 CD-1 mice were infected intranasally with 5×10^3 pfu with the rPR8-HK-NS-wt, rPR8-HK-NS-R227K and rPR8-HK-NS-V226I+R227K viruses. Lungs were collected at days 1, 3, 5 and 7 pi and the viral titer was assessed by plaque assay. Values are shown as average of 3 replicates +/- standard deviation for each value.

5.2 The effect of V226I and R227K mutations on the A/Brevig Mission/1/1918 NS gene rescued onto the A/HK/1/68 backbone

To further assess the virulence associated with 226I and 227K mouse adaptive mutations, the A/Brevig Mission/1/1918 (H1N1) NS gene, that possesses the I and K amino acids in the 226 and 227 sites respectively, was rescued onto A/HK/1/68 (H3N2) backbone. To assess the roles of these mutations the 226I and 227K sites of the 1918-NS gene were mutated back to consensus and then rescued onto the A/HK/1/68 (H3N2) backbone. Groups of 3 CD-1 mice were infected intranasally with 5×10^6 pfu in a 50 μ l volume with the rHK-1918NS and the three mutants (rHK-1918NS-I226V, rHK-1918NS-K227R and rHK-1918NS-I226V+K227R). The rHK-1918NS virus was not virulent in CD-1 mice it did not result in any mortality ($LD_{50} > 10^{7.2}$ pfu) the virus also did not result in a severe disease as the maximum body weight loss recorded was ~15 % by day 3 pi. Furthermore, mutating the I226V or K227R back to consensus did not attenuate the avirulent rHK-1918NS virus as observed by similar body weight loss (Figure 30) and $LD_{50} > 10^{7.2}$ pfu for both rHK-1918NS-I226V and rHK-1918NS-K227R viruses. However, when both 226 and 227 sites were mutated back to consensus, the virus rHK-1918NS-I226V+R227K resulted in significant body weight loss compared to the rHK-1918NS virus ($P < 0.001$) and the $LD_{50} = 10^{7.0}$ pfu (**Figure 30**). Therefore we can conclude that the 226I and 227K NS1 residues are associated with a loss of virulence when expressed on the A/HK/1/68 (H3N2) and A/Brevig Mission/1918 (H1N1) NS genes in the context of A/PR/8/34 (H1N1) and A/HK/1/68 (H3N2) backbones respectively. It is possible that the combination of H3N2 and H1N1 genes does not

represent a suitable setting to observe the 226I and 227K associated virulence traits due to epistatic effects due to genetic interactions.



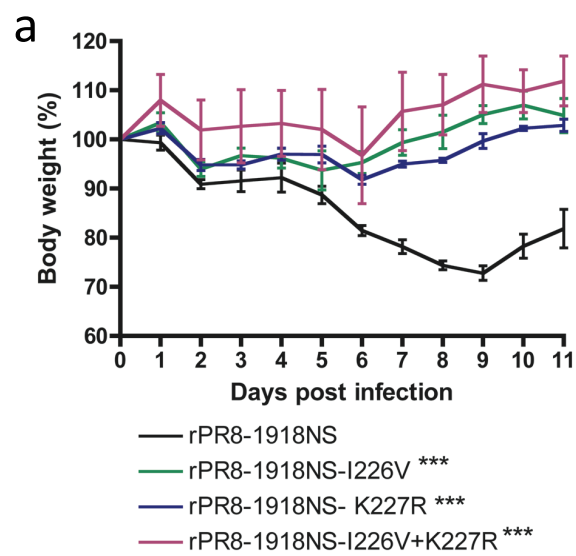
— rHK-1918NS
 — rHK-1918NS-I226V
 — rHK-1918NS-K227R
 — rHK-1918NS- I226V+K227R ***

CD-1 mice
 intranasal infection
 5×10^6 pfu/ml
 *** $P < 0.001$

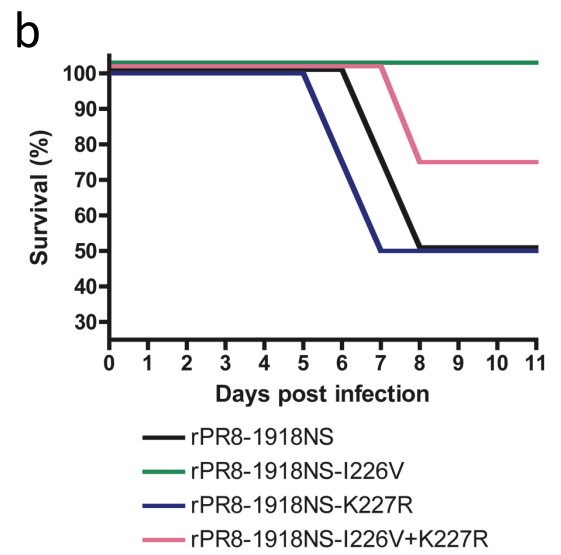
Figure 30 The NS1-MA C-terminal residues 226I and 227K in the A/Brevig Mission/1/18 (H1N1) NS1 decrease disease severity in CD-1 mice. Groups of 3 CD-1 mice were infected intranasally with 5×10^6 pfu with the rHK-1918-NS, rHK-1918-NS-I226V, rHK-1918-NS-K227R and rHK-1918-NS-I226V+K227R viruses. Body weight loss was calculated as body weight was recorded daily. Values are shown as average of 3 replicates +/- standard deviation for each value (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; two-tailed paired student's t-test between days 1 and 10).

5.3 The V226I and R227K are associated with enhanced virulence and disease severity in the A/Brevig Mission/1/1918 NS gene onto the A/PR/8/34 backbone

To further explore whether the 226I and 227K NS1 residues possess adaptive properties, the effect of these residues was determined on its parental H1N1 (A/Brevig Mission/1/1918) NS gene in the context of H1N1 backbone (A/PR/8/34). We hypothesize that the close ancestral relationship H1N1 of the NS and the structural genes should create a more natural environment that allows us to better understand the contribution of 226I and 227K to virulence and adaptation. Therefore the 1918-NS gene rescued onto the A/PR/8/34 (H1N1) backbone as well as rPR8-1918NS-I226V, rPR8-1918NS-K227R and rPR8-1918NS-I226V+K227R mutants were used to intranasally infect groups of 3 and 4 CD-1 mice at 10^4 and 10^5 pfu/ml dose respectively in a 50 μ l volume. The rPR8-1918NS virus possessing the 226I and 227K sites caused a severe disease expressed by > 25% body weight loss 9 days following infection with 10^4 pfu/ml that resulted in an $LD_{50} = 10^{4.65}$ pfu (Figure 31 a,b). Mutating the I226V and/or K227R sites resulted in an increase in the LD_{50} (rPR8-1918NS-I226V: $10^{5.2}$ pfu, rPR8-1918NS-K227R: $10^{5.0}$ pfu and rPR8-1918NS-I226V+K227R: $10^{4.9}$ pfu) and a decrease in body weight loss significantly when compared to the rPR8-1918NS ($P < 0.001$) (Figure 31 a,b). Therefore on the rPR8 backbone, we have demonstrated that the 226I and 227K residues of the 1918 NS are responsible for a small but significant increase in virulence and disease severity.



Dose of infection: 10^4 pfu/ml
 CD-1 mice
 Paired t-test
 *** $P < 0.001$



Dose of infection: 10^5 pfu/ml
 CD1 mice

Figure 31 The NS1-MA C-terminal residues 226I and 227K in the A/Brevig Mission/1/18 (H1N1) NS1 increase virulence and disease severity in CD-1 mice. **a.** Groups of 3 CD-1 mice were infected intranasally with 10^4 pfu with the rPR8-1918-NS, rPR8-1918-NS-I226V, rPR8-1918-NS-K227R and rPR8-1918-NS-I226V+K227R viruses. Body weight loss was calculated as body weight was recorded daily. Values are shown as average of 3 replicates +/- standard deviation for each value (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; two-tailed paired student's t-test between days 1 and 11). **b.** Groups of 4 CD-1 mice were infected intranasally with 10^5 pfu with the rPR8-1918-NS, rPR8-1918-NS-I226V, rPR8-1918-NS-K227R and rPR8-1918-NS-I226V+K227R viruses. Survival was monitored for 14 dpi.

5.4 Increased virulence due to the 226I and 227K in the 1918-NS gene are associated with improved growth in the mouse lungs

To assess whether the 226I and 227K associated adaptive properties observed through enhanced virulence and body weight loss also correlate with an enhanced growth in the mouse lungs, viral growth of the rPR8-1918NS and the three mutants (rPR8-1918NS-I226V, rPR8-1918NS-K227R and rPR8-1918NS-I226V+K227R) was compared in CD-1 mice. Groups of 15 CD-1 mice (3 mice/time point) were infected intranasally with 5×10^3 pfu of each of the four recombinant viruses. Mouse lungs were collected 1, 3, 5, 7 and 8 dpi and viral titers in the lung homogenates were determined by plaque assay. The rPR8-1918NS virus possessing 226I and 227K NS1 residues reached a peak titer of 2.5×10^7 pfu/g at 3 dpi and interestingly the lung viral titer decreased by 1.45 log by day 8 pi (8.85×10^5 pfu/g). The rPR8-1918NS-I226V showed increased viral growth significantly between days 1 and 7 pi ($P < 0.01$) whereas at day 8 pi viral growth decreased by > 3.5 logs compared to the rPR8-1918NS (226I+227K) virus (Figure 32a). Both rPR8-1918NS-K227R and rPR8-1918NS-I226V+K227R viruses reached similar lung viral titers compared to the rPR8-1918NS (days 1 to 5 pi) ($P = 0.1$ and $P = 0.52$ respectively paired student's t-test), however at later time points, the K227R and I226V+K227R decreased viral growth by ~ 2 logs and ~ 4 logs at days 7 and 8 pi respectively compared to the rPR8-1918NS (Figure 32a). This leads us to the conclusion that the K227R NS1 mutation decreases viral growth from days 5-8 pi (as observed by rPR8-1918NS-K227R and rPR8-1918NS-I226V+K227R). In addition, the viruses possessing the 227K residue (rPR8-1918NS and rPR8-1918NS-I226V) not only have the ability to grow efficiently in mice lungs between day 1 and 5 pi, but viral growth is sustained

in mice lungs up to day 7 pi. Moreover, when the NS1 227K is combined with the 226I 1918-NS1-residue (in rPR8-1918NS virus) viral growth was prolonged for another day (day 8 pi) compared to the rest of the mutants (Figure 32a). Therefore the 227K mutation is needed to sustain viral growth in the lungs (between days 5 and 7 pi) while the combination of 226I and 227K 1918 NS1 residues prolong virus growth in the mice lungs up to 8 dpi.

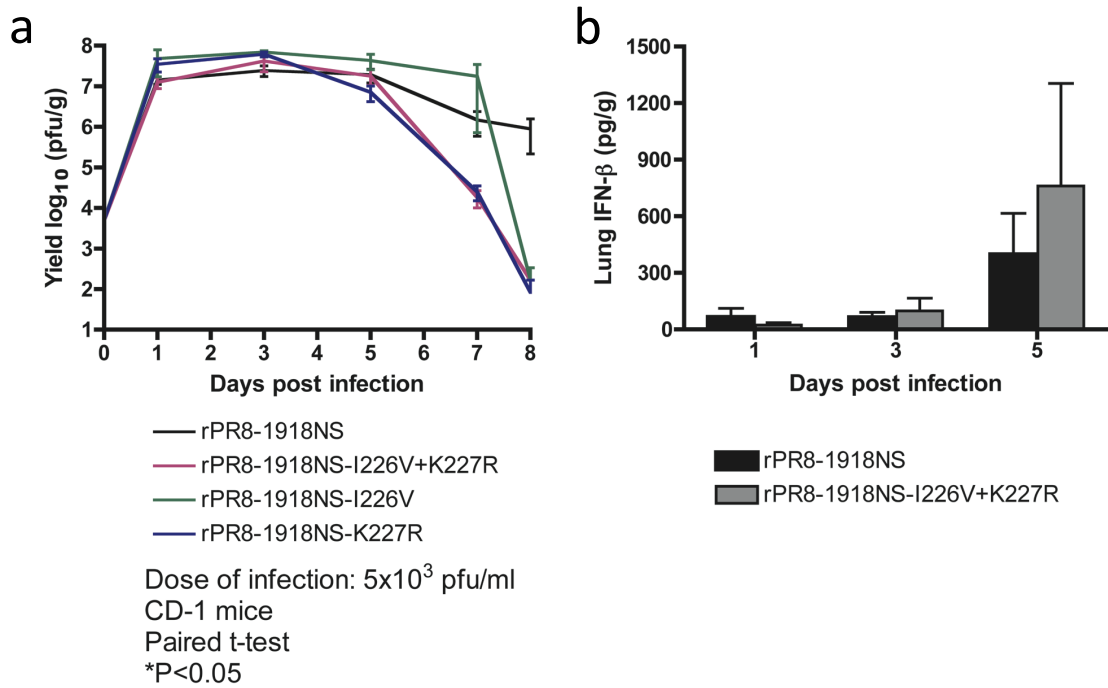
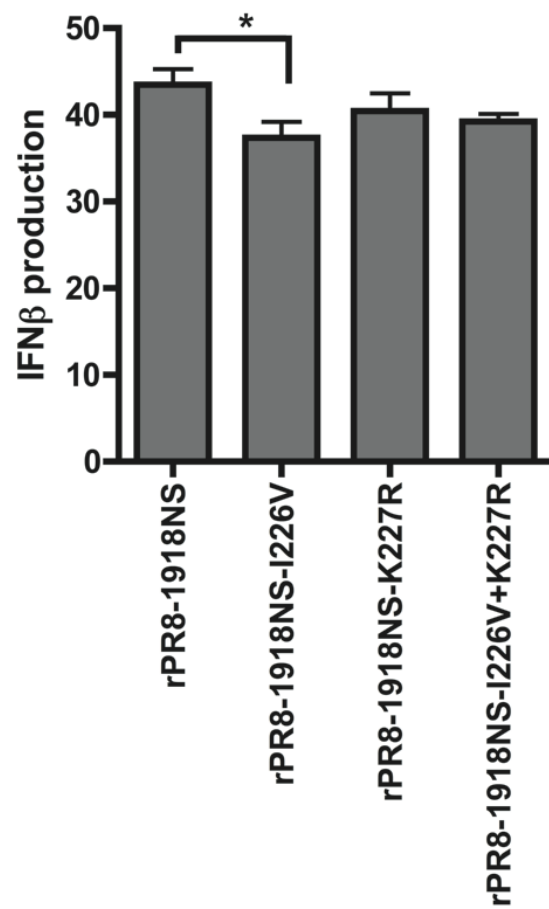


Figure 32 **226I and 227K mutations in the 1918-NS gene prolong viral growth in CD-1 lungs but do not affect IFN- β levels in the lung homogenates.** **a.** Groups of 12 CD-1 mice were infected intranasally with 5×10^3 pfu of the rPR8-1918-NS and the three mutants (rPR8-1918-NS-I226V, rPR8-1918-NS-K227R and rPR8-1918-NS-I226V+K227R). Lungs were collected at days 1, 3, 5 and 7 pi and viral titers were assessed by plaque assay of the lung homogenates. **b.** Groups of 18 CD-1 mice were infected intranasally with 5×10^3 pfu of the rPR8-1918-NS and the rPR8-1918-NS-I226V+K227R. The levels of IFN- β lung homogenates were determined by ELISA at days 1, 3 and 5 pi. Values are shown as average $\pm$ standard deviation.

5.5 NS1 C-terminal mutations and IFN- β levels in the infected lung

To determine whether the sustained growth observed by 226I+227K mutations resulted from a better ability to inhibit IFN- β , the IFN- β levels in lung homogenates were assessed by ELISA. Groups of 6 CD-1 mice were infected intranasally with 5×10^3 pfu of the rPR8-1918-NS and the rPR8-1918NS-I226V+K227R. Lungs were collected at days 1, 3 and 5 pi and the IFN- β levels from the lung homogenates were determined by ELISA. The IFN- β levels were comparable between both viruses: rPR8-1918NS and rPR8-1918NS-I226V+K227R throughout the course of the experiment ($P > 0.05$ student's t-test, equal variance). However, the mutations back to the consensus sequence I226V+K227R tended to increase the IFN- β levels in the lungs at day 5pi although not to a statistically significant level compared to the rPR8-1918NS (Figure 32b).



IFN β production *in vitro*
M1 cells
MOI=2
Supernatant collection 24 hpi
* P<0.05

Figure 33 Interferon production in M1 cells. Monolayers of M1 cells were infected with the each of the recombinant viruses rPR8-1918NS, rPR8-1918NS-I226V, rPR8-1918NS-K227R and rPR8-1918NS-I226V+K227R at MOI=2. Twenty-four hours post infection, the supernatants were collected and the level of IFN- β production was assessed by ELISA. Values are shown as average +/- standard deviation (*p<0.05, **p<0.01, *** p<0.001; two-tailed student's t-test).

5.6 226I and 227K and IFN- β production following infection in mouse cells

To further determine whether the enhanced virulence and body weight loss associated with 226I and 227K residues on the 1918NS was associated with IFN- β inhibition, we determined the IFN- β antagonism ability of the rPR8 virus possessing the 1918NS and the three mutants (rPR8-1918NS-I226V, rPR8-1918NS-K227R and rPR8-1918NS-I226V+K227R) *in vitro*. The four recombinant viruses were used to infect monolayers of mouse M1 cells at an MOI=2. Cell supernatant was collected 24 hpi and the IFN- β levels were determined by ELISA. The rPR8-1918NS virus induced low levels of IFN- β (43 pg/ml) while each of the K227R containing recombinants (rPR8-1918NS-K227R and rPR8-1918NS-I226V+K227R) induced similar IFN- β levels respectively compared to rPR8-1918NS virus (40 and 35 pg/ml respectively) whereas the rPR8-1918NS-I226V decreased IFN- β induction significantly to 37 pg/ml ($P<0.05$ student's t-test equal variance) (Figure 33). The comparable IFN- β levels induced by both the rPR8-1918NS virus possessing the 226I+227K residues as well as the rPR8-1918NS-I226V+K227R was also seen *in vivo* (Figure 32b).

5.7 The 226I and 227K 1918 NS1 residues and different aspects of IFN- β antagonism

The rPR8-1918NS-I226V decreased IFN- β induction significantly 24h following infection in murine cells ($P<0.05$ student's t-test equal variance). To have a better understanding of the mechanism by which 1918NS1-I226V inhibits IFN- β production, different aspects of NS1 IFN inhibition were examined at the pre-transcriptional level (by assessing IFN- β promotor activation) as well as the post-transcriptional level (CPSF-30 binding). The CPSF binding sites have been mapped to the NS1 103, 106, 144 and 184-188

sites by [155, 158, 159] and by residues spanning 98-180 as recently published by [160] therefore, we assessed whether the C-terminus NS1 mutations affected NS1 binding to the F2F3 domain of CPSF30 in the context of 1918-NS gene.

Briefly, 1918-NS and 1918-NS-I226V, 1918-NS-K227R and 1918-NS-I226V+K227R gene segments as well as FLAG-tagged CPSF-F2F3 (FLAG-F2F3) fragment were expressed *in vitro* in the presence of ³⁵S-methionine and cysteine. Equal expression of each NS protein as well as FLAG-F2F3 was verified by SDS-Page electrophoresis followed by autoradiography (Figure 34a). In an α -FLAG pulldown assay, *in vitro* translated and ³⁵S-labeled H5N1-NS and the three mutant NS proteins were mixed with FLAG-F2F3 or H₂O as a control and precipitated using Protein G Dynabeads (Invitrogen, Burlington, Ontario) pre-incubated with α -FLAG M2 antibody. Following incubation and washing, bound proteins were eluted in SDS buffer and subject to SDS-Page electrophoresis followed by autoradiography (Figure 34b). The results show that the 1918-NS gene is a strong CPSF-30 binding partner and all the 1918NS mutants show similar strong NS1 binding affinity to F2F3 fragment *in vitro*. Therefore we can conclude that the C-terminus of the 1918 NS1 gene did not affect CPSF-F2F3 binding. Furthermore, the decrease in IFN- β induced by the rPR8-1918NS-I226V observed following infection (Figure 33) is not due to a better CPSF binding as demonstrated. Therefore a different aspect of IFN- β inhibition at the pre-transcriptional level was assessed. As described earlier (section 4.7) monolayers of 293T cells were transfected with the 1918-NS bidirectional plasmid as well as the 1918-NS-I226V, 1918-NS-K227R and 1918-NS-I226V+K227R along with a firefly reporter plasmid under the control of human IFN- β and IRF-3 promoters. The IFN- β and IRF-3 promoters transcriptional activities were compared.

The 1918-NS gene possessing the 226I+227K sites inhibited IFN- β transcription compared to the empty control vector whereas only the I226V mutation decreased IFN- β promoter activation significantly compared to the 1918-NS ($P < 0.01$ student's t-test equal variance) (Figure 34c). Furthermore the IFN- β transcription inhibition observed by the 1918-NS1-I226V gene did not correlate with a decrease in IRF-3 promoter activation (Figure 34d). As a conclusion, the presence of 227K in combination with the consensus amino acid I226V in the 1918 NS1 gene resulted in a significant decrease in IFN- β protein levels, which is also observed at the level of IFN- β transcription *in vitro*.

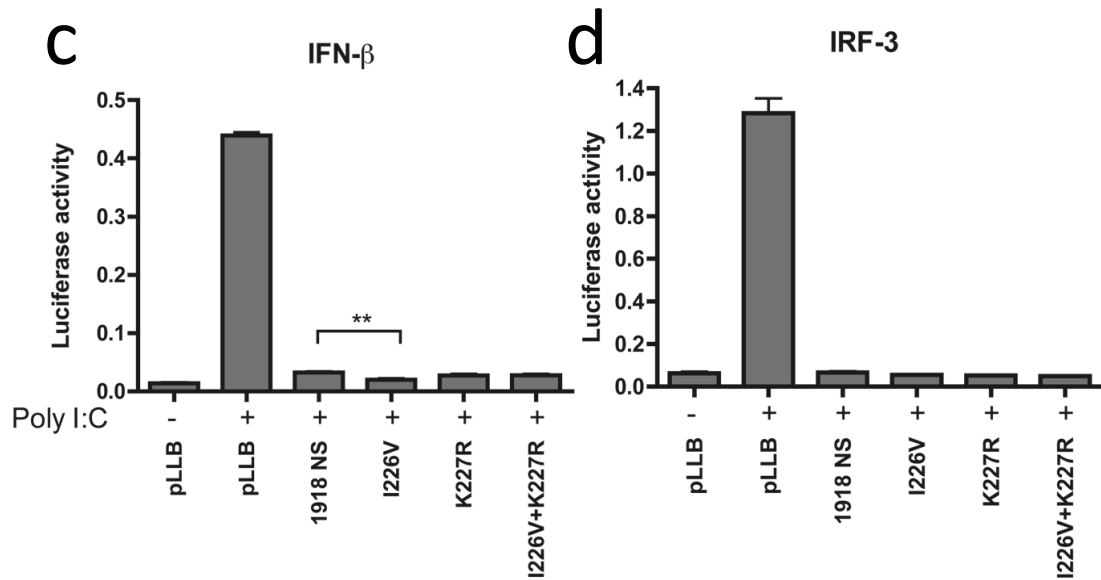
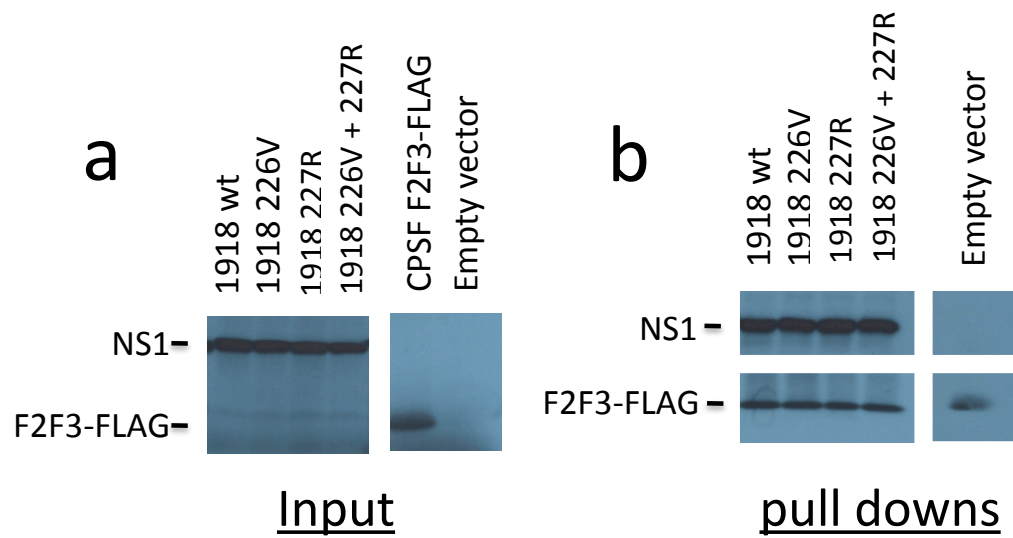


Figure 34 Effect of the 226I and 227K residues in the 1918-NS gene on IFN- β production at the pre-transcriptional (IFN- β and IRF-3 promoter transcriptional activation) and post-transcriptional levels (CPSF-30 F2F3 segment binding affinity *in vitro*).

a. 1918-NS1 proteins (wt and mutants) as well as CPSF30-F2F3-FLAG were expressed by *in vitro* coupled transcription and translation of expression plasmids or empty vector in the presence of ^{35}S labeled methionine and cysteine and expression was evaluated by autoradiography. Radiolabeled NS1 proteins or empty vector were mixed with radiolabeled CPSF30- F2F3-FLAG and subject to pull-down using α -FLAG M2 antibody bound to Protein G Dynabeads then SDS-PAGE electrophoresis and autoradiography. **b.** Representative pull-down of 2 independent replicates. **c.** IFN- β promoter activation was measured in human 293-T cells in triplicate for the 1918-NS-wt and the three NS mutant (I226V, K227R and I226V+K227R) expression plasmids using a dual-luciferase reporter assay. Each of the NS expression plasmids along with the firefly luciferase p125 under the control of the human IFN- β promoter as well as the renilla luciferase control pRL-SV40 reporter plasmids were transfected in 293T cells. Sixteen hours following transfection, IFN- β promoter activation was stimulated by poly I:C transfection. The luciferase activities were measured following a 24h incubation period. **d.** The same experiment was repeated using the firefly reporter plasmid under the control of the human IRF-3 promoter. Data represent the means $\pm$ SD (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; two-tailed student's t-test) of firefly luciferase activity relative to renilla.

6. Discussion

The ability to predict the emergence of influenza viruses with pandemic potential has become of increased concern because of the increasing number of outbreaks of pathogenic influenza viruses in poultry and animals [257]. It is therefore essential to investigate the evolutionary steps required by IAV to become virulent and adapt to new species. Our laboratory has employed adaptive evolutionary studies of the human prototype A/HK/1/68 (H3N2) in a mouse model. Mouse passaging of the avirulent prototype A/HK/1/68 (H3N2) resulted in the emergence of mouse adapted populations possessing mutations in most of the IAV genes, all except PB1-F2 [203]. In total 18 MA variants possessed either single or double mutations in the NS1 gene (Table 1). Eleven mutations were detected in the NS1 gene that also resulted in three overlapping mutations in the NEP gene with one found only in the NEP (E108K) (Table 1). Adaptive mutations result in increased replicative functions and therefore have properties of positive selection, which results in increased prevalence of the mutations in the population or are often seen to be independently selected in different experiments and therefore demonstrate parallel evolution. Among the eleven NS1 mutations, parallel or convergent evolution was observed for the D125G mutation (selected in both MA10 and MA11 populations) while parallel evolution was identified for the M106V and M106I occurring in MA41 and MA51 populations respectively (Table 1). Results showed that ten out of eleven NS1 mutations have been selected in multiple clones. Each of the F103L, M106V and M106I sites have been selected in three clones (MA20b-c-d, MA41-2-3- and MA51-2-3 respectively) within individual mouse-adapted populations and the NS1 mutations V23A, L98S and V180A were each selected in two MA clones (MA20-20a, MA52-

3, MA62-3 respectively) therefore indicating that these NS1 mutations are positively selected [203]. In addition, some of the NS1 mutations have been selected in adjacent sites in independent populations i.e. M124I and D125G (MA43 and MA102+MA111 respectively) as well as the V226I and R227K NS1 mutations (MA103 and MA112 respectively) further supporting the involvement of these NS1 sites in virus adaptation and therefore enhanced virulence. The importance of these NS1-MA mutations was further elucidated when similar NS1 mutations were selected in separate adaptation experiments as well as in highly pathogenic or pandemic IAV viruses. The NS1 D125G mutation was independently selected following 10 serial passages of another H3N2 prototype A/Aichi/2/68 in BALB/c mice [258]. In addition, 23 serial lung-to-lung passages of the duck isolate A/Duck/HK/702/79 (H9N2) in quail resulted in the M106T NS1 mutation. The mutation was maintained after 10 more lung-to-lung passages in chicken, which again supports the observation that the M106 site is required for virus adaptation within a new host [259]. Convergent evolution was also seen for, the NS1 F103L and M106I mouse-adapted mutations that were also selected in the “potentially pandemic” avian poultry adapted H9N2 virus as well as the highly pathogenic poultry adapted H5N1 IAV [243]. Both H9N2 and H5N1 poultry adapted viruses have acquired the ability to cross the species barrier and infected humans, a trait that again raises the possibility that the NS1 F103L and M106I mutations are required for host adaptation and increased virulence for multiple virus types and host species. The adjacent C-terminal mutations V226I and R227K selected upon mouse adaptation have also been selected in the pandemic A/Brevig Mission/1/1918 (H1N1) virus. The NS1 pattern of

selection further elucidates the importance of these NS1 sites in adaptation and association with enhanced virulence.

In this study we employed reverse genetics to investigate the adaptive properties of each of the NS1-MA mutations. We further focused on determining the replicative potential, virulence and IFN antagonism properties of the F103L+M106I and V226I+R227K mutations in NS1 genes of various origins in different background contexts. We have observed evidence of positive and negative epistasis that contributed to the adaptive properties of NS1-MA mutations in specific IAV genetic backgrounds.

6.1 NS1-MA mutations enhanced viral replication *in vitro*, disease severity and virulence

In general, the HK MA variants exhibited increased virulence seen at the level of decreased LD₅₀, disease severity, growth *in vitro* and *in vivo* as well as lung tropism [33, 160, 196, 202, 203]. Similarly we have demonstrated in this study that the NS1 MA mutations were associated with enhanced adaptive properties when expressed on the A/HK/1/68 (H3N2) NS genome segment rescued onto the A/PR/8/34 (H1N1) backbone (Table 4). Among the thirteen mutants that increased viral growth and plaque diameter *in vitro*, eleven mutants enhanced virulence either by inducing increased mortality ranging from 20 to 100% (Table 4 and Figure 12) or by increasing disease severity significantly in BALB/c mice (Table 4 and Figure 13). Similar phenotypes were observed by the NS1 mutations on different backbones and in different hosts. Parallel gain-of-function effects were previously observed by the same NS1-MA mutations when rescued onto the A/HK/1/68 backbone [160]. The V23A, F103L, M106I, L98S, M06I+L98S, M106V, V180A, R227K and D125G NS1 mutations enhanced virulence by decreasing the LD₅₀ or enhancing disease severity and

viral replication in both CD-1 and BALB/c mice when expressed in the contexts of both A/HK/1/68 and A/PR/8/34 backbones respectively. In addition, the increased replicative and virulence properties of two double F103L+M106I and M124I+D125G NS1 mutants were only determined in the A/PR/8/34 backbone (Table 4). We have therefore seen strong signs of positive epistasis for the V23A, F103L, M106I, L98S, M06I+L98S, M106V, V180A, R227K and D125G NS1 mutations when expressed in the HK and PR8 backbones, which might be characteristic of strongly adaptive mutations. However, the presence of M124I NS1 mutation did not result in gain-of-function phenotypes in the A/PR/8/34 backbone (rPR8-HK-NS-M124I and rPR8-HK-NS-M106V+M124I) (Table 4) while the M106V+M124I mutations demonstrated increased virulence properties as observed by enhanced disease severity and IFN antagonism *in vivo* when expressed on the A/HK/1/68 backbone showing gain-of-function of both mutations but the M106V mutation seems to be providing a compensatory function and thus demonstrating positive epistasis for the M124I [160]. The presence of the NS1 M106V as well as the rest of the MA mutations in the seven structural genes may be required to allow for functional or structural gene interactions due to epistatic effects with the NS1 gene possessing the M124I NS1 mutations and therefore to observe its adaptive properties. On the other hand, two NS1 mutations demonstrated a selective advantage associated with one viral backbone (HK vs PR8) and a specific mouse host strain (CD-1 vs BALB-c). The D2N NS1 mutation resulted in 80% mortality and the V226I NS1 mutant increased viral growth and IFN antagonism properties in CD-1 mice when expressed on the A/HK/1/68 (H3N2) backbone. The negative effects of V226I and D2N NS1 mutations observed on the PR8 backbone can be compensated by other combination of mutations

present in the HK backbone. Therefore we have here demonstrated two examples of negative epistasis that can be alleviated to show positive epistasis by a different combination of backbone genes.

Table 4 Adaptive patterns of the rPR8 viruses determined in MDCK cells and BALB/c mice in comparison to the rPR8-HK-NS-WT virus

rPR8-HK-NS VIRUSES	PLAQUE DIAMETER ¹	<i>IN VITRO</i> GROWTH ²	% MORTALITY IN BALB/C MICE	DISEASE SEVERITY IN BALB/C MICE ³
WT	1	1	0	1
V23A	1.47	1.68	0	1.24
F103L	1.24	1.39	40	1.32
M106V	1.36	2.52	100	2.19
M106V+M124I	1.18	0.53	0	0.72
M106I	1.57	2.48	20	0.78
L98S+M106I	1.29	1.25	80	1.96
V180A	1.52	1.83	60	1.38
D2N	1.52	1.78	0	1.01
D125G	1.37	2.04	40	1.53
V226I	1.05	0.5	0	1.04
R227K	1.87	4.22	20	1.17
L98S ^Ψ	1.6	2.56	40	1.61
M124I ^Ψ	1.03	2.73	0	0.70
F103L+M106I ^Φ	1.59	2.5	20	0.84
M124I+D125G ^Φ	1.68	5.78	0	1.52
V226I+R227K ^Φ	1.75	5.13	0	0.87

^Ψ Mutations selected in pairs in the MA experiments are cloned separately to determine the effect of each mutation alone

^Φ Mutations cloned together as they map to the same binding site in the NS1 gene segment

¹ indicates fold change with respect to rPR8-HK-NS wildtype

² indicates fold change with respect to rPR8-HK-NS wildtype at the maximum time point yield (48hpi)

³ Indicates fold change with respect to rPR8-HK-NS wildtype at the maximum body weight loss for each virus

6.2 Understanding the mechanism of NS1-MA mutations enhanced growth and virulence

Increased viral replication, plaque diameter and virulence by various NS1 MA mutations prove that these mutations are mediating new or enhanced functions that result in the observed adaptive properties. In this study we have explored different aspects of NS1 multifunctionality. At the level of IFN antagonism, none of the NS1-MA mutants increase the level of IFN- β promoter inhibition when compared to the A/HK/1/68 (H3N2) NS protein (Figure 14). Moreover, some of the NS1 mutations (M106I, F103L+M106I, M106V and V180A) that have been associated with enhanced adaptive properties (Tables 3 and 4, Figures 10, 11, 12, 13) actually increase IFN- β promoter activation compared to the HK-NS wt protein (Figure 14). This non-inhibitory effect also correlated with an increase in the levels of IFN- β protein measured by ELISA following infection of mouse cell lines by the rPR8 viruses expressing the M106I and F103L+M106I mutations (Figure 20). However, when the same NS1 MA mutations were expressed onto the A/HK/1/68 (H3N2) backbone, most of the NS1 MA mutations resulted in significant lung IFN- β inhibition as recently published by [160]. Therefore we concluded that although the NS1 recombinant proteins' ability to inhibit IFN- β transcriptional activation was reduced in human cells, this property was separable from an increased viral ability to inhibit IFN- β production *in vivo*. In fact we confirmed that the polymerases (PB2, PB1 and PA) as well as the NP, NS1 and NS2 all contribute to IFN- β promoter activation inhibition by IFN- β promoter transcriptional activation analyses by the A/HK/1/68 (H3N2) (Figure 25a). However, when expressed in combinations, the three A/HK/1/68 polymerase proteins (PB2+PB1+PA) exhibit synergy at

inhibiting IFN- β promoter activation *in vitro* ($P=0.047$ when compared to PA which was the best polymerase subunit for inhibiting IFN- β activation) (Figure 25a). This is consistent with previous reports where the heterotrimer PB2, PB1 and PA of A/PR/8/34 origin also exhibited synergy at inhibiting IFN- β transcription activation when expressed in combination as observed by [46, 260].

Furthermore the three HK polymerase proteins when combined with the HK-NS-M106I recombinant protein reduced IFN- β promoter activation although not to a statistically significant point compared to the level of IFN- β inhibition mediated by HK-NS-M106I alone (0.27 ± 0.09 vs. 0.41 ± 0.16 respectively) (Figure 25b). Additionally the level of IFN- β promoter transcriptional activation inhibition mediated by HK-NS M106I- +3 POL proteins was not as demonstrated when the 3 POL proteins were expressed together with HK-NS protein (0.129 ± 0.04 for HK-NS vs. 0.124 ± 0.03 for 3POL+HK-NS) (Figure 25b). Therefore the rHK virus expressing the HK-NS-M106I mutation (as published by [160]) was capable of enhancing IFN- β inhibition *in vivo* in BALB/c mice compared to the rHK virus possessing the HK-NS gene; a trait that is possibly mediated by the heterotrimer polymerase complex.

Moreover, our team has recently proved that most of the NS1 MA mutations enhance the rate of viral protein synthesis *in vitro* [160]; therefore we speculate that NS1 is mediating a better inhibition of IFN- β synthesis presumably by making more NS1 proteins as well as viral polymerases in infected cells compared to the WT virus. Because all of these proteins are IFN antagonists, a greater abundance of these proteins is expected to be associated with greater levels of antagonism of IFN production.

6.3 103L and 106I in the HK-NS1 protein enhance virulence and viral replication in various hosts

Here we demonstrated that both the IAV NS1 F103L and M106I mutations were adaptive mutations that increased replicative abilities in cells of multiple species (murine, canine and human) as well as virulence and replication in the mouse lung (Figure 15). Although we observed a loss of ability to inhibit IFN- β promoter activation as well as IFN- β induction on the PR8 backbone for the HK-NS1 F103L and M106I mutations, this property was separable from an enhanced ability for protein synthesis and viral replication by the HK-NS gene. Therefore, there appeared to be a counterbalance between the inhibitory effects of IFN- β induction and enhanced overall function seen at the level of replication and virulence for both of these mutations. In our experiments, the F103L and M106I mutations enhanced IFN- β induction only when transferred onto the foreign, A/PR/8/34, genetic background (interestingly this backbone is normally associated with PR8 NS1 that possesses similar mutations at these positions, 103S and 106I). This was also reported for the A/HK/156/97 (H5N1) NS gene that also possessed these, F103L and M106I, mutations and did not induce IFN- β on its native backbone but did when transferred onto the A/Udorn/1/72 (H3N2) backbone [216]; that was correlated with the loss of CPSF-30 binding and thus inhibition of host gene expression.

The different abilities to inhibit IFN- β induction among viruses, that possessed the same combinations of mutations, 103L and 106I versus 103F and 106M, but that differed in their NS1 genes (A/HK/1/68 (H3N2), A/HK/156/97 (H5N1) or A/Ck/Bj/1/95 (H9N2)) as well as virus backbones (A/PR/8/34 and A/WSN/33) (Figure 20), indicates that inhibition of IFN- β

induction is not only controlled by the NS1 gene but also by epistatic interactions with the other virus gene products. It has been reported that the A/HK/156/97(H5N1) was able to suppress IFN- β induction in the context of its NS1 gene, possessing F103L and M106I mutations, due to properties of its NP and PA genes [161]. This is consistent with the recently described roles for PA, PB1 and PB2 genes in blocking IFN induction by binding to the mitochondrial antiviral signaling protein (MAVS) [46, 260] but suggest that a functional interaction with NS1 protein is also involved in these (or other undefined) processes which are downstream from the RIG-I signal that is inhibited by NS1 [153]. Thus the F103L and M106I mutations were not associated with increased IFN- β induction either in A/HK/156/97(H5N1) virus [161] or for the MA20c and MA51 mouse-adapted viruses in which these mutations were selected (unpublished data), indicating that these mutations were selected in a genetic context that maintains suppression of IFN- β induction. Increased IFN- β induction was also suppressed for the H9N2-NS gene on the WSN backbone, and the H5N1-NS gene on the PR8 backbone where it was demonstrated that in the absence of increased IFN- β induction the F103L and M106I mutations mediated a large increase in extent of lung infection and virulence. We have therefore demonstrated that both F103L and M106I mutations were multifunctional, including loss of ability to suppress IFN- β in some contexts but also including gain-of-function that increased replicative abilities and virulence. Multifunctional adaptive mutations have been previously seen in the HA gene for individual adaptive mutations that affect both pH of fusion and receptor binding [196] suggesting that strongly adaptive mutations affect more than one function.

6.4 F103L and M106I mutations enhance protein synthesis

The increased protein synthesis of the F103L NS1 mutant was clearly seen for the M1 and NS1 proteins at all times in MDCK cells whereas the M106I mutation increased protein synthesis at 2 hpi but not at later times, and where the double mutant was not significantly enhanced relative to rPR8-HK-NS-wt (Figure 17a). This may have been due to the increased induction of IFN- β as seen for mouse cells infected with these mutants. Thus there was an enhancement of protein synthesis due to each mutation alone but that this was being suppressed by the increased amounts of IFN- β that were simultaneously expressed for F103L and M106I in combination, thus indicating the critical role of NS1 in inhibition of IFN- β induction.

6.5 The F103L and M106I mutations increase virulence of human and avian NS1 genes

Increased virulence by the NS1 F103L and M106I mutations was observed in various NS1 genes of human, avian and mouse origins. Both HK-NS1 F103L and M106I mutations enhanced the level and duration of replication in the mouse lung where viral titers were increased up to 10^5 fold at 5 dpi compared to rPR8-HK-NS-wt (Figure 15c). Even though the rPR8-HK-NS-wt virus showed poor BALB/c lung infection patterns, each mutation in the HK-NS1 gene enhanced the ability to infect clusters of cells in the alveoli of infected mice (Figure 16). The extent of infection as monitored by virus yield suggested that more cells were infected in the lung for all the mutant viruses but that both mutations in combination were not more effective in the PR8 backbone than the F103L mutation alone, presumably because of the increased amount of induced IFN- β by this mutant. Consistent with the staining, each of the NS1 F103L and M106I mutations showed marginally increased

virulence by $>10^{0.4}$ and $>10^{0.2}$ fold respectively however, when expressed in combination the MA mutations were not as effective at increasing virulence ($>10^{0.2}$ fold) compared to the F103L mutation (Table 5). Previous studies of variation in LD₅₀ showed that the assay has a standard deviation of $\pm 10^{0.3}$ indicating that the observed values are at the edge of significance however they may be actually significantly greater because of the unmeasurable LD₅₀ of the PR8-HK-NS-wildtype virus. Thus the increase in virulence was quite small because it is being compared to the rPR8-HK-NS-WT virus that did not result in mortality indicating that this genetic backbone was not in a linear response range for detecting differences in virulence and thus underestimated the differences in virulence. The large increase in replication in mice lung in combination with the trend to increased mortality suggests an important role of the F103L and M106I NS1 mutations in virulence that was even observed on a wildtype virus with properties of attenuated growth, virulence and lung tropism.

The presence of the F103L and M106I in combination in a more compatible context demonstrated their extensive abilities to increase virulence, viral infectivity and replication *in vivo*. The 103L+106I residues in the H5N1-NS gene were associated with >100 fold and $\sim 10^7$ fold increase in viral growth in mice lungs by days 5 and 7 pi respectively in CD-1 mice (Figure 23a). The same residues in the avian H9N2-NS were also associated with >10 fold and >150 fold increased yield seen in lungs titers by days 5 and 7 pi respectively in BALB/c mice compared to the respective wildtype possessing 103F+106M mutations (Fig. 26b). The 103L+106I residues of the human H5N1-NS and avian H9N2-NS genes increased virulence dramatically by decreasing LD₅₀ $>10^{3.75}$ and $>10^{1.0}$ fold respectively (Table 5). We have

demonstrated that these mutations were critical for the enhanced virulence of the A/HK/156/97 (H5N1) and A/Ck/Bj/1/95 (H9N2) NS1 genes such that reversion to L103F and I106M resulted in an IAV that was very restricted in its ability to infect lung tissues and replicate in CD-1 and BALB/c mice lungs respectively (Figure 22, 23a and 26). These mutations were not associated with increased IFN- β induction in M1 cells when present on the A/Ck/Bj/1/95 H9N2-NS gene on the WSN background, or on the A/HK/156/97 H5N1-NS gene on the A/PR/8/34 backbone when compared to the respective rWSN-WT and rPR8-WT viruses respectively (Figure 20) indicating that the ability to block IFN- β induction was maintained by these avian and human NS genes on the WSN or PR8 backgrounds respectively. In addition, this maintenance in IFN- β production was in part associated with the presence of the 103L+106I residues since mutating these residues back to consensus; the L103F+I106M mutations resulted in a significant increase in IFN- β production in the rPR8-H5N1-NS-L103F+I106M virus (Figure 20). Similarly we have also demonstrated a reduced IFN- β production *in vivo* due to the F103L + M106I mutation when standardized for amount of growth (Figure 23 a and b). Therefore the increase in viral growth, virulence, and lung tissue tropism associated with the 103L+106I residues may have been, at least in part, due to a better ability of the H5N1 NS protein to inhibit lung IFN- β levels at early times post infection but that the increased infection of lung tissue thereafter eventually induced high levels of IFN- β that are associated with toxic levels leading to cytokinemia as has been described in human H5N1 infections [12]. This effect is consistent with a greater ability to resist inhibition by IFN- β and paralleled the large effect of the PR8 NS1 gene on expanding tissue tropism that was observable in PR8 infected STAT1 knockout mice when compared to

virus that had the NS1 gene deleted in the PR8 NS1 null mutant [149]. It would be of great interest to further confirm whether the L103F+I106M mutations parallel the 103L+106I mediated adaptive properties in immunocompromised systems such as STAT-1 knockout mice or whether these mutations would have less effect in the absence of an IFN response.

Table 5 Effects of 103 and 106 mutations on various human, avian and mouse adapted NS1 genes in BALB/c mice

NS1 GENE		103 &106 MUTATIONS	BACKBON E	GROWTH <i>IN VIVO</i> ¹	DISEASE SEVERITY ²	LD ₅₀ (LOG10)	INCREASED VIRULENCE ³
A/HK/1/68 (H3N2)	Human	F+M	A/PR/8/34 (H1N1)	1	1	>7.2	--
		F103L		4.34	1.32	6.8	>0.4
		M106I		8.43	0.78	7.0	>0.2
		F103L+I106M		4.12	0.84	7.0	>0.2
A/HK/156/9 7 (H5N1)	Human	L+I	A/PR/8/34 (H1N1)	84.56	25.7	2.75	>3.75
		L103F		nd ⁴	21.6	3.0	3.5
		I106M		nd	21.1	3.6	2.9
		L103F+I106M		1	1	>6.5	--
		L+I	A/HK/1/68 (H3N2)	nd	6.85	>7.2	--
		L103F		nd	1.5	>7.2	--
		I106M		nd	3.34	>7.2	--
		L103F+I106M		nd	1	>7.2	--
A/Ck/Bj/1/9 5 (H9N2)	Avian	L+I	A/WSN/33 (H1N1)	102.38	nd	5.5	>1
		L103F+I106M		1	nd	>6.5	--
A/PR/8/34 (H1N1)	Mouse adapted	S+I	A/PR/8/34 (H1N1)	nd	6.04	3.5	>1
		S103F		nd	5.01	4.1	0.4
		I106M		nd	3.35	3.9	0.6
		S103F+I106M		nd	1	>4.5	--

¹ Indicates fold change with respect to the 103F+106M-containing virus at the maximum viral growth for each virus

² Indicates fold change with respect to the 103F+106M-containing virus at the maximum body weight loss for each virus

³ Increased virulence observed by the difference in log₁₀ LD₅₀ between L+I and F+M containing viruses

⁴ Not determined

6.6 103L and 106I enhance virulence in mouse adapted viruses

Our comprehensive study of 103L and 106I in various NS genes (human, avian and mouse) as well as several backbones (HK, PR8 and MA20) on multiple hosts (CD-1 and BALB/c mice) demonstrated that the Leu and Ile on 103 and 106 sites are necessary and sufficient for a virus to adapt and become virulent in a new host.

The 103S and 106I residues of the A/PR/8/34 NS were associated with enhanced virulence and disease severity in both BALB/c and CD-1 mice (Fig. 27 a, b, c and d). In addition when the 106I and the 103L+106I were expressed on another mouse adapted virus the rMA20, the mutations also exhibited enhanced mortality and body weight loss (Fig. 28). Simultaneously, the reversion to 103F+106M on the MA20 backbone resulted in decreased disease severity in CD-1 mice (rMA20-HK-NS Fig 28). However the F103L NS1 MA mutation decreased virulence of the MA20 virus. The F103L NS1 mutation was selected initially on the MA20c virus, which possessed several additional mutations including a PA E133G mutation [202] that is not expressed in the MA20 sequence. Therefore, we speculate that genetic interactions between the cognate PA and NS1 proteins might be required for a better viral fitness in CD-1 mice.

Similarly, negative epistasis interactions were observed when the S103F and I106M mutations were expressed on a mixed genetic background of high and low virulent PR8 genes derived from 2 PR8 lineage viruses [256]. The 103L and 106I demonstrated decreased growth and virulence properties when expressed on the reassortants possessing the A/HK/156/97 (H5N1) the H5N1 NS gene on the A/Udorn/1/72 backbone [216, 217]. Genetic incompatibilities between the NS gene and the structural IAV genes in the described

reassortments presumably affect NS gene interactions with the seven structural genes [92] and therefore result in negative epistatic interactions.

6.7 Mechanism of action of NS 103L and 106I ability to antagonize IFN- β

The NS1 103L and 106I mutations demonstrated an increased ability to antagonize IFN- β activity in the HK-NS1 gene observed by the enhanced ability to grow in mouse epithelial cells following IFN- β pre-treatment when compared to rPR8-HK-NS-wt (Figure 18b). Increased growth of these mutants was also greater at early times in MDCK and mouse M1 cells (Figures 15a and 18a) before IFN- β had been induced in the infected cell cultures. Simultaneously the L+I and S+I residues in H5N1 and PR8 NS1 genes respectively were associated with a more pronounced increase in various aspects of virulence including viral growth, body weight loss as well as lung spread and tissue tropism in IFN- β controlled environments.

The NS1 protein can mediate multiple mechanisms to inhibit IFN- β transcription, production as well as IFN mediated signaling. To understand how various NS genes are mediating IFN- β antagonism we have employed multiple *in vitro* assays covering the pre-transcriptional and post-transcriptional IFN- β antagonism pathways mediated by NS1 protein. It has been demonstrated by our laboratory and elsewhere that the 103F and 106M consensus sites have been associated with a strong binding affinity to CPSF-30 F2F3 fragment *in vitro*. The NS gene of all three viruses A/Brevig Mission/1/18 (H1N1), A/Udorn/72 (H3N2) as well as A/HK/1/68 (H3N2) possessing the 103F+106M sites show a strong binding affinity to the F2F3 fragment [160, 203, 216].

However, although CPSF binding has been shown to result in inhibition of IFN and other host gene expression [154], the NS genes of A/PR/8/34 (H1N1) and A/HK/156/1/97 (H5N1) possessing S+I and L+I on their 103 and 106 sites respectively have lost CPSF-30 binding yet have been associated with low or undetectable IFN- β levels. A/PR/8/34 (H1N1) viruses maintained the ability to block IFN induction by preventing pre-transcriptional activation of IRF-3, NF κ B and c-Jun/ATF-2 as reviewed [88]. Therefore we can first conclude that IFN- β production and various aspects of virulence do not correlate with CPSF-30 binding. On the other hand, we also demonstrate that the 103L+106I residues of H5N1-NS are only partly mediating IFN- β inhibition to result in partial IFN- β transcriptional activation (Fig 24a,b).

Given that the 103 and 106 amino acids also map to regions of interaction between NS1 and cellular translational initiation factors eIF4GI and PABPI and result in increased protein synthesis as recently published by [160], leads to the hypothesis of increased NS1-mediated initiation complex formation on IAV mRNA for enhanced viral gene expression due to mutations F103L and M106I. Enhanced viral gene expression through increased RNA polymerase activity has been shown for these NS1 mutations which will result in increased protein synthesis of polymerase subunits [160] and possibly increased viral polymerase proteins following infection might be another pathway that results in IFN- β transcriptional inhibition.

It is also possible that these mutations affect other functions of NS1 that are mediated by interaction with other viral or host proteins. Several studies have identified multiple host factors that interact with NS1 protein [88], including the recent identification of nine PR8 viral proteins and 20 host proteins that bind NS1 protein, all of which may be modified in

their interaction due to adaptive mutations [92]. Future studies are needed to assess the role of the adaptive NS1-MA mutations on interactions with other viral factors.

6.8 The C-terminus mutations and their adaptive properties

The adaptive properties of the V226I and R227K NS1 mutations selected respectively in the MA variants MA103 and MA112 are of particular interest since they have also been additionally selected in the pandemic virus A/Brevig Mission/1/18 virus. The V226I mutation expressed on the HK-NS was not associated with adaptive properties *in vitro* (i.e. plaque size and viral replication in canine and murine cells) when rescued onto either the A/HK/1/68 (H3N2) [160] or the A/PR/8/34 (H1N1) backbones (Table 4). However *in vivo* the V226I NS1 mutation showed enhanced viral replication and IFN inhibition properties when rescued onto the A/HK/1/68 [160]. While, the R227K HK-NS mutation was associated with enhanced replication *in vitro*, plaque size and 20% mortality in BALB/c mice when rescued onto the A/PR/8/34 (H1N1) (Tables 4 and 6) as well as enhanced viral replication and IFN inhibition in CD-1 lungs associated with the A/HK/1/68 genetic background [160]. Simultaneously, when expressed on the A/Brevig Mission/1/1918 (H1N1) NS in the PR8 background, the 226I+227K residues were associated with a moderate increase in virulence (increase in LD₅₀~10^{0.3} pfu) and a significant increase in disease severity *in vivo* as well as prolonged growth in CD-1 mice lungs (from days 5 to 8 pi) (Figures 31 and 32).

The moderate increase in virulence observed by the 226I and 227K might be at least in part associated with the incompatibility between the NS gene and the IAV backbone that it was associated with in this experiment. Our experiments showed that the 1918 NS gene expressed on the A/PR/8/34 (H1N1) backbone decreased the rPR8wt LD₅₀ by > 1 log (LD₅₀ of

rPR8 virus = $10^{3.5}$ pfu while LD₅₀ of rPR8-1918NS virus = $10^{4.65}$ pfu). Similarly the 1918NS also attenuated the mouse-adapted (rWSN) viral replication and virulence in mice as reported by [245]. In 2006, a study has shown that the A/Brevig Mission/1/18 (H1N1) NS gene only contributed to virulence when expressed with the seven 1918 IAV structural genes [261]. Simultaneously the 1918NS virus was associated with enhanced virulence in macaques when expressed in combination with the surface 1918 antigens [13]. Therefore, we have further confirmed that essential epistatic interactions between the 1918 IAV genes and the genetic backbone confer enhanced virulence.

Further studies have shown that the 1918NS1 gene enhanced virulence was associated with a general IFN gene transcription inhibition [244]. When rescued onto the H1N1 USSR/90/77 backbone, the 1918NS gene enhanced viral replication and pathology through a delay in interferon and lung cytokine production in ferrets [262]. Similarly, our studies have shown that the 1918NS protein is a strong binding partner to CPSF-30 F2F3 segment. At the same time, the 1918NS1 (226I+227K) protein mediates a strong IFN- β inhibition at the pre-transcriptional level by limiting IFN- β and IRF-3 transcriptional activities. In addition, the 1918 NS1 227K residue was associated with limited IFN- β transcriptional activation that culminated in a significant decrease in the total IFN- β levels 24 hpi (Figure 33). Alternatively, a recent study described a new mechanism through which the NS1 C-terminal residues 226-ARSK-229 of H3N2 strain possessing 226A and 227R residues limit the antiviral response by mimicking a host-histone protein [263]. The NS1 ARSK C-terminal 226-229 (ARSK) residues are capable of binding a host transcriptional elongation complex (PAF1C) and therefore block specifically host antiviral genes transcription. The newly described

mechanism therefore explains the antiviral activity mediated by multiple NS1 genes [263] and suggests that the V226I and R227K mutations in our studies may be affecting this process. Further studies are required to examine the roles of the V226I and R227K mutations in affecting histone mimicry and host gene expression.

6.9 Epistatic interactions determine virulent properties

In this study we have repeatedly demonstrated that NS is a virulence factor by showing the enhanced virulence and replicative properties of the A/PR/8/34 (H1N1), A/HK/156/97 (H5N1), A/Ck/Bj/1/95 (H9N2) and A/Brevig Mission/1/1918 (H1N1) NS genes on the mouse adapted PR8 and WSN backbones. However the NS1 associated virulence can be contradictory depending on three essential factors:

a) The compatibility of the NS1 gene with the viral backbone is necessary to allow for inter-genic epistatic interactions between the IAV genes and NS1, more specifically between NS1 and the polymerase proteins to further improve viral polymerase activity and therefore enhance viral fitness [160]. In this study we have demonstrated that the virulence properties of the H5N1 and 1918 NS genes were only detected when interactions with the A/PR/8/34 structural IAV genes were permitted while virulence was completely halted when the structural genes were replaced by the human A/HK/1/68 IAV strain.

b) The intragenic interactions within the NS1 gene are also important determinants of the virulence properties of specific residues within an NS1 gene. As demonstrated in our study, 226I and 227K residues enhanced virulence, disease severity and growth properties of the 1918-NS protein. However, when the same mutations were introduced to the HK-NS

rescued onto the same PR8 backbone, virulence was reduced possibly due to uncharacterized intra-genic NS1 interactions.

c) Third, host susceptibility plays an important role in determining virulence properties as observed by BALB/c vs. CD-1 differences; i.e. this reflects the importance of NS1 interactions with various host factors and constitutes another level of epistasis that exists between the virus and host.

Therefore, It is imperative to understand all three genetic interactions described above to assess adaptive properties of IAV genes. In this thesis I have examined the biological properties of adaptive mutations using genetic approaches that demonstrate multiple instances of both positive and negative epistasis using a number of virus backbones and genes.

Table 6 Effects of 226 and 227 mutations on various human NS1 genes

NS1 GENE	226 & 227 MUTATIONS	BACKBONE	GROWTH <i>IN VIVO</i> ¹	DISEASE SEVERITY ²	LD ₅₀
A/HK/1/68 (H3N2)	V+R	A/PR/8/34 (H1N1)	1	1	>7.2
	V226I		nd ³	1.04	>7.2
	R227K		0.006	1.17	7
	V226I+R227K		0.27	0.87	>7.2
A/BM/1/98 (H1N1)	I+K	A/PR/8/34 (H1N1)	5311	25.63	4.65
	I226V		1	1	5.2
	K227R		0.5	4.3	5.0
	I226V+K227R		1	1	4.92
	I+K	A/HK/1/68 (H3N2)	nd	0.87	>7.2
	I226V		nd	0.22	>7.2
	K227R		nd	0.59	>7.2
	I226V+K227R		nd	1	7

¹ Indicates fold change with respect to the 226V+227R-containing virus at day 8pi

² Indicates fold change with respect to the 226V+227R-containing virus at the maximum body weight loss

³ Not determined

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Appendix 1: List of primers used for site directed mutagenesis

Mutations introduced into the HK-NS1 WT gene

M124I	Forward: CCA GGC AAT CAT AGA TAA GAA CAT CAT Reverse: CAT GAT GTT CTT ATC TAT GAT TGC CTG G
L98S	Forward: CAT GAC TAT TGA GGA ATC GTC AAG GGA CTG GT Reverse: ACC AGT CCC TTG ACG ATT CCT CAA TAG TCA TG
F103L+M106I	Forward: GAC TGG CTC ATG CTA ATT CCC AAG Reverse: CTT GGG AAT TAG CAT GAG CCA GTC
D2N	Forward: AAT GAA TTC TAA CAC TGT GTC AAG Reverse: CTT GAC ACA GTG TTA GAA TTC ATT
D125G	Forward: CAG GCA ATC ATG GGT AAG AAC ATC Reverse: GAT GTT CTT ACC CAT GAT TGC CTG
M124I+D125G	Forward: GAC CAG GCA ATC ATA GGT AAG AAC ATC ATG Reverse: CAT GAT GTT CTT ACC TAT GAT TGC CTG GTC
R227K	Forward: TGG CGA GAA CAG TTA AGT CAA AAG Reverse: CTT TTG ACT TAA CTG TTC TCG CCA
V226I+R227K	Forward: TGG CGA GAA CAA TTA AGT CAA AAG TTC G Reverse: CGA ACT TTT GAC TTA ATT GTT CTC GCC A

Mutations introduced into the NS1 gene of the A/Brevig Mission/1/1918 (H1N1)

I226V:	Forward: GAG AAC AGT TAA GTC AGA AG Reverse: CTT CTG ACT TAA CTG TTC TC
K227R:	Forward: GAG AAC AAT TAG GTC AGA AG Reverse: CTT CTG ACC TAA TTG TTC TC
I226V+K227R:	Forward: TGG CGA GAA CAG TTA GGT CAG AAG TTT GAA G Reverse: CTT CAA ACT TCT GAC CTA ACT GTT CTC GCC A

Mutations introduced into the NS1 gene of the A/Hong Kong/156/1997 (H5N1)

L103F: Forward: GAC TGG TTC ATG CTC ATT CCC AAG
Reverse: CTT GGG AAT GAG CAT GAA CCA GTC

I106M: Forward: CTG GTT AAT GCT CAT GCC CAA GCA G
Reverse: CTG CTT GGG CAT GAG CAT TAA CCA G

L103F+I106M: Forward: TCA AGG GAC TGG TTC ATG CTC ATG CCC AAG CAG
Reverse: CTG CTT GGG CAT GAG CAT GAA CCA GTC CCT TGA

Mutations introduced into the NS1 gene of the A/PR/8/34 (H1N1)

S103F: Forward: GGA CTG GTT CAT GCT CAT ACC CAA G
Reverse: CTT GGG TAT GAG CAT GAA CCA GTC C

I106M: Forward: GTC CAT GCT CAT GCC CAA GCA GAA AG
Reverse: CTT TCT GCT TGG GCA TGA GCA TGG AC

S103F+I106M: Forward: AGG GAC TGG TTC ATG CTC ATG CCC AAG CAG A
Reverse: TCT GCT TGG GCA TGA GCA TGA ACC AGT CCC T

Appendix 2: List of primers used for PCR and RT-PCR

Universal primers for segments 1, 2, 3 and 6

RTG GGG GAG CGA AAG CAG G

Universal primers for segments 4, 5, 7 and 8

RTA GGG GAG CAA AAG CAG G

Segment 8 specific primers

Forward Kc19: GGG GAG CAA AAG CAG GGT GAC AAA G

Reverse Kc11: GGT TAT TAG TAG AAA CAA GGG TGT TTT TTA TC

Appendix 3: List of reagents

Luria-Bertani (LB) Media

Per litre
10 g Bacto-tryptone
5 g Bacto-yeast extract
10 g NaCl
Adjust pH to 7.5 with NaOH and autoclave

LB Agar plates

Prepare LB media as described, just before autoclaving add 15g agar/litre. Autoclave, allow to cool to 55°C add then add 500 µg/µl ampicillin. Pour plates and allow to dry and harden

1.54 M NaCl

Add 90 g to 1 litre distilled water

0.5 M Dibasic sodium phosphate (500 ml stock)

Add 35.49 g to 500 ml distilled water

0.5 M monobasic sodium phosphate (500 ml stock)

Add 35 g to 500 ml distilled water

200 mM phosphate buffer

Add 50 ml monobasic sodium phosphate to 150 ml dibasic sodium phosphate, until pH equals 7.2. Monitor pH while adding monobasic sodium phosphate. Bring volume to 500 ml by adding distilled water

Antibody buffer

Add 3g bovine serum albumin to 100 ml of 10 mM PBS while stirring.
Add 0.3 ml 100% Triton-X with stirring. Bring pH to 7.2 with NaOH.

Anti-fade mounting media

90% glycerol, 10% PBS, 1mg/ml p-phenylenediamine

Appendix 4: Curriculum Vitae

EDUCATION

University of Ottawa, Ottawa Ontario Canada 2007-2012

Ph.D Microbiology and Immunology

Thesis "Identification of mutations in the NS1 gene that control Influenza A virus virulence in the mouse model"

Supervisor: Dr. Earl G. Brown

American University of Beirut, Beirut Lebanon 2006

M.Sc. Animal Sciences

Thesis "Studies on Newly Emerging H9N2 Avian Influenza Outbreak in Poultry of Lebanon "

Supervisor: Dr. Elie Barbour

Lebanese University, Beirut Lebanon 2004

Diploma in Agricultural Sciences

Thesis "Effect of Prebiotics, Avilamycin and Flavomycin on the gut flora of broilers raised under University Conditions "

Supervisor: Dr. Nada Usayran

PUBLICATIONS

Selman M., **Dankar SK**, Jia JJ, Forbes NE and Brown EG. Adaptive mutation in Influenza A viral NS gene is linked to host switching and induces a novel protein by alternative splicing. *Emerging Microbes and Infection*. 2012 In Press

Forbes NE, Ping J, **Dankar SK**, Jia JJ, Selman M, Keleta L, Zhou Y, Brown EG. Multifunctional adaptive NS1 mutations are selected upon human influenza virus evolution in the mouse. *PLoS One*. 2012; 7(2):e31839.

Ping J, Keleta L, Forbes NE, **Dankar S**, Stecho W, Tyler S, Zhou Y, Babiuk L, Weingartl H, Halpin RA, Boyne A, Bera J, Hostetler J, Fedorova NB, Proudfoot K, Katzel DA, Stockwell TB, Ghedin E, Spiro DJ, Brown EG. Genomic and protein structural maps of adaptive evolution of human influenza A virus to increased virulence in the mouse. *PLoS One*. 2011; 6(6):e21740.

Dankar SK, Wang S, Ping J, Forbes NE, Keleta L, Li Y, Brown EG. Influenza A virus NS1 gene mutations F103L and M106I increase replication and virulence. *Virology*. 2011 Jan 12; 8:13.

Ping J, **Dankar SK**, Forbes NE, Keleta L, Zhou Y, Tyler S, Brown EG. PB2 and hemagglutinin mutations are major determinants of host range and virulence in mouse-adapted influenza A virus. *J Virol*. 2010 Oct; 84(20):10606-18.

Barbour, EK, Sagherian VK, Sagherian NK, **Dankar SK**, Jaber L, Usayran NN and Farran MT. Avian Influenza outbreaks in poultry of Lebanon and transmission to the neighboring farmers and swine. *Veterinaria Italiana*. 2006 Apr-Jun; 42(2): 77- 85.

SCHOLARSHIPS AND AWARDS

Admission Scholarship 06/2010-08/2011
University of Ottawa, Ontario, Canada

American Society for Virology Travel Award 07/2010
American Society for Virology, Montana, Bozeman, US

Poster Day, Third Prize
PhD Microbiology and Immunology 05/2008
Department of Biochemistry, Microbiology and Immunology
University of Ottawa, Ontario, Canada

Bourse Internationale de la Francophonie 09/2007-05/2010
University of Ottawa, Ontario, Canada

Graduate Assistantship 09/2004-05/2006
American University of Beirut, Lebanon

ORAL AND POSTER PRESENTATIONS

Canadian Pandemic Preparedness Meeting: Outcomes, Impacts and Lessons Learned, CIHR, Montreal, Canada — 11/2010 — “ Identification of mutations in the NS1 gene that control Influenza virus virulence” —Poster presentation

American Society for Virology 29th annual meeting, Montana, Bozeman, USA— 07/2010 — “ Identification of mutations in the NS1 gene that control Influenza virus virulence” — Oral presentation

RNA club, CHEO Research Institute, Ottawa, Ontario, Canada — 01/2010 — “Evolution of Influenza A virus: NS1 virulence and pathogenicity” — Oral presentation

TEACHING EXPERIENCE

Mentor for undergraduate students— University of Ottawa, Ottawa, Ontario, Canada — 2009-2011

Supervised Honor's student (Mohammed Selman) at Earl Brown's Lab during the academic year 2010-2011. Thesis Title: "Effects of NS1 mutations on host range and virus fitness in mouse-adapted influenza A virus"

Supervised undergraduate students (Myriam Kimpton and Shelly Luu) for the period of Summer 2009 at Earl Brown's Lab, University of Ottawa, Ontario, Canada.

Let's Talk Science Volunteer Program— Instructor/ Volunteer— Immaculata High School, Ottawa, Ontario, Canada — Fall and Winter 2010

Preparing experiments (Chemistry magic show, DNA and forensics) for high school students to improve science literacy and turning kids on to science.

Teaching Assistant — American University of Beirut — 09/2004- 03/2006

Assisting in teaching, preparing lectures and exams for undergraduate courses "Animal Microbiology" and "Physiology and Anatomy".