

Multifunctional adaptive NS1 mutations are selected upon Influenza A virus evolution in the mouse

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Abstract

Influenza A virus (IAV) can evolve from low virulence in animal hosts to become highly virulent in humans. Pandemic Influenza A viruses such as the 1918 Spanish Influenza caused over 50 million deaths worldwide. However the genetic determinants of IAV host adaptation and virulence are largely uncharacterized. The IAV NS1 protein is a multifunctional interferon antagonist and a known virulence factor. We hypothesized that NS1 mutations selected upon IAV evolution to a novel host contribute to host adaptation by mechanisms involving increased gene expression and IFN antagonism. To this end, I phenotypically characterized the NS1 mutations selected upon adaptation of A/Hong Kong/1/1968 (H3N2) (HK-wt) to increased virulence in the mouse. Sequencing the NS genome segment of mouse-adapted variants revealed eleven mutations in the NS1 gene and four in the overlapping NEP gene. Using the HK-wt virus and reverse genetics to express recombinant HK NS1 mutant viruses, I demonstrated that all NS1 mutations were adaptive and enhanced virus replication (up to 100 fold) in mouse cells and/or lungs. All but one NS1 mutant was associated with increased virulence measured by survival and weight loss in the mouse. Ten of twelve NS1 mutants significantly enhanced IFN- β antagonism to reduce the level of IFN- β production relative to HK-wt in infected mouse lungs at 1 day post infection, where nine mutants induced viral yields in the lung that were $\geq$ HK-wt (up to 16 fold increase). Eight of 12 NS1 mutants had decreased binding affinity to the cleavage and polyadenylation specificity factor (CPSF30). The majority of mutant NS1 genes demonstrated increased viral polymerase activity and viral protein production in mouse cells. Viral protein production and viral growth were also assessed in human and canine cell

lines; however these adaptive phenotypes were more robust in infected mouse cells.

Adaptive NS1 mutations also increased cytoplasmic cellular localization of the NS1 protein in infected cells in a host cell-specific manner. Evaluation of phenotypic trends associated with the NS1 mutants demonstrated an inverse correlation between CPSF30 binding affinity and viral polymerase activity enhancement. This study demonstrates that NS1 is a multifunctional virulence factor subject to adaptive evolution.

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List of Symbols

α	alpha, anti
β	beta
Δ	delta
$^{\circ}$	degree
$\pm$	plus or minus

List of Abbreviations

1918 NS	NS gene segment of A/Brevig Mission/1/1918 (H1N1)
2'5' –OAS	2'5' oligoadenylate synthetase
^{35}S	radioactive isotope of sulphur
aa	amino acid
Arg	arginine
ATP	Adenosine 5'-triphosphate
bp	base pair
CDK	cyclin-dependent kinase
CFIA	Canadian Food Inspection Agency
CPSF30	30 kDa subunit of cleavage and polyadenylation specificity factor
CRK	CT10 regulator of kinase
CRM1	chromosome region maintenance 1
cRNA	complementary RNA
Cys	cysteine
cytopl.	cytoplasm
deINS1	Influenza A Virus lacking the NS gene segment

dH ₂ O	distilled water
DNA	deoxyribose nucleic acid
dsRNA	double stranded RNA
eIF2 α	eukaryotic initiation factor 2 alpha
eIF4GI	eukaryotic initiation factor 4 GI
ERK	extracellular signal related kinase
H & E	hematoxylin and eosin
h	hour
H, HA	hemagglutinin
H3	histone 3
HK	Hong Kong; A/Hong Kong/1/1968 (H3N2) IAV strain
HK-wt	HK wild-type strain
hnRNP-F	heterogeneous nuclear ribonucleoprotein F
HPAI	highly pathogenic avian influenza
hStaufen	human isoform of the yeast protein Staufen
IAV	influenza A virus
IF	immunofluorescence
IFN	interferon
Ile, I	isoleucine
IRF	IFN regulatory factor
ISG	IFN stimulated gene
Leu, L	leucine

Lys, K	lysine
M, m	mock-infected with PBS
M1	matrix protein 1
MA	mouse-adapted
MA20	mouse-adapted HK virus following 20 serial lung-to-lung passages
MA21	mouse-adapted HK virus following 21 serial lung-to-lung passages
MDA5	melanoma differentiation associated protein 5
Met, M	methionine
MLD ₅₀	median lethal dose 50
mRNA	messenger RNA
Mx1	myxovirus (influenza virus) resistance gene 1
N, NA	neuraminidase
N/A	not available
ND	not determined
ND10	nuclear domain 10
NEP	nuclear export protein
NES	nuclear export signal
NF-κB	nuclear factor- kappa B
NLS	nuclear localization signal
NoLS	nucleolar localization signal
NP	nucleoprotein
NP40	nonyl phenoxypolyethoxylethanol 40

NS1	nonstructural protein 1
NS3	nonstructural protein 3
nt	nucleotide
NXF1/TAP	nuclear RNA export factor 1/tip associated protein
PA	polymerase acidic protein
PABP1	poly(A) binding protein 1
PABPN1	poly(A) binding protein nuclear 1
PACT	PKR activating protein
PB1	polymerase basic protein 1
PB1-F2	PB1- frame 2
PB2	polymerase basic protein 2
PBS	phosphate buffered saline
PFU	plaque forming units
pH1N1	pandemic H1N1 IAV strain that emerged in 2009
Phe, F	phenylalanine
pi	post infection
PI3K	phosphatidylinositol-3-kinase
PKC	protein kinase C
PKR	protein kinase R
PTM	post-translational modification
RHA	RNA helicase A
RIG-I	retinoic acid-inducible gene 1

RNA	ribonucleic acid
RNP	ribonucleoprotein
s	second
SD	standard deviation
SDS	sodium dodecyl sulfate
SE	standard error
SG	stress granule
SH3	SRC homology 3 domain
snRNA	small nuclear RNA
SPF	specific pathogen free
STAT-1	signal transducer and activator of transcription 1
SUMO	small ubiquitin-like modifier
TLR	toll-like receptor
TREX	transcription export complex
TRIM-25	tripartite motif-containing protein 25
ts	temperature sensitive
U	units
UTR	untranslated region
vRNA	viral RNA
wt	wild-type

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1. Introduction

1.1. Preface

Highly pathogenic avian influenza (HPAI) H5N1 Influenza A viruses (IAV) present a global threat to world health. Since 2000, the World Health Organization has documented cases of IAV transmission from infected avian or poultry sources to humans, which result in a high incidence of severe viral pneumonia and often death. As of July 2012, HPAI H5N1 has caused 607 confirmed infections in humans, of which 358 cases induced mortality (59% mortality rate) (194). However, this virus has not yet fully evolved to the human host, as it is incapable of human-to-human transmission. If the virus undergoes genetic changes resulting in human adaptation, the world population may be at risk of infection with a highly virulent pandemic strain reminiscent of the 1918 Spanish Influenza, which was estimated to have killed 50 million worldwide (68). It is therefore necessary to understand the genetic determinants of IAV host range and virulence in order to predict the emergence of strains with pandemic potential.

The non-structural 1 (NS1) protein is expressed by IAV during virus infection and is a multifunctional interferon (IFN) antagonist (54) that contributes to virulence of IAV in animal models including the mouse and ferret (18, 110). The NS1 protein functions in numerous roles during virus infection; NS1: i) suppresses the IFN-regulated host response to IAV infection; ii) limits IFN production; iii) enhances viral gene expression; iv) down-regulates host gene expression; and v) suppresses the host cell apoptotic response to virus infection. Ultimately these functions allow the virus to evade the host immune response and replicate to robust levels. NS1 is capable of such a range of functions because it binds

numerous cellular factors, all other IAV proteins, as well as RNA. NS1 cellular binding partners include proteins involved in the IFN response, post-transcriptional factors, nuclear export machinery, spliceosome and translational factors, and apoptosis regulators (54).

The NS1 protein is a known IAV virulence factor. Several studies in addition to the research presented in this thesis have shown mutations and deletions in the NS1 protein increase IAV virulence and alter host range (See section 1.2.4). Although NS1 engages in numerous functions during virus infection, it is not clear which functions are the primary modulators for adaptive advantages. In order to identify and phenotypically characterize genetic determinants for IAV virulence and host adaptation, we and others have employed experimental evolution in a novel host to map adaptive genetic mutations (9-11, 63, 65, 66, 119, 198, 201). To this end, we forced the evolution of the human isolate A/Hong Kong (HK)/1/1968 (H3N2) (HK-wt) to increased virulence in the mouse by serial lung-to-lung passage (10, 11, 34, 71, 140, 141). Using reverse genetics, expression of each individual resulting mutant mouse-adapted (MA) genome segment on the parental HK-wt virus genetic backbone resulted in increased disease severity, including the NS1 V23A mutation that decreased the median lethal dose 50% (MLD₅₀) by 6 fold compared to the HK-wt virus (140). NS1 gene mutations F103L and M106I selected upon HK virus mouse adaptation were found to be convergent with virulence determinants in the HPAI H5N1 NS1 gene from A/HK/156/1997, and expression of these mutations in the HK-wt NS1 gene, the NS1 gene of A/HK/156/1997 (H5N1) and also A/Ck/Beijing/1/1995 (H9N2) increased virulence in vivo in the mouse (18). My PhD research phenotypically characterized NS1 mutations selected upon mouse adaptation of the HK-wt virus and demonstrated that expression of MA NS1

mutations was sufficient to drive host adaptation and enhance virulence in the mouse (34). To introduce the scope of my research, the remainder of this section will provide a brief overview of IAV, focusing on replication, host range, virus evolution, epidemiology, and the NS1 protein.

1.2 Influenza A Virus

Influenza A Virus (IAV) belongs to the *Orthomyxoviridae*, and has a negative sensed, single stranded segmented RNA genome. Influenza A, B, and C viruses, as well as Thogotovirus (transmitted by ticks) and Isavirus (infectious salmon anemia virus) account for the five genera of the *Orthomyxoviridae*. Influenza virus strains are classified according to their genus (A, B, or C), the species from which the virus was isolated (omitted if human), location of isolate, the reference number of the isolate, the year of isolation, and, for IAV, the subtypes of the hemagglutinin (H) and neuraminidase (N) surface proteins. For example, the parental virus used in my research is classified as A/Hong Kong/1/1968 (H3N2). Influenza A and B viruses possess eight RNA genome segments; while Influenza C virus possesses only seven RNA genome segments (132). Host range is distinct for each genus of Influenza viruses, where A, B, C, and Thogotovirus viruses cause disease in humans (see section 1.2.4 for further detail on IAV host range).

1.2.1 IAV Structure

IAV are enveloped pleomorphic viruses that acquire their lipid membrane upon budding from the host cell. The viral envelope is studded with surface proteins hemagglutinin (HA), neuraminidase (NA), and M2. HA is the IAV receptor protein; following recognition of sialic acid residues on the surface of host cells, HA also functions as the pH-

dependent fusion protein required for virus entry, and also has roles in particle formation and virus budding from host cells (161, 162). The NA protein is an integral membrane glycoprotein responsible for cleaving sialic acid molecules from the surface of the host cell, thus allowing the virus to be fully released from the host cell, which prevents accumulation and clumping of nascent virions at the cell surface (161). The final surface protein is the M2 protein, which functions as an ion channel, facilitating virus uncoating as well as virus maturation (139). The viral genome exists as 8 genome segments, which encode at least 11 viral proteins. The viral RNA (vRNA) never exists as naked nucleic acid; instead it forms a ribonucleoprotein (RNP) complex, consisting of vRNA, the viral polymerase proteins polymerase basic protein 2 (PB2), polymerase basic protein 1 (PB1) and polymerase acidic protein (PA). The most numerous RNP protein, the nucleoprotein (NP), coats the entire length of the vRNA (125). The matrix protein 1 (M1) lies below the viral envelope and forms a matrix with the viral RNPs by binding RNA in a non-sequence specific manner. IAV has two non-structural proteins; the NS1 protein and polymerase basic protein- frame 2 (PB1-F2), which are only expressed in cells during virus infection (See section 1.2.2 for details).

1.2.2 IAV Life Cycle

IAV enters the host cell by endocytosis; this is initiated when the virus particle is bound to sialic acid molecules at the host cell surface by the HA protein (162), then the virus is engulfed internally by an endocytic compartment (100). The endosome increases in acidity, which triggers a conformational change in the HA protein, resulting in exposure of the fusion peptide, which leads to fusion with the endosomal membrane (100, 162). Endosomal acidification allows diffusion of protons into the virion via the M2 ion channel,

which facilitates uncoating of the RNP complex (139). The RNP then translocates to the host cell nucleus through nuclear pores by interaction of the three NP nuclear localization signals (NLS) with host nuclear pore machinery (importin α family members) (16).

Once inside the nucleus, the negative sense vRNA is transcribed into mRNA by a primer-dependent mechanism, which, by exploitation of host transcription products, are capped and then polyadenylated by a viral RNA polymerase dependent process (112). In a primer-independent reaction, the vRNA is also transcribed by the viral polymerase into a full length positive sense copy, called complementary RNA (cRNA), which serves as an intermediate to amplify vRNA for viral replication (also primer-independent) (112). Like vRNA, cRNA is encapsidated by NP, which is synthesized in the cytoplasm by host translational machinery, then shuttled back to the nucleus through the nuclear pore complex by interaction with cellular importin $\alpha 1/\alpha 2$ proteins (186). The protein subunits that form the viral polymerase complex each perform a specialized function during transcription. The PB1 protein is responsible for nucleic acid elongation, while PB2 has a unique role in mRNA transcription initiation. IAV is unable to cap its own mRNA, although the virus relies on cap-dependent mRNA translation. Therefore the virus has evolved to exploit the host to circumvent this obstacle by engaging in “cap snatching” (112). PB2 binds and recruits the 5' cap on pre-mRNA host transcripts, while PA is responsible for cleaving the cap, which allows it to serve as a primer for viral mRNA transcription (23). Viral mRNA is polyadenylated by PB1, which finalizes its transcripts by the addition of a stretch of adenine residues. The non-structural protein PB1-F2 also plays a role in viral polymerase activity. The PB1-F2 protein, which is expressed by most influenza viruses due to an alternative

initiation site in the +1 reading frame during translation of the PB1 mRNA, has been found to enhance viral polymerase activity indirectly by interacting with the full length PB1 protein (107). However, enhanced viral polymerase activity associated with PB1-F2 expression is strain specific and has not been shown to affect virus replication rates (108).

The virus also relies on the host spliceosome machinery to express the two viral transcripts encoded on gene segment 7 (M1 and M2 proteins) and two or three transcripts on gene segment 8 (NS1, nuclear export protein (NEP), and non-structural protein 3 (NS3; synthesized upon NS1 D125G (GAT-GGT) mutation, described further in section 3.4.11). The ratio of spliced (M2, NEP and NS3) to unspliced (M1, NS1) transcript abundance is tightly regulated by the viral polymerase, the cellular spliceosome, as well as by the rate of transcript nuclear export. Moreover, the NS1 transcript as well as the NS1 protein have been found to influence splicing by cis and trans functions, respectively, including regulation of nucleocytoplasmic transport of spliced mRNAs (1, 2, 39, 148, 149) (See section 1.3.4 for further details).

Viral gene expression is temporally regulated, where early gene expression consists of the genes encoding NP and NS1 proteins, while late gene expression is characterized by the genes encoding the matrix protein M1 as well as surface proteins HA and NA. The polymerase proteins show less temporal regulation and are synthesized early and throughout infection. One factor that has been associated with the switch from early to late gene expression is the 3' sequence of the vRNA promoter, which differs by one nucleotide at position 4 between gene segments encoding polymerase proteins (C4) and the remaining 5 gene segments (U4) (82). Mutational analysis has shown that the C4 gene segments are

subject to diminished transcription and increased replication compared to the U4 gene segments (83), which explains the observation of less polymerase mRNA and proteins in infected cells compared to other viral genes/proteins (164) (Figures S6-S10). The switch from early to late gene expression also drives the virus toward replication, as M1 protein accumulation down regulates viral transcription (136), while promoting the export of RNPs from the nucleus to the cytoplasm, the site of virus particle assembly (105). Moreover, a recent study by Perez et al. (2010) showed IAV small RNA transcripts also drive the switch from transcription to virus replication (137).

Since IAV requires host factors for viral protein synthesis, viral mRNA must be exported from the nucleus into the cytoplasm. Viral mRNA export is dependent on interaction with cellular mRNA export machinery, specifically the nuclear RNA export factor 1/tip associated protein complex (NXF1/TAP). However the extent to which viral mRNA export depends on NXF1/TAP is viral transcript-dependent. Interestingly, the transcript for one of the most abundant viral proteins, NP, was found to export to the cytoplasm by a process completely independent of NXF1/TAP (145).

Cellular translation factors are also hijacked to favour viral protein synthesis over cellular protein synthesis due to numerous actions of the NS1 protein. The NS1 protein also inhibits cellular gene expression by binding host factors involved in post-transcriptional processing, the cleavage and polyadenylation specificity factor 30 KDa subunit (CPSF30) (76, 78, 121, 124) and the polyA binding protein nuclear 1 (PABPN1) (15), and thus inhibits host message maturation. In addition, NS1 limits the abundance of host mRNA in the cytoplasm by binding to NXF1/TAP which inhibits its function in nuclear export (151). NS1 also directly

enhances viral protein synthesis by binding and recruiting cellular translation factors eukaryotic initiation factor 4 G1 (eIF4G1) and poly(A) binding protein 1 (PABPI) to the 5' untranslated region (UTR) of viral mRNA, which is also a binding target for NS1 (3, 12). The role of NS1 in enhanced viral gene expression is further discussed in section 1.3.4.

In order to drive assembly of new virions, IAV translocates nascent RNP to the cytoplasm, which is mediated by the M1, NEP, and NP proteins. M1 interacts with the RNP as well as vRNA itself and facilitates its disassociation from the nuclear matrix (120), while NEP interacts with nucleoporins as well as the nuclear export receptor chromosome region maintenance 1 (CRM1) (123). RNP nuclear export is also driven by its own nucleoprotein; NP encodes three nuclear export signals (NES), one of which has been found to function in a manner dependent of CRM1 (196). Virus assembly occurs along the apical cellular membrane to allow budding from the external cell surface. The M1 protein facilitates the interaction between the cytoplasmic tails of the surface proteins HA, NA, and M2 with the RNPs (120). The nascent virus buds from the host plasma membrane in an ATP-dependent process driven by the M1 protein (46). The bud is then pinched off by NA-induced cleavage of HA-bound sialic acid residues by the NA, and virus progeny is ultimately released in the extracellular environment.

1.2.3 Mechanisms driving IAV genetic diversity

Influenza A viruses are perpetually evolving in a battle to replicate while circumventing host antiviral responses. The virus accumulates mutations through a process termed *genetic drift*, where sequence variations are introduced into the virus quasispecies pool by IAV low-fidelity polymerase that are either trapped or selected in an attempt to

evade host immune system recognition while preserving important structural and functional protein characteristics. However, changes in the environment drive the selection of biological variants with increased replicative fitness (19). When a population of a magnitude large enough to account for all possible single nucleotide polymorphisms ($\geq 3 \times 10^5$ PFU for IAV) is faced with environmental changes, such as serial passage in a novel host, adaptive evolution occurs at a maximal rate (44). Current adaptive evolution theory states that when a population is under strong selective pressure due to changes in the environment, the first mutations selected provide the greatest advantage i.e. most pronounced increase in replicative fitness (reviewed in (129)).

This theory is supported by the differential rate of evolution among viral proteins. The HA and NA surface glycoproteins are most exposed to the host immune system and thus highly antigenic. Therefore HA and NA evolve at an accelerated rate compared to other viral proteins. Sequence analyses of IAV strains have noted an increased ratio of coding: silent mutations in the HA protein compared to viral polymerase proteins, which are highly conserved and under little pressure from the host antiviral immune response (8, 118, 189). Natural selection predicates deleterious and non-advantageous mutations to be selected against as they cannot compete within the quasispecies viral population.

Since IAV possesses a segmented genome, the virus also evolves by a process called *genetic shift* or *reassortment*. Reassortment refers to the event that occurs upon infection of a cell by more than one virus species, where nascent progeny receive viral gene segments from more than one parental viral genetic origin. Reassortment between two virus isolates can hypothetically result in 2^8 (256) genetically distinct progeny, however as

IAV infects as a quasispecies in vivo, natural selection dictates which resulting recombinant progeny can outcompete other strains and propagate to a titre high enough to transmit to a new host. Reassortment events have been linked to the emergence of numerous pandemic strains of IAV, often where an antigenic surface protein has originated from a non-human virus lineage and is thus immunological naïve in the human host (45). Through both genetic drift and genetic shift, seasonal IAV infection is always one step ahead of recognition by the adaptive immune response. As a result, influenza vaccines have to be tailor-made on an annual basis to provide protection against that year's circulating strains.

1.2.4 IAV Host Range

Influenza A viruses have an extensive host range, however virus sampling and phylogenic analyses has indicated the primary viral reservoir is in aquatic birds (189); all known virus subtypes (H1-H17; N1-10) have been isolated from aquatic birds with the exception of H17N10, which was recently isolated from bats and has not been shown to have originated from avian hosts (177). IAV strains have been isolated from a wide range of other hosts, including swine, equine, canine, feline, poultry, sea mammal and humans. However, a given strain is restricted in its host range due to numerous factors, some which are well understood, others that remain unknown. Numerous researchers have sought to define the genetic determinants of IAV adaptation to a novel host.

Genetic mutations in viral polymerase proteins, surface proteins, and the multifunctional IFN antagonist protein, NS1, have been linked to virus adaptation to a novel host. The D701N mutation of the PB2 protein was associated with mouse adaptation of avian H5N1 and seal H7N7 viruses (37, 92). Mouse adaptation of the human pandemic

isolate A/Hong Kong/1/1968 (H3N2) also strongly selected for the PB2 D701N mutation, where 25 of 39 mouse-adapted virus isolates selected for this mutation (141), which was associated with increased viral polymerase activity and virulence in the mouse (140, 141). The 701 residue lies within an adaptive hot-spot; 3-D protein modeling demonstrated 701 is adjacent to another PB2 residue associated with host adaptation, 627.

Numerous reports have shown the amino acid at position 627 of the PB2 protein is a critical determinant of IAV host range. While avian IAV generally possess a glutamic acid residue at this site, human viruses instead have a lysine residue (except for 2009 pandemic H1N1 virus (pH1N1) that has 627E). The PB2 E627K mutation has been associated with enhanced virus replication, virulence, tissue tropism, and transmission of IAV in mammalian hosts (58, 59, 168). However, virus adaptation to a novel host is often a phenomenon characterized by epistasis, where the phenotypes are affected or dependent on other mutations in the viral genome. In such instances epistatic mutations in more than one viral gene either additively or synergistically result in host adaptation. Hence the discovery that the novel 2009 pH1N1 isolate possessed the avian-type E627 PB2 signature initially baffled scientists. Moreover, pH1N1 PB2 mutation to 627K resulted in increased viral polymerase activity but did not increase replication or pathogenesis *in vivo* in the ferret lung (62), and attenuated virus replication both *in vivo* in mice and *in vitro* (67, 200). It has been shown that a pair of PB2 mutations, 590S and 591R, can suppress the negative influence of the PB2 627E mutation in mammalian cells on pH1N1 virus replication (96). A recent report highlighted the importance of an additional PB2 residue, 271A, in pH1N1 adaptation to

humans (197). These studies show one single mutation is often insufficient to explain evolution of a zoonotic virus to humans.

As the HA protein is the most highly immunogenic and antigenically variable IAV protein, it is not surprising the protein is constantly under evolutionary pressure to evolve and escape host immune response recognition. Moreover, HA recognizes sialic acid residues on the apical surface of polarized cells and these receptors differ in sugar linkages in a host-dependent manner. Seasonal IAV strains currently circulating in the human population preferentially bind α -2,6-linked sialic acid, which is found on human cells lining the upper respiratory tract, in the bronchioles, trachea, and nasal turbinates. In contrast, avian influenza strains preferentially bind α -2,3-linked sialic acid, which is found on avian epithelial cells lining mucosal surfaces including the gastrointestinal and respiratory tract. The lower respiratory tract of humans, i.e. the alveolar region, also is lined with cells expressing α -2,3-linked sialic acid on their surfaces. Hence, in the case of HPAI H5N1 infection in humans, pathology is characterized by increased alveolar inflammation and incidences of viral pneumonia compared to seasonal IAV, which induces tracheobronchitis. Notably, cells lining the respiratory tract of swine contain both α -2,3 and α -2,6 -linked sialic acid, therefore swine are permissive to both human and avian IAV isolates and can serve as “mixing vessels” for avian and human strain reassortment events. Such an event was responsible for the emergence of the novel pH1N1 virus in 2009 (181).

Preferential binding to a specific sialic acid sugar linkage alone does not determine HA host specificity. Research conducted by Liya Keleta, a previous member of Earl Brown’s laboratory, found that not only the linkage but the adjacent sugar moieties determined HA

host specificity (71). Mutations that affect HA cleavage (and exposure of the fusion peptide), glycosylation, and pH of fusion also have been found to influence tissue tropism, virulence, and host range (71, 106, 141, 182, 184).

Mutations and deletions in the NS1 protein have also been associated with increased IAV virulence and host adaptation. HPAI H5N1 viruses isolated from waterfowl, poultry, and humans in Southeast Asia since 2000 possess a deletion from nucleotide positions 263–277 in the NS gene (48, 49, 85), resulting in a NS1 deletion from residues 80 to 84 that enhances virulence in both chicken and mice (98). Other HPAI H5N1 isolates such as A/Goose/Guangdong/1/96 and A/Duck/Guangxi/27/03 possess NS1 mutations V149A and P42S that induce high virulence in chickens and mice, respectively (85, 93). A recent study employing a novel sequence/phylogeny analysis methodology proposed roles for NS1 sequence variations in determining IAV host range (126). The NS gene segment of the 1918 Spanish Influenza (1918 NS) contributed to the virulence of the virus by enhancing the ability to antagonize the antiviral IFN response to the virus. Expression of the 1918 NS on the A/WSN/1933 (H1N1) virus by reverse genetics blocked the expression of IFN-stimulated genes (ISGs) more efficiently than expression of the parental WSN NS gene segment upon in vivo infection in the mouse (42). In addition, my research has shown that NS1 mutations selected upon human IAV adaptation to the mouse are sufficient to induce virulence in a novel host, which was associated with increased interferon antagonism and viral gene expression (34). The genetic determinants associated with IAV host adaptation have yet to be fully elucidated, neither in this summation nor in scientific exploits. However research thus far has shown that IAV host adaptation is a complex process involving acquisition of

advantageous mutations and/or genetic reassortment events, allowing the virus to transmit, replicate, and avoid recognition of host responses upon infection in a novel host.

1.2.5 Experimental models of IAV host adaptation

Experimental evolution of IAV in a lab-controlled setting has allowed scientists to study the genetic determinants of IAV host adaptation and to begin to understand how IAV isolates gain the ability to infect, transmit and induce pathology in a novel host. Influenza A virus has been experimentally evolved to the mouse (9-11, 34, 37, 65, 71, 119, 140, 141, 153, 166, 198, 201), ferret (63, 66), duck (159, 167), and also chicken (24, 144, 165, 173). Each model has its own limitations and benefits, which will be discussed for the two mammalian models, the ferret and mouse.

The ferret in vivo IAV model has several advantages and disadvantages to other model organisms, which will be discussed later. As mentioned, sialic acid receptors of the ferret respiratory tract closely resembles those lining the human respiratory tract. In addition, a recent study highlighted the similarities between ferret and human HPAI H5N1 neuropathology including infected cell types and the severity of meningoencephalitis (135). However, ferrets are expensive, as is their housing costs, they are only available as outbred lines, and serial passage in the ferret lung is quite laborious.

An example of experimental evolution of an IAV strain to the ferret is HPAI H5N1 virus. Infection with HPAI H5N1 results in high (59%) mortality in humans (194). Infection is caused by direct contact with contaminated poultry or avian sources, as there has been no reported human-to-human HPAI H5N1 transmission. Therefore this virus has not fully evolved to the human host and this possibility presents a threat of pandemic proportion.

Recently, two studies forced the evolution of HPAI H5N1 to render the virus transmissible between ferrets (63, 66).

Since news of this research reached the press, these reports have created waves of controversy and an international press embargo delayed their publication (117). The ferret is generally accepted to be the animal model that most closely resembles human respiratory infection associated with seasonal IAV (180), and ferret adaptation of a virus already known to induce a high percentage of mortality in humans (194) was perceived by the global media more as a bioterrorism threat than as a predictive tool to study the emergence of pandemic strains from natural viral reservoirs.

In Imai *et al.* (2012), researchers generated a series of reassortant human viruses expressing the HA protein from an HPAI H5N1 strain, where the HA gene segment was subject to random mutagenesis. HA mutations that altered receptor specificity to favour α -2,6-linked sialic acid binding as well as mutations that increased HA stability were found to increase transmissibility of the H5N1 HA-expressing virus to the ferret (66). In Herfst *et al.* (2012), HPAI H5N1 was first subjected to site-directed mutagenesis then serially passaged in the ferret lung in parallel with the wild-type HPAI H5N1, which culminated in the ability of the mutant virus to transmit efficiently from ferret to ferret, although the virus did not induce mortality following transmission (63). Serial passage in the ferret drove the selection of five IAV mutations, four in the HA protein and one in the PB2 protein, however the mechanism by which these mutations result in efficient mammalian transmission remains to be elucidated.

The mouse in vivo model of IAV host adaptation, compared to the ferret model, is cost-effective, genetically defined, and can be performed in small rodent BSL2 animal facilities. However, the mouse lung resembles an avian lung in receptor specificity in that it is lined with cells expressing α -2,3-linked sialic acids. Therefore, the mouse model does not allow for examination of IAV pathogenesis in a manner parallel to the pathogenesis observed in the human host. With this in mind, the mouse does not naturally harbour IAV and therefore is an immunologically naïve host to IAV originating from avian and mammalian sources alike. This system is therefore satisfactory for the study of IAV adaptation to a novel host.

1.2.6 IAV Epidemiology in Humans

Influenza virus is estimated to infect 10-25% of Canadians each year (60). Of that population, 2,000 to 8,000 individuals die as a result of influenza and its complications annually, depending on the severity of the seasonal strains (142). Influenza epidemiology typically consists of seasonal influenza, which annually occurs in the fall and winter seasons. Seasonal influenza symptoms include mild tracheobronchitis, accompanied with sore throat, nasal congestion, fever, chills, body aches, and overall fatigue (13). The illness is self-limiting and resolves without intervention within 1-2 weeks. However, immunocompromised individuals, those with underlying chronic conditions of the heart and lung, as well as the young and elderly, are at greater risk for more severe complications, including viral pneumonia and bacterial pneumonia. The majority of mortalities associated with seasonal IAV infection are from these at-risk populations.

Influenza A virus is also associated with pandemic influenza, which has occurred throughout history, and likely was the cause of the sickness accounted by Hippocrates in 412 BCE (69). Pandemics are defined as disease outbreaks that affect a large geographic area (>1 continent) in a short period of time. Influenza A virus pandemics are typically characterized by high levels of morbidity and mortality (primarily due to viral pneumonia).

1.2.7 Pandemic Influenza

The most severe pandemic recorded in history was the Spanish Influenza pandemic of 1918 (A/Brevig Mission/1/1918 (H1N1)), which was estimated to have killed over 50 million worldwide (68). This pandemic as well as later pandemics can be attributed to the evolution of a zoonotic IAV strain to high virulence in humans. The 1918 virus has been phylogenically traced back to an avian lineage, however interestingly the actual first transmission to human is speculated to have involved contact with swine (17). It is speculated that primary transmission of the avian virus to the pig resulted in adaptive mutations that allowed transmission to humans. Phylogenic analysis on the origin of the 1918 pandemic strain has demonstrated the virus actually was introduced to a mammalian host 1-2 decades prior to 1918 (26, 163), then the pandemic strain emerged from a reassortment event between viruses of human and mammalian origin; most likely this occurred upon co-infection in the pig (163).

There have been several other influenza pandemics in the past century. In 1957, Asian influenza caused an estimated 1 million deaths worldwide, where mortality was highest in the young. The virus originated from Southern China, but quickly spread throughout Asia then to Europe and North America. Phylogenetic analyses showed the virus

resulted from reassortment between avian and human lineage viruses, where the HA (H2) and NA (N2) as well as the PB1 gene segments were of avian origin (70, 152). At this point in history, humans were immunologically naïve to H2 and N2 viral antigens, which explains the heightened pathogenicity of Asian influenza.

In 1968, emergence of a novel H3N2 strain completely replaced the H2N2 strains circulating since 1957. The virus was first isolated in Hong Kong, and is therefore known as Hong Kong influenza. The virus spread worldwide and, like the Asian influenza of 1957, the greatest mortality risk was for the young. The total deaths attributed to the Hong Kong pandemic were estimated to about 1 million. The H3 surface protein originated from an avian strain (152), however since N2 subtyped viruses were already in circulation, humans already had partial immunity to the virus. Another shared feature between the 1957 and 1968 pandemic strains is both have an avian origin PB1 gene segment (70).

Most recently, a novel H1N1 virus emerged as a pandemic IAV strain. This virus (pH1N1) was first isolated in March 2009 in Mexico, and by June 2009 the World Health Organization officially declared that the epidemic had fulfilled the criteria of an influenza pandemic (14). However, the virus was not as virulent as initially feared, and the number of deaths resulting from the pandemic remained minimal. In the United States, an estimated 1 in 2000 individuals infected with pH1N1 died (80). Although the finalized estimates of pH1N1 mortality are still being assessed by the World Health Organization (193), a recent study estimated the global mortality resulting from pH1N1 within the first 12 months of its circulation to be between 100,000 and 400,000 (21). As with the 1957 and 1968 pandemics, the pH1N1 strain preferentially affected young adults, with a clustering of

severe and fatal cases in adults between the ages of 30 and 50 years; moreover 80% of deaths in the first 12 months were in people below the age of 65 (21). The majority of pH1N1 cases resulted in mild respiratory infection; however there was also documentation of lower respiratory tract illness and gastrointestinal symptoms primarily in children and pregnant women (127). Notably, the majority of severe cases occurred in individuals with underlying medical conditions (134).

The pH1N1 virus is now known to have emerged from a triple reassortment event, where the NA and M gene segments have been traced to a Eurasian swine genetic lineage, and the HA, NP, and NS gene segments originate from a classical swine lineage. The PB2, PA, and PB1 genes are from the swine triple reassortant lineage, which emerged around 1998 when the classical swine influenza virus underwent reassortment with a H3N2 human strain and an American avian influenza virus (41). Although IAVs most typically cause mild seasonal influenza, the potential for zoonotic strains to adapt to the human host remains a possibility, and with it the emergence of novel pandemic strains.

1.3 The NS1 protein

The NS1 protein is a non-structural viral protein of 215-237 amino acids (27); it is expressed during viral infection but is not packaged in nascent virus particles. NS1 is encoded by the NS1 gene, which is encoded by the full length gene product of the NS gene segment, the smallest of the IAV genome segments. The NS gene segment also encodes the nuclear export protein (NEP; previously termed NS2). The NS1 and NEP proteins share the 5' leader sequence preceding the start codon followed by 27 coding nucleotides; however the NEP mRNA is then spliced out of frame from the NS1 mRNA, resulting in the expression

of two proteins with 10 conserved N-terminal amino acids (79). The N-terminal region of NS1 (aa 1-73) comprises the RNA binding domain, while the remainder of the protein is known as the effector domain (Figure 1). The distal C-terminus (from aa 207) is less conserved in length and in sequence, and known as the disordered tail region. Phylogenetic analysis of the evolution of the NS1 protein has shown that prior to the 1940's, NS1 existed as a 230 aa protein, however upon a single nucleotide mutation that abrogated a stop codon, the protein lengthened to 237 aa, which remained the dominant form of the protein for four decades, at which time the mutation was reversed (54). In addition to this historical trend, NS1 proteins with truncated C-terminal tails (~15–30 aa truncation) are common (171). The effector domain and the RNA binding domain are connected by a short linker domain, which possesses a conserved sequence among IAV strains. Although the direct function of this domain is unknown, alanine mutagenesis resulted in attenuated virus replication and altered NS1 localization and deletion of aa 80-84 resulted in increased virulence of the 2003 H5N1 lineage (as reported earlier) (87). NS1 exists as a homodimer, and both the RNA and effector domains are involved in dimerization (122) that results in higher order tubular and filamentous structures (7). Dimerization is required for RNA binding, and double stranded RNA (dsRNA) is bound to a NS1 dimer in a 1:1 ratio (188). NS1 binds RNA by interaction of its N-terminal basic amino acids (Arg-38 and Lys-41 are two key residues) with the negatively charged nucleic acid, therefore binding occurs in a RNA sequence-independent manner. NS1 binds numerous cellular and viral proteins, and this binding occurs within discrete regions in both the RNA binding and the effector domains.

NS1 binding partners and their role in virus infection will be discussed in subsequent sections.

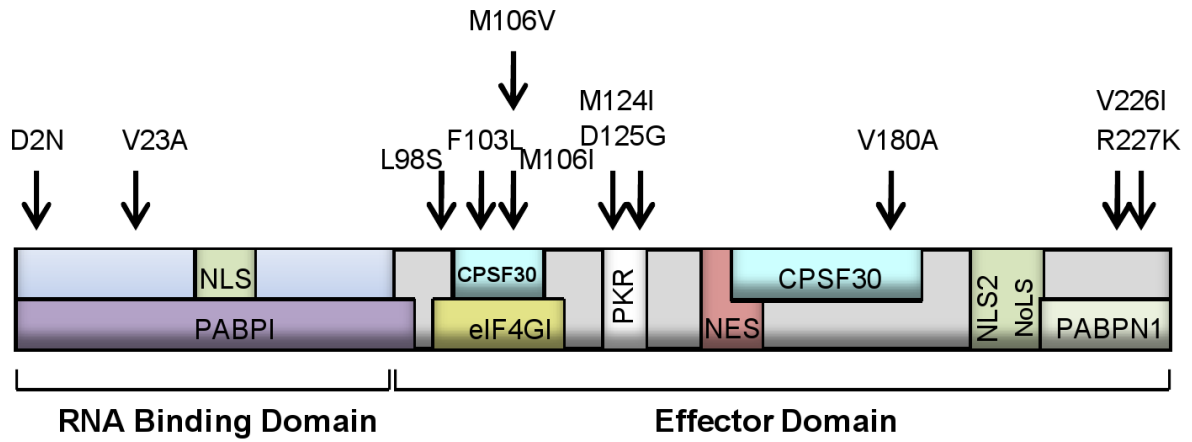


Figure 1. NS1 protein map. Location of NS1 mutations selected upon adaptation of the A/Hong Kong/1/1968 (H3N2) influenza A virus strain to the mouse are depicted by black arrows. NS1 binding domains to cellular factors are indicated within and below the protein diagram (34).

1.3.1 Expression, modification, and cellular distribution of the NS1 protein

NS1 is expressed during early virus gene expression; NS1 mRNA is the first viral mRNA expressed along with NP, and NS1 protein is detectable by 2.5 hours following infection in vitro (155). The NS1 gene transcript is much more abundant than the spliced NEP transcript, and the ratio of the two transcripts is estimated at 10:1 (79). The NS1 protein is one of the most highly expressed IAV proteins, and undergoes numerous post-translational modifications (PTMs); these two factors contribute to the ability of the NS1 protein to engage in numerous functions.

Several reports have shown NS1 undergoes SUMOylation, a reversible PTM mediated by the small ubiquitin-like modifier (SUMO) protein (130, 131, 195). In one report, a NS1 protein of an HPAI H5N1 strain was found to be modified by the human SUMO1 protein at Lys-219 and Lys-221. Abrogation of SUMOylation by site-directed mutagenesis was found to have a pronounced impact on NS1 function. NS1 lacking SUMOylation sites demonstrated decreased stability, and virus expressing SUMOylation-deficient NS1 proteins suppressed host gene expression to a lesser extent, and ultimately attenuated virus replication (195). Immunoprecipitation assays employing HA-tagged NS1 proteins of various IAV strains demonstrated the majority of NS1 proteins undergo SUMOylation (195). Modification of the NS1 protein by SUMOylation thus positively enhances NS1 at a structural and functional level.

NS1 has also been found to undergo phosphorylation in its C-terminal region by various cellular kinases. NS1 is phosphorylated by the cellular protein kinase C (PKC) (102) in a strain-specific manner (138). Abrogation of PKC phosphorylation during virus infection

reduces viral polymerase activity and diminishes virus replication; however PKC also phosphorylates PB1 and PB1-F2 and the contributions of NS1 phosphorylation to viral polymerase activity and virus replication are not known (102, 116). The NS1 of A/Udorn/72 (H3N2) undergoes phosphorylation at aa Thr-215 by a kinase belonging to the cyclin-dependent kinase (CDK)/ extracellular signal related kinase (ERK) family, and abrogation of this modification by alanine substitution reduced virus replication in a mechanism independent of IFN antagonism (53). Therefore NS1 phosphorylation enhances viral replication in a mechanism that may involve enhancement of viral polymerase activity.

NS1 is also reported to be modified by ISG15, a ubiquitin-like modifier that is induced by IFN α/β expression (175, 199). Unlike NS1 phosphorylation and SUMOylation, ISGylation was found to suppress, not enhance, protein function and ultimately virus replication. ISG15 modifies target proteins through the sequential action of three enzymes, E1, E2, and E3 (Herc5), which are all induced by IFN α/β expression. ISG15 conjugation suppresses IAV infection in vivo; where ISG15 knockout mice are more susceptible to both influenza A and B virus infection (84). Through the enzymatic action of Herc5 (175), ISG15 targets NS1 at Lys-41 (199), which is within both the RNA binding domain and a NLS that interacts with cellular importin- α to translocate NS1 to the nucleus (109). ISGylation at Lys-41 was found to block NS1 interaction with importin- α , however RNA binding affinity was not affected (199). The IFN-induced modification of NS1 by ISG15 was found to inhibit viral replication in vitro and virulence in mice in vivo (199).

NS1 is found in both the cytoplasm and the nucleus of infected cells. Like all IAV proteins NS1 is synthesized in the cytoplasm by host translational machinery. Nascent NS1

proteins rapidly localize to the nucleus of infected cells; this has been recently validated by real-time visualization of NS1 by bi-arsenically labeling live cells (89). NS1 possesses two NLS, NLS1 at aa 34-38 and NLS2 at aa 203-237 (47), which interact with the cellular protein importin- α (109). Key residues involved in nuclear localization are Arg-35, Arg-38 and Lys-41 for NLS1, and Lys-219, Arg-220, Arg-231 and Arg-232, (109), as well as Lys-221 (56) for NLS2. Since it is common for NS1 proteins to possess C-terminal truncations, not all NS1 proteins possess NLS2. NS1 also possesses a nucleolar localization site (NoLS) within NLS2, with key basic residues Arg-224 and Arg-229 (109). In addition to the nucleolus, NS1 has been reported to co-localize with nuclear domain 10 (ND10), which are sub-nuclear structures that have been implicated as sites of viral replication (150). Although ND10 structures are more frequently observed and increase in size upon IAV infection, the actual role of this cellular site in IAV replication is unknown. Several reports have shown inhibition of NS1 nuclear localization by alanine substitution at key NLS residues 38 and 41 resulted in accumulation of NS1 in the cytoplasm, which led to increased IFN α/β production and attenuation of virus replication (25, 114). Therefore the ability of the NS1 protein to translocate to the nucleus and engage in functions such as suppression of host gene expression and limitation of IFN induction are essential for optimum virus replication.

NS1 also engages in numerous functions in the host cell cytoplasm, including inhibition of IFN-stimulated antiviral proteins, suppression of the host cell apoptotic response, and enhancement of viral protein synthesis. Several reports have shown NS1 present in the cytoplasm of infected cells immediately following infection (89), which can be attributed to nascent NS1, and also at later times during infection (156). NS1 possesses a

nuclear export signal (NES) in the effector domain from aa 137-147, however the secondary structure of NS1 in the region adjacent to the NES is hypothesized to typically “mask” this site (90). It currently is not known whether a cellular or viral factor is involved in “unmasking” the NES, or if alternative mechanisms are involved. Another possibility is nascent NS1 protein may remain in the cytoplasm, which may be due to interaction with binding factors that abrogate the ability of NS1 to bind importin- α binding.

Several reports using various viruses and cells have shown differing patterns of cellular localization of the NS1 protein. Since the NS1 localization was first documented (47), NS1 was regarded as a primarily a nuclear viral protein. Subsequent reports verified this notion, however in 1998, a publication by Li *et al.* brought the cellular localization of NS1 back into question. In this report, the researchers not only investigated whether naturally occurring C-terminal truncations of the NS1 protein as well as host cell lineage affected NS1 localization, but also whether the fixation procedure played a role (91). Previous studies, including Greenspan *et al.* (1988), employed acetone fixation prior to immunofluorescence (IF) imaging (47). However, as NS1 is a small protein, it could hypothetically be extracted by acetone. Acetone and formaldehyde fixation were compared in two parallel IF experiments, and it was found that only formaldehyde-fixed cells were able to allow NS1 to be stained in the cytoplasm. Therefore previous reports could have indeed been underestimating the amount of NS1 localized to the cytoplasm.

NS1 localization has been demonstrated to be strain-specific as well as host cell-specific (90, 109, 183). In addition, NS1 binding affinity for nuclear factors has also been considered a factor in the protein’s localization. NS1 residues Phe-103 and Met-106 have

been shown to be required for NS1 binding to the cellular post-transcriptional processing factor CPSF30 (76). NS1 truncations that delete these residues resulted in cytoplasmic localization of the NS1 protein (20, 56). Whether or not the ability to bind CPSF30 directly affects nuclear localization of NS1 has yet to be determined.

Recent studies have focused on differences in NS1 localization to the nucleolus. In Volmer *et al.* (2010), a panel of cell lines (primary and immortalized) derived from varying mammalian and avian hosts was used to assess NS1 localization of four IAV isolates with differing C-terminal NLS2 sequences. Their findings indicated the mechanism of nucleolar localization of NS1 in avian cells differed from that of mammalian cells (183). However, the role of NS1 in the nucleolus is unknown, as is its implications in virulence and viral replication. Li *et al.* (2011) found that alanine substitutions at Leu-69 and Leu-77 within the highly conserved NS1 linker region disabled localization to the nucleolus, and dramatically reduced the ability of NS1 to limit IFN induction in vitro (87). Although many researchers have studied intracellular localization of the NS1 protein, there has yet to be a report on the effect of host adaptation on this property.

1.3.2 NS1: Interferon antagonist

The NS1 protein became known as an IFN antagonist when researchers used reverse genetics to rescue a virus lacking the NS gene segment. This delNS1 virus replicated similar to its wild-type counterpart in Vero cells, a cell line deficient in IFN signalling. However, the virus was markedly attenuated in IFN-competent cells, and induced high level expression of IFN and IFN-stimulated genes (40). Since this report, researchers have found that NS1

antagonises the IFN response at numerous levels, including direct and indirect effects that limit IFN production and suppress the action of antiviral proteins downstream of IFN.

NS1 inhibits IFN production at the level of the cytoplasmic viral RNA sensor retinoic acid-inducible gene 1 (RIG-I), both by directly binding RIG-I to block IFN mRNA transcription activation (51, 111, 128) and by binding the ubiquitin ligase tripartite motif-containing protein 25 (TRIM25) that activates RIG-I antiviral signalling (38). NS1 also binds dsRNA, which limits RIG-I activation. NS1 also blocks the signal transducer and activator of transcription 1 (STAT-1) signalling pathway induced by type I IFN, which results in the suppression of ISG transcription (76). In a recent study that determined the effect of NS1 truncations on global transcriptional profiles in primary human lung cells, full-length NS1 was found to suppress expression of type I IFN genes as well as genes involved in NF- κ B signalling and antigen presentation (176). IFN production is indirectly limited by NS1 as NS1 engages in numerous mechanisms to suppress host gene expression (see section 1.3.3).

Upon virus infection, viral factors such as dsRNA and envelope glycoproteins activate type I IFN production through pattern recognition receptors including toll like receptors (TLR) 3 and 7 as well as cytoplasmic signalling receptors RIG-I and melanoma differentiation associated protein 5 (MDA5). Receptor binding leads to activation of transcriptional activators including IFN regulatory factor (IRF) 3 and 7 and NF κ B, which bind to and activate transcription of type I IFN genes (6). IFN production leads to the activation of >100 ISGs, including protein kinase R (PKR), 2'5'- oligoadenylate synthetase (OAS), myxovirus (influenza virus) resistance gene 1 (Mx1), ISG15, IRF7, RIG-I, MDA5, and STAT-1

(6). Just as IFN has evolved to trigger multiple antiviral responses, NS1 has followed close in pursuit and counteracts the action of numerous IFN-induced effector proteins.

The cellular kinase PKR serves as a key antiviral defence mechanism that targets viral translation. Once activated by dsRNA, PKR phosphorylates eukaryotic initiation factor eIF2 α , which leads to abrogation of cellular translation machinery. To circumvent this blockade in virus gene expression, NS1 directly binds PKR as well as dsRNA, which prevents dsRNA-induced activation of PKR (86, 115, 174). However, dsRNA binding is not required for NS1 to inhibit PKR, as PKR activating protein (PACT)-mediated (dsRNA-independent) PKR activation is still blocked by the NS1 protein (86). The antiviral effector protein 2'5' -OAS is also activated by dsRNA. 2'5'-OAS acts to polymerize ATP into 2'-5' oligoadenylate chains, which activate RNase L, leading to inhibition of virus replication by the degradation of cytoplasmic RNA (160). NS1 evades 2'5'-OAS action by binding dsRNA and preventing its activation (114). It is hypothesized that the primary function of the NS1 RNA binding domain is to outcompete 2'5'-OAS in RNA binding (114). By blocking the antiviral action and signalling of PKR, 2'5'-OAS, RIG-I, and TRIM-25, the NS1 protein suppresses the host antiviral response to IAV infection, which supports viral replication.

1.3.3 NS1 suppresses host gene expression

IAV competes with the host cell to express its genome at the level of: i) mRNA capping, ii) mRNA nucleocytoplasmic export, and iii) protein translation. The NS1 protein increases the virus's advantage over the host cell by: i) limiting production of mature host mRNA, ii) targeting viral mRNA to nuclear export machinery, and iii) targeting viral mRNA to cellular translation initiation factors. Each of these three mechanisms will be discussed.

NS1 down regulates host gene expression by suppressing production of mature host mRNA by binding to and inhibiting cellular post-transcriptional processing factors, CPSF30 (76, 121, 124), PABPN1 (15, 88), and U6 small nuclear RNA (snRNA) (99, 143, 187). CPSF30 binds to and cleaves the AAUAAA poly(A) signal at the 3' end of cellular pre-mRNA, which precedes addition of the 3' poly(A) tail (103). The PABPN1 protein stimulates the addition of the poly(A) tail to the 3' end of pre-mRNA, which is synthesized by the poly(A) polymerase. The poly(A) tail is important for the nuclear export, translation, and stability of mRNA. U6 snRNA is one of the cellular RNAs involved in the spliceosome complex, and interacts with pre-mRNA at the 5' splice site in association with U2 snRNA, which catalyzes RNA splicing (35). Cellular mRNA polyadenylation and splicing are critical reactions required for mRNA maturation.

The IAV does not require host cell mechanisms for polyadenylation; the IAV polymerase complex is multifunctional, and capable of adding a long stretch of A's to the 3' end of nascent mRNA by reiterating the 6 U template at the end of the gene. Therefore the virus can afford to target this host process to suppress host gene expression. NS1 inhibits CPSF30 by binding two zinc finger motifs (F2 and F3) in the 30 kDa subunit of CPSF (CPSF30-F2F3). Reports have varied in identifying the region to which NS1 binds CPSF30-F2F3 (76, 121, 124). The first report of the protein: protein interaction indicated the NS1 effector domain was responsible for binding CPSF30-F2F3 (121), while later reports have identified a binding domain centered around aa 186 (124), and identified two critical aa residues, Phe-103 and Met-106 (76, 179). In addition to enhancing viral polymerase activity, the association of NS1 with IAV polymerase has been implicated in NS1 binding affinity to

CPSF30. The interaction of NS1 with cognate viral polymerase proteins was found to be required for CPSF30 binding to the A/Hong Kong/483/97 (H5N1) NS1 protein that does not possess Phe-103 and Met-106 aa residues, which are instead Leu-103 and Ile-106 (179). As different IAV strains were used in the biochemical analyses of NS1:CPSF30-F2F3 binding, the site that determines CPSF30-F2F3 binding and the requirement of expression of cognate polymerase proteins may be strain-specific.

The inhibition of CPSF30 action by NS1 is not absolute, as mature cellular mRNA is still detected in IAV infected cells (121). NS1 engages in a second mechanism to limit mRNA processing by binding to PABPN1 (15, 88). The C-terminus of NS1 is responsible for PABPN1 binding, specifically aa 223–237 (88). As a result, host pre-mRNAs that contain truncated poly(A) tails accumulate in the nuclei of IAV infected cells (15). The NS1-mediated inhibition of host post-transcriptional processing factors CPSF30 and PABPN1 limit host mRNA maturation, which provides an advantage to viral mRNA in competition for access to host translation machinery.

NS1 also limits host mRNA maturation at the level of RNA splicing (36, 99, 143, 187). During host transcript splicing, U6 snRNA binds to the 5' splice site in a sequence independent fashion. There are several forms of U6 snRNA to complement the variation in 5' and 3' splice sites; the most common splice sites are GU/AG, however a small population of spliced mRNA possess AU/AC (35). Interestingly, NS1 only binds to and inhibits the action of the U6 snRNA specific for AU/AC splice sites (187). NS1 binds the RNA species in a manner conserved with polyadenylated RNA and dsRNA binding; the NS1 dimer forms a 6 helical motif at its RNA binding domain, which exposes positively charged amino acids that

interact with the negatively charged RNA (95, 187). U6 snRNA is unable to form a complex with U2 or U4 snRNA when bound by NS1 (143). The virus also requires host splicing machinery to splice its own M and NS genes. However, the ratio of spliced to unspliced transcript is quite low (~10%); it is possible that suppression of host splicing machinery limits the amount of viral spliced mRNA to a level that is physiologically beneficial for virus replication.

NS1 also engages in an additional mechanism to limit host mRNA in the cytoplasm by indirectly and directly suppressing host mRNA nuclear export. NS1 indirectly suppresses cellular mRNA export from the nucleus by binding polyadenylated mRNA and sequestering it in the nucleus (36). NS1 directly suppresses host mRNA export by binding to NXF1/TAP and forming an inhibitory complex with the nuclear export machinery (151). Therefore despite the inhibitory actions of NS1 on polyadenylation, some host mRNA successfully manage to obtain a poly(A) tail, but NS1 ensures the nuclear export of these mature transcripts is suppressed. However, IAV mRNA also must be exported from the nucleus. In a recent study by Read *et al.* (2010), viral mRNA nuclear export was found to depend on NXF1 to a degree that was viral transcript specific. Notably, early gene transcripts lacking introns showed the greatest independence to NXF1 in nuclear export. The two smallest gene segments, 7 (M) and 8 (NS) both demonstrated a dependence on the helicase UAP56, a component of the transcription export complex (TREX), for export of their respective mRNAs (145). Currently it is not known how viral mRNAs are capable of utilizing the NXF1 export pathway while its activity is being suppressed by the NS1 protein.

By limiting host gene expression through the actions of the NS1 protein, the host cell is rendered more permissive to IAV infection. The production of antiviral cytokines such as type I IFNs is suppressed, as well as the expression of genes stimulated by IFN production. In addition, the cellular translation machinery is more accessible to IAV mRNA.

1.3.4 NS1 regulates viral gene expression

Several reports have implicated the NS1 protein in viral RNA polymerase activity. Co-immunoprecipitation experiments demonstrated the NS1 protein interacts with the viral polymerase protein complex (104). In a recent report, this association was attributed to a direct interaction between the NP and the NS1 proteins, via the NS1 RNA binding domain (147). Research on the NS1 protein using temperature sensitive (ts) NS1 mutants showed NS1 plays a regulatory role in viral RNA synthesis (33, 192), which is IAV strain specific (33) and is associated with specific aa residues at site 123-124 (115).

The interaction of NS1 with several cellular proteins contributes to enhancement of viral polymerase activity. NS1 binds heterogeneous nuclear ribonucleoprotein F (hnRNP-F), and this interaction plays a role in viral transcription. The depletion of hnRNP-F in infected cells increased viral RNA polymerase activity and ultimately viral replication (81). A recent report showed that the NS1 protein interacts with RNA helicase A (RHA) in a RNA binding-dependent manner, and this interaction enhanced viral polymerase activity at the level of mRNA transcription (94). In addition, the research presented in this thesis demonstrates adaptive NS1 mutations selected upon IAV evolution to a novel host enhance viral polymerase activity (34).

The NS1 protein also regulates viral mRNA production at the level of splicing. NS1 was found to alter the accumulation of spliced mRNAs from the M gene segment (148), however the role of NS1 in NS gene segment 8 splicing is less clear. Some reports have demonstrated NEP transcript splicing is independent of NS1 function (99, 149), while other studies have shown NS1 blocks the splicing of its own transcript (36, 39). Strain-specific differences in the ability of NS1 to regulate NS gene segment transcript splicing may contribute to these discordant findings.

NS1 also enhances viral gene expression at the level of protein synthesis. Early studies of ts mutants showed mutations in the NS gene segment led to reduced levels of viral protein synthesis (57, 192). However, this phenomenon was virus-specific, as host mRNA translation was not up-regulated by the actions of the NS1 protein (22). Since these preliminary findings, research on NS1 binding partners has led to the current model of NS1-directed enhancement of viral mRNA translation: NS1 directly binds cellular translation initiation factors and recruits them to the 5' UTR of viral mRNA. The RNA binding domain of NS1 allows NS1 to directly bind the conserved 5' UTR of viral mRNA (133). NS1 binds the eukaryotic translation initiation factors PABP1 and eIF4GI using two distinct binding domains; residues 1-81 bind PABP1; while residues 81-113 bind eIF4GI (3, 12). The two cellular factors bind NS1 in a non-competitive manner and enhancement of viral mRNA translation occurs by the formation of a heterotrimeric complex of NS1, PABP1, and eIF4GI at the 5'UTR of viral mRNA and subsequent recruitment of the eukaryotic translation machinery to the translation start codon (12).

1.3.4.1 NS1 inhibits stress granule formation and enhances viral protein synthesis

NS1 also enhances viral protein synthesis by an additional complimentary mechanism. During viral infection as well as other cellular stresses, the host cell initiates a stress response, which leads to formation of stress granules (SGs) as a mechanism of halting cellular translation. Stress granules are comprised of abortive translation initiation complexes and untranslated mRNA (75). Following stress recovery, the cell must disassemble SGs to allow protein translation to resume. The dsRNA binding protein, Staufen 1 (the human isoform of the yeast protein Staufen, also known as hStaufen) is recruited to SGs and hypothesized to re-stabilize the polysomes within these structures (75). Interestingly, the NS1 protein has been found to bind Staufen 1 both in vitro and in vivo, and this interaction was found to localize to polysomes in virus infected cells (32). In a recent study, the NS1 protein was found to inhibit SG formation by a PKR-dependent mechanism (72). These reports suggest that NS1 counteracts the innate immune defense of SG formation by binding to Staufen 1, which positively influences SG disassembly and leads to activation of previously stalled translation initiation complexes, which are then recruited by NS1 to the 5' UTR of viral mRNA by interaction with translation factors eIF4GI and PABPI.

1.3.5 NS1 suppresses host cell apoptotic signalling

Upon virus infection, the host cell is triggered to initiate programmed cell death, or apoptosis, as a means to limit spread of the pathogen within the organism. Like other viruses, IAV has evolved to counteract this defense mechanism. NS1 binds to and activates the p85 β regulatory subunit of phosphatidylinositol-3-kinase (PI3K) (29, 30, 52, 157, 158), triggering a cell signalling cascade that results in Akt phosphorylation and inhibition of pro-

apoptotic factors, such as caspase-3 and caspase-9 (28). Ultimately the Akt pathway promotes cell survival and differentiation while suppressing apoptotic signalling.

Furthermore, the 1918 NS1 as well as several avian-origin NS1 proteins encode a Src homology 3 domain-binding motif (SH3) between aa 212 and 216, by which they interact with the CT10 regulator of kinase (CRK) and CRKL adaptor proteins. However this motif was absent in A/WSN/1933 (H1N1) and A/Udorn/1972 (H3N2). NS1 interactions mediated by the SH3 binding domain were associated with increased PI3K signalling (61). Down-regulation of apoptosis is advantageous for IAV replication; inhibition of the PI3K/Akt pathway during infection results in reduced viral growth (157).

1.4. Rationale and approach

Influenza A viruses are animal and human pathogens, where human infections are initiated as a zoonosis emanating from viruses with various host ranges that stem from aquatic waterfowl. In humans, the virus presents itself annually as seasonal influenza, which although primarily characterized by mild self-limiting tracheobronchitis, still results in mortality for the immunocompromised. The emergence of pandemic influenza strains have been documented throughout history, where the virus has evolved from animal origins to become capable of high mortality in an immunologically naïve human host. However, the genetic determinants of IAV host range and virulence are currently unknown. To this end a human isolate A/Hong Kong/1/1968 (H3N2) (HK) was experimentally evolved to high virulence in the immunologically naïve mouse. Previous reports have shown mouse-adapted (MA) mutations in the PB1 and HA proteins as well as two mutations, F103L and M106I, in the NS1 protein are associated with host adaptation and increased virulence of

the HK virus. The NS1 protein is a well characterized IAV virulence factor, which engages in multiple roles during virus infection culminating in the suppression of the host innate immune response and in the enhancement of virus replication. To further investigate the role of the 11 NS1 mutations selected upon mouse adaptation, I expressed single or double NS1 mouse-adapted mutations on the HK NS gene segment and employed reverse genetics to generate recombinant HK viruses. I then performed numerous in vivo and in vitro experiments to determine the effect of MA NS1 mutations on virulence, virus replication, virus gene expression, binding to CPSF30-F2F3, IFN antagonism and intracellular localization of the NS1 protein. With this information, I was then able to assess relationships between adaptive phenotypes and NS1 mutation sites. This work will lead to a better ability to predict the emergence of IAV strains with pandemic potential.

2. Materials and Methods

2.1 Cell lines

MDCK cells (Madin-Darby canine kidney; Health Canada), M1 cells (Mouse kidney epithelium; ATCC), and A549 cells (human lung carcinoma; ATCC) were maintained in minimum essential medium (MEM). 293-T cells (Human embryonic kidney; ATCC) were maintained in Dulbecco's minimal essential medium (DMEM) (Gibco, Invitrogen). Both MEM and DMEM were supplemented with L-glutamine (2 mM), Penicillin (100 U/ml), Streptomycin (100 ug/ml) and fetal bovine serum (FBS) (10%). All cells were incubated at 37°C in the presence of 5% CO₂.

2.2 Mice

All mouse infections in this study were performed on 19–21 gram female CD-1 mice (Charles River Laboratories, Montreal, Quebec, Canada). For infections with influenza A viruses, viruses were diluted to specified PFU in 50-100 uL PBS then used to intranasally inoculate mice under halothane anesthesia (3.5% halothane in O₂) by injection of diluted virus into nasal cavity with a 200 uL pipet. All efforts were made to minimize suffering, and in all experiments, mice were humanely euthanized by CO₂ narcosis upon experimental endpoint (> 30% body weight loss accompanied by respiratory distress).

2.3 Viruses

The H3N2 human influenza isolate A/Hong Kong/1/1968 (HK-wt) was obtained from the Laboratory Center for Disease Control (Health Canada, Ottawa, Canada). The H1N1 human influenza isolate A/Puerto Rico/8/34 was obtained by reverse genetics of the 8 viral

gene segments expressed on PLLB plasmids gratefully obtained from R. Webby (St. Jude's Children's Research Hospital, Memphis). The lab adapted influenza isolate A/WSN/33 (H1N1) was obtained by reverse genetics of the 8 viral gene segments expressed on pHH21 plasmids obtained from Yoshihiro Kawaoka (University of Madison, Wisconsin). Viruses were plaque purified in MDCK cells and grown in the allantoic cavity of 10-day-old specific pathogen free (SPF) chicken embryos (Canadian Food Inspection Agency (CFIA), Ottawa).

2.4 Mouse adaptation of A/Hong Kong/1/1968 (H3N2)

Mouse-adapted (MA) variants of A/Hong Kong/1/1968 (H3N2) were selected after 20 or 21 passages (MA20 or MA21) in the CD-1 mouse lung (11, 141). **MA20 viruses:** Briefly, 10^5 PFU was diluted in 50 μ L PBS and used to intranasally inoculate CD-1 mice. Following 3 days, the mice were culled and lungs were surgically removed, resuspended in PBS then homogenized by sonication (2 minutes on ice). The lung homogenates were then subject to centrifugation at 250 G for 5 minutes to pellet debris. Following 10 fold dilution in PBS, the virus extracts were then used to inoculate the next group of CD-1 mice (50 μ L volumes per mouse). The process was repeated for a total of 20 serial passages. Following final passage, lung extract was prepared then titred by plaque assay on MDCK monolayers and serially plaque purified on MDCK cells to generate six MA20 clones. Virus stock was propagated in either MDCK cells or in the allantoic fluid of 10 day old SPF chicken embryos (CFIA, Ottawa). **MA21 Viruses:** MA21 viruses were selected by 10 independent parallel mouse adaptation experiments in CD-1 mice (141). Briefly, 10 HK-wt clones were obtained by plaque purification and virus stock was propagated in the allantoic fluid of 10 day old SPF chicken embryos (CFIA, Ottawa). Serial passage in the mouse lung was performed for a total of 21

passages as per the previous protocol, with the following modifications. Initial inoculation of HK-wt virus was performed with undiluted stock (approx. 10^6 PFU dose). Following inoculation, mice were culled after 1 day for passages 1-10 (n=1), after 2 days for passages 11-15 (n=2), and after 3 days for passages 16-21 (n=2). During each passage, lungs were suspended in 1 mL PBS then subject to sonication, followed by filter sterilization using 0.22 μ m Millex-GV PVDF (Millipore, Cork, Ireland). Following final passage, each of the 10 lung extracts were subject to two sequential plaque purifications on MDCK cells to generate 3 biological clones. All MA variants derived from the 10 independent MA21 population clones were designated as HK(clone #) MA (mouse passage #)-(clone #) (Table 1).

2.5 Plasmids

PLLB NS: The circular DNA of A/Hong Kong/1/1968 genome segment 8 was inserted into the bi-directional pLLB vector by ligation-independent cloning (97). To generate PLLB MA NS plasmids, each MA NS mutation was introduced into the HK NS gene (Genbank accession no. CY033005) by site-directed mutagenesis. **pET17b CPSF30-F2F3-FLAG:** The F2F3-FLAG pET17b plasmid under T7 promoter control was constructed using homologous recombination (34). Briefly, pCAGGS CPSF30-F2F3-FLAG (obtained gratefully from L. Martinez-Sobrido, Mt. Sinai School of Medicine) was cloned using primers specific to CPSF-F2F3-FLAG with ends homologous to the pET17b sequence upstream of the T7 promoter. The F2F3-FLAG construct was subject to homologous recombination with pET17b using DH5 α *E. coli* competent cells. Positive colonies were subject to sequencing to verify proper insertion of the construct. The CPSF30-F2F3 gene was of human origin, however the F2F3

		Mutation in indicated protein at indicated aa position													
		NS1										NEP			
Virus	Clone no. MA no.	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	20	-	A	-	-	-	-	-	-	-	-	-	-	-	-
MA20A	20a	-	A	-	-	-	-	-	-	-	-	-	-	-	-
MA20B	20b	-	-	-	L	-	-	-	-	-	-	-	-	-	-
MA20C	20c	-	-	-	L	-	-	-	-	-	-	-	-	-	-
MA20D	20d	-	-	-	L	-	-	-	-	-	-	-	-	-	-
MA20E	20e	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HK1MA21	1 11	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HK1MA21	2 12	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HK1MA21	3 13	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HK2MA21	1 21	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HK2MA21	2 22	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HK2MA21	3 23	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HK4MA21	1 41	-	-	-	-	V	-	-	-	-	-	-	-	-	-
HK4MA21	2 42	-	-	-	-	V	-	-	-	-	-	-	-	-	-
HK4MA21	3 43	-	-	-	-	V	I	-	-	-	-	-	-	-	-
HK5MA21	1 51	-	-	-	-	I	-	-	-	-	-	-	-	-	-
HK5MA21	2 52	-	-	S	-	I	-	-	-	-	-	-	-	-	-
HK5MA21	3 53	-	-	S	-	I	-	-	-	-	-	-	-	-	-
HK6MA21	1 61	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HK6MA21	2 62	-	-	-	-	-	-	-	A	-	-	-	P	-	-
HK6MA21	3 63	-	-	-	-	-	-	-	A	-	-	-	P	-	-
HK8MA21	1 81	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HK8MA21	2 82	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HK8MA21	3 83	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HK9MA21	1 91	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HK9MA21	2 92	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HK9MA21	3 93	N	-	-	-	-	-	-	-	-	-	N	-	-	K
HK10MA21	1 101	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HK10MA21	2 102	-	-	-	-	-	-	G	-	-	-	-	-	-	-
HK10MA21	3 103	-	-	-	-	-	-	-	-	I	-	-	-	-	-
HK11MA21	1 111	-	-	-	-	-	-	G	-	-	-	-	-	-	-
HK11MA21	2 112	-	-	-	-	-	-	-	-	-	K	-	-	S	-
HK11MA21	3 113	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HK12MA21	1 121	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HK12MA21	2 122	-	-	-	-	-	-	-	-	-	-	-	-	-	-
HK12MA21	3 123	-	-	-	-	-	-	-	-	-	-	-	-	-	-

Table 1. Mutations in the NS gene segment selected upon human IAV isolate A/Hong Kong/1/1968 (H3N2) evolution in the mouse. Left column: Mouse-adapted virus name, where viruses selected after 20 passages are named MA20 then clone group (MA20a), and viruses selected after 21 passages are named HK(sister clone number)MA21(plaque pick number). NS1 as well as NEP gene mutations are listed at the amino acid level at residues indicated on the top row of the table.

portion of CPSF30 is completely conserved in the mouse (141). All of the plasmid constructs were sequenced to ensure the absence of unwanted mutations. **phPOL1-NP-LUC:** The phPOL1-NP-LUC plasmid was constructed by insertion of the firefly luciferase gene flanked by NP non-coding regions. Briefly, the insert was amplified by PCR using template DNA pGL3 Basic (Promega, Fisher Scientific, Nepean, Ontario) and the following primers:

Forward: 5'TATTCGTCTCAGGGAGCAAAAGCAGGGTAGATAATCACTCACTGAGTGACATCAAAATCATGGAAGACGCCAAAAACATA-3' Reverse:

5'ATATCGTCTCGTATTAGTAGAAACAAGGGTATT TTTCTTTACACGGC GATCTTTCCG-3'.

The resulting PCR product was then digested by BsmBI and cloned into BsmBI linearized pHH21 vector. **PRL-SV40:** The PRL-SV40 plasmid was commercially procured (Promega).

2.6 Virus rescue

To generate recombinant Hong Kong NS1 mutants (rHK NS1 mutants), each pLLB MA NS plasmid (segment 8) was incorporated with pLLB plasmids expressing gene segments 1-7 of the parental HK-wt genome. Reverse genetics was performed using an 8 plasmid system (92, 97). Briefly, plasmid DNA and Lipofectamine 2000 (Invitrogen) were mixed (1 ug: 2 uL) and incubated at room temperature for 30 minutes. Following incubation, transfection was performed on 90% confluent 293-T cells seeded in 6-well plates coated with poly (D) lysine. At 16 hours post transfection, Opti-MEM (Gibco, Carlsbad, CA) containing 1 ug/mL TPCK trypsin (Thermo Fisher Scientific, Waltham, USA) was used to supplement the cells. Two days later, virus was propagated by inoculation of 10 day old SPF chicken embryos (CFIA, Ottawa) with the cell supernatant. Positive virus rescue was verified by hemagglutinin assay using chicken red blood cells (CFIA, Ottawa). The NS1 gene segment sequence was verified

to ensure unwanted mutations. Briefly, virus RNA was extracted from 140 uL of allantoic fluid used to propagate each rHK NS1 mutant using the QIAamp Viral RNA Mini Kit (Qiagen, Mississauga) followed by reverse transcription and sequencing (34).

2.7 Nucleotide sequencing

Nucleotide sequencing of MA viral genomes: MA viruses were grown in the allantoic cavity of SPF chicken embryos (CFIA, Ottawa) then viral RNA was extracted from 140 uL of centrifuged allantoic fluid using the QIAamp Viral RNA Mini Kit (Qiagen, Mississauga) and full length virus genome segments were amplified and sequenced using universal IAV primers at the J. Craig Venter Institute (Rockville, MD) as part of the Influenza Genome Sequencing Project (National Institute for Allergies and Infectious Diseases).

NS sequencing: Following successful virus rescue, the NS gene segment of rHK NS mutants was sequenced to verify absence of unwanted mutations. Viral RNA was extracted as per the previous protocol, and NS gene segment sequencing was performed at the StemCore Facility (OHRI, Ottawa) using primers specific to the 5' and 3' ends of the NS gene segment.

2.8 Genbank accession numbers

HK-wt gene segments 1-7: CY033001– CY033008. HK-wt NS: CY044265; MA20 NS (V23A): CY044273; MA20C NS (F103L): CY045720; MA41 NS (M106V): HM641216; MA43 NS (M106V + M124I): CY033125; MA51 NS (M106I): HM641215; MA53 NS (M106I + L98S): CY044281; MA63 NS (V180A): CY045736; MA93 NS (D2N): CY033533; MA103 NS (V226I): JN896299; MA111 NS (D125G): CY033541; MA112 NS (R227K): CY033549.

2.9 In vivo infection assays

2.9.1 MLD₅₀

To determine the MLD₅₀ of MA viruses with NS1 mutations as well as the rHK NS MA virus expressing the NS1 mutation D2N, female CD-1 mice were intranasally infected with 10-fold dilutions of virus, ranging from 10¹ to 10⁷ PFU dose (n=3-5 per dose per virus), diluted to a 50 uL final volume in 1 x PBS. Survival, body weight loss and body temperature were monitored for 14 days following infection, and mice exhibiting symptoms exceeding the humane endpoint were euthanized by CO₂ narcosis.

2.9.2 Survival assay

The mouse survival response to rHK NS mutant infection was determined by intranasal infection with 5 x 10⁶ PFU dose in 50 uL final volume (n=5-6), as per the previous protocol. However the titre of the NS1 mutant M106V + M124I was too low to assess at this dose, so the experiment was performed using 2.5 x 10⁶ PFU dose for this virus. Survival, body weight loss, and body temperature were monitored as per the previous protocol.

2.9.3 Lung immunofluorescence and pathology

Mice were infected with a 1 x 10⁵ PFU dose of the specified rHK NS mutants or rHK-wt virus (n=4 per virus) as per the previous protocol, and at 2 and 6 days following infection, two sets of lungs were collected, venously perfused with 1 x PBS, inflated, then fixed with 3.7 % formaldehyde for 24 hours at 4° C. The buffer was then replaced with 20% sucrose, incubated at 4° C overnight, then frozen and cut into 7 uM sections. For detection of viral antigen, frozen sections were then stained as per the previous protocol (71). Briefly,

lung sections were first made permeable by a 5 minute incubation with cell culture grade trypsin diluted 1/20 in 1 x PBS. Then sections were washed twice with 10 mM PBS and incubated overnight with rabbit anti-HK virus polyclonal antibody diluted 1/1000 in antibody buffer (10 mM PBS containing 3% bovine serum albumin and 0.3% Triton X-100). The sections were then washed with 10 mM PBS, then incubated 2 hours with Cy3-conjugated donkey-anti rabbit IgG (Jackson ImmunoResearch laboratories Inc., ME) diluted 1/800 in antibody buffer. The slides were then washed with 10 mM PBS, and nuclei were stained by incubation with 100 ml Hoechst (0.2 mg/ml). The slides were then observed using fluorescent microscopy using an Olympus BX50 microscope 20X objective lens. To determine extent of lung pathology, frozen sections were stained with H&E then observed under light microscopy. Images were obtained by a Nikon microscope using a 10X objective lens. All images were processed in a parallel manner using Adobe Photoshop 7.0 (34).

2.9.4 Virus growth in mouse lung

Mice were infected with 5×10^3 PFU dose of the specified rHK NS mutants or rHK-wt virus (n=12 per virus) as per the previous protocol, then at 1, 3, 5, and 7 days post infections, 3 sets of lungs were collected for each virus infection. Mice were euthanized by CO₂ narcosis, then surgically removed lungs were diluted 1:5 in 1X PBS and homogenized by tissue homogenizer. Virus titre was quantified by plaque assay on MDCK cell monolayers.

2.9.5 Virus yield and IFN- β induction in the mouse lung

Mice were infected with 1×10^5 PFU dose of the specified rHK NS mutants or rHK-wt virus (n=6-12 per virus) as per the previous protocol, then at 1 and 3 days post infections, 3-6 sets of lungs were collected for each virus infection. Mice were euthanized by CO₂

narcosis, then surgically removed lungs were diluted 1:5 in 1 x PBS then homogenized by tissue homogenizer (metal bead method) and virus titre was quantified by plaque assay on MDCK cell monolayers. Lung homogenates were also quantified for IFN- β concentration by murine IFN- β ELISA using the manufacturer's protocol (PBL biosciences, New Jersey, USA) with subtraction of mock PBS-infected lung values.

2.10 In vitro infection assays

2.10.1 Virus growth in vitro

Virus growth was determined in MDCK, A549, and M1 cell lines. Briefly, confluent monolayers were washed twice with 1x PBS then infected at a multiplicity of infection (MOI) of 0.02 with specified rHK NS or rWSN mutants or rHK-wt virus (n=3). Following a 45 minute incubation at 37° C, monolayers were supplemented with serum-free 1 X MEM and TPCK trypsin (Thermo Fisher Scientific, Canada) at the final concentration of 0.5 ug/mL (M1 cell infection), 1 ug/mL (MDCK cell infection), or 0.3 ug/mL (A549 cell infection). Supernatants were collected at the specified hours post infection, and virus titre was determined by plaque assay on MDCK monolayers performed in duplicate.

2.10.2 Rate of virus protein synthesis

The rate of virus protein synthesis was determined by the pulse labeling of infected cells with ³⁵S-labeled Cys and Met. Cells (M1, A549 or MDCK) were infected at MOI=2 as per the previous protocol, in the absence of trypsin. At the specified times following infection, cells were washed with 1X PBS then the media was replaced with serum-free Met- Cys- DMEM (Gibco, Carlsbad, USA) containing 80 mCi/mL ³⁵S-labeled Cys and Met (Express tag,

Perkin Elmer, Canada) for a 1 hour pulse at 37° C, before the cell lysate was collected in 0.5 mL 1X SDS (sodium dodecyl sulphate) buffer. Radiolabeled proteins were then separated by SDS PAGE electrophoresis and detected by autoradiography (34).

2.10.3 Quantification of accumulated virus protein

Accumulated virus protein was quantified by Western blot analysis followed by densitometry (See section 2.13) of bands representing viral proteins. Briefly, cells (M1, A549 or MDCK) were infected at MOI=2 as per the previous protocol, in the absence of trypsin. At the specified times following infection, cell lysate was collected in 0.5 mL 1X SDS buffer. Proteins were then separated by SDS PAGE electrophoresis and transferred to PVDF membrane (Millipore) by a semi-dry transfer apparatus (Bio-rad). Immunoblots were probed with rabbit antiserum raised against purified A/WSN/33 virus or recombinant A/HK/1/68 NS1 protein. Antibody binding was detected with alkaline phosphatase (AP) labeled goat-anti-rabbit secondary antibody followed by AP incubation, or HRP-conjugated goat-anti rabbit antibody (Sigma, Burlington) followed by incubation with SuperSignal West Pico chemiluminescent substrate (Pierce) and exposure to film.

2.10.4 Pulse chase assay

M1 confluent monolayers were washed twice with 1X PBS then infected in triplicate with the specified rHK NS1 mutants as well as rHK-wt (MOI = 2) for 30 min at 37°C. Cells were supplemented with serum-free MEM in the absence of trypsin. At 8 hours post infection (hpi), the cells were washed with 1X PBS then pulse labeled with serum-free Met-Cys- DMEM (Gibco, Carlsbad, USA) containing 80 mCi/mL ³⁵S-labeled Cys and Met (Express tag, Perkin Elmer, Canada) for one 1 hour at 37°C. Cell lysates was either collected in 0.225

mL SDS buffer (pulse samples) or washed twice with PBS then the media was replaced with serum-free MEM supplemented with an additional 15 mg/L cold methionine and cysteine (chase samples). Chase sample cell lysates were collected at 1 and 3 hours following pulse labeling (10 and 12 hpi) in 0.225 mL SDS buffer. Radiolabeled proteins were then separated by SDS PAGE electrophoresis and visualized by autoradiography. For quantification of M1 and NS1 band intensity, each gel was exposed to a PhosphorScreen (GE Health Sciences) that was then read on a Typhoon Image Analyzer (GE Health Sciences). Relative band intensity was quantified using Imagequant software (GE Health Sciences) and calculated as the average $\pm$ standard deviation of the ratio of M1 to NS1 protein in each condition. The experiment was performed in triplicate, with representative data shown. Statistical analysis was performed, however no virus induced statistically significant ($p > 0.05$) difference in the ratio of M1:NS1 for pulse compared to chase (34).

2.10.5 Quantification of viral mRNA and vRNA by Q-PCR

Confluent monolayers of M1 cells were infected in triplicate with rHK NS mutant or rHK-wt viruses (MOI = 2), incubated for forty-five minutes at 37°C, then cells were washed once with PBS and supplemented with fresh serum-free MEM. Following 8 hpi, the cells were washed twice with 1X PBS, then the total RNA was isolated using RNeasy Protect Mini Kit (Qiagen). 500 ng of total RNA, which corresponds to equal cell equivalents, was reverse transcribed using an oligo(dT) primer for viral mRNA and an universal RTA12 primer for vRNA, respectively. Real-time PCR (qPCR) was performed with SYBR GreenER qPCR SuperMix (Bio-Rad Laboratories (Canada) Ltd., Mississauga, Ontario) and the Bio-Rad Chromo 4 Real-Time PCR Detector (Bio-Rad Laboratories (Canada) Ltd., Mississauga,

Ontario). Briefly, 2 ml of 10 fold diluted cDNA was added to the qPCR reaction mixture [10 ml SYBR GreenER qPCR SuperMix (2X), 1 ml each of forward and reverse gene specific primer (5 pM), 7.5 ml ddH₂O]. The cycle conditions of qPCR were 95°C 3 min, followed by 40 cycles of 95°C 30 s, 55°C 30 s and 72°C 30 s, then extended at 72°C for 3 min. Results were analyzed by DD CT methods and normalized with respect to the β -actin gene. Each experiment was performed for three biological repeats and two technical replicates. Real-time PCR primers for HK virus NS1, NP, and M1 mRNA and vRNA were matched in PCR efficiency (within $\pm 10\%$) with primers specific to mouse β -actin forward: AAATCGTGCGTGACATCAAA, and mouse β actin reverse: AAGGAAGGCTGGAAAAGAGC, and were as follows: NP forward: TGGAGAGGTGAAAATGGACG, NP reverse: TTTCCTGGGTTCGACTTTC, NS1 forward: ACCATTGCCTTCTCTCCAG, NS1 reverse: TCCCATTCTCATTACTGCTTCC, M1 forward: GTACGTTCTCTCTATCGTCCC, M1 reverse: GTCTTGTCTTTAGCCATTCCA (34).

2.10.6 IFN induction in vitro

Confluent monolayers of M1 cells were infected in triplicate with rHK NS mutant or rHK-wt viruses (MOI = 2), incubated for forty-five minutes at 37°C, then cells were washed once with PBS and supplemented with fresh serum-free MEM in the absence of trypsin. Following 24 hpi, a 1 mL aliquot of the cell supernatant was collected then assayed for murine IFN- β concentration by ELISA (PBL biosciences, New Jersey, USA) according to the manufacturer's protocol.

2.10.7 Confocal microscopy

To observe the localization pattern of NS1 in infected cells, M1 or A549 cells were seeded to 60-80% confluency on 8 chamber immunoslides then infected with rHK NS1 mutants or rHK wt virus, as well as prototype mouse-adapted viruses A/PR/8/34 and A/WSN/33, at an MOI=3 in the absence of trypsin. At 16 hpi, the cells were washed with 1X PBS, then the immunochamber was removed. The cells were permeabilized in 0.1% Triton X in PBS for 5 minutes then washed with 10 mM PBS and fixed in 3.7% formaldehyde for 10 minutes. Following two washes in 10 mM PBS, the cells were incubated with rabbit anti- HK NS1 polyclonal antibody, or mouse anti- NS1 monoclonal antibody (as specified in the figure legend) overnight at 4° C. The cells were washed twice with 10 mM PBS then the slides were incubated with secondary Cy3-conjugated donkey anti-rabbit or donkey anti-mouse IgG, respectively (Jackson ImmunoResearch laboratories Inc., ME), for 2 hours at room temperature. Following two washes, the nuclei were labeled by DAPI (300 ng/mL) incubation in the dark for 15 minutes. Excess stain was removed by 3 washes with dH₂O. Cover slides were mounted with Dako mounting medium (Dako, Denmark) as per manufacturer's protocol. The cells were viewed using a Zeiss LSM 510 META/AxioVert 200 Confocal Microscope 63X oil immersion objective lens. Representative images were processed in parallel (GNU Image Manipulation Program 2.0).

2.10.8 Quantification of NS1 in subcellular compartments of infected cells

Confluent monolayers of M1 or A549 cells were infected (n=2-3) with rHK NS mutant or rHK-wt viruses as well as the prototype MA viruses A/PR/8/34 and A/WSN/33 (MOI = 2), as per the previous infection protocol, in the absence of trypsin. At 16 hpi, the cells were

washed once with ice cold 1X PBS then lysed with ice cold NP-40 lysis buffer (50 mM Tris HCl pH 8.0, 150 mM NaCl, 10% glycerol, 1% NP-40, 1 mM EDTA), and scraped to collect adherent cells. Cells were fractionated as per a recently published protocol (172). Briefly, an aliquot of the cell lysate was obtained to represent the whole cell lysate, and the remainder was centrifuged for 2 minutes in a table top centrifuge at 13.5K RPM. The supernatant containing the cell cytoplasm was then collected. The nuclear pellet was washed with NP-40 lysis buffer and re-centrifuged. The supernatant was discarded and the pellet was resuspended in 50 uL NP-40 lysis buffer. Whole cell, cytoplasmic, and nuclear fractions were then mixed with 4X Laemlli sample buffer and boiled for 3 minutes prior to protein separation by SDS-PAGE gel electrophoresis. Immunoblots were probed for NS1 (polyclonal rabbit anti-NS1), histone H3 (rabbit anti-histone H3, CT, pan clone A35; Millipore, Billerica, MA, USA) and β -tubulin (mouse anti-tubulin α ; Sigma, Burlington, ON). Antibodies were detected by incubation with HRP conjugated goat anti-rabbit or goat anti-mouse antibody (Sigma, Burlington, ON) followed by SuperSignal West Pico chemiluminescent substrate (Pierce, Thermo Fisher Scientific, Nepean, ON) and exposure to film.

2.11 RNA polymerase assay

To compare the effect of mutant NS gene segments on viral polymerase activity, a luciferase reporter minigenome assay was used as per the previously published protocol (141). Briefly, 96 well plates of 293-T cells were transfected with 0.06 ug of phPOL1-NP-LUC reporter plasmid, 0.06 ug of the internal control luciferase expression plasmid PRL-SV40 (Promega, Madison, WI, USA), 0.06 ug of each pLLB HK-wt PB2, PB1, PA and NP, and 0.06 ug of either HK-wt or mutant pLLB NS plasmids or empty pLLB vector, in the presence of 0.5 uL

lipofectamine 2000 in 100 uL Opti-MEM (Invitrogen, Burlington, Ontario) (141). **NS gene segment mutagenesis:** The HK-wt NS plasmid was mutated using PCR mutagenesis to express NS1 protein alone by disrupting the 3' NS2/NEP splice site with a synonymous mutation as well as introducing a stop codon at position 14 in the NS2 reading frame using the following primers:

NS1-forward: GCCTTCTCTTCCCGGACATACTATAGAGGATGTCA

NS1 reverse: TGACATCCTCTATAGTATGTCCGGGAAGAGAAGGC.

To express NS2/NEP protein alone, the HK-wt NS plasmid was mutated using PCR mutagenesis to delete the NS intron using the following primers:

NS2-forward: ACACTGTGTCAAGTTTTTCAGGACATACTATTGAGGATGTC

NS2 reverse: GACATCCTCAATAGTATGTCCTGAAAACCTTGACACAGTGT.

At 48 hours post transfection, luminescence was quantified using the Promega Dual-Glo Luciferase Assay System with a Glomax Multi Detection System, Model 9301-010 (Fisher Scientific, Nepean, Ontario) according to the manufacturer's protocol. Data represent the average $\pm$ SD of relative luciferase activities (ratio of firefly and renilla luciferase luminescence) (n=3-4) (34).

2.12 CPSF30 F2F3: NS1 binding assay

The CPSF30-F2F3 fragment (domain responsible for the binding of CPSF to NS1) as well as each HK-wt and mutant NS1 protein were radiolabeled and synthesized as per a previously published method using the TnT T7 Quick-Coupled Transcription/Translation System (Promega) (101). The F2F3 fragment was synthesized as a flag-tagged construct in the pET17b vector under the control of the T7 promoter. All NS mutants and HK-wt NS

expression were also expressed with the T7 promoter in bi-directional pLLB plasmids. While on ice, 0.5 ug plasmid DNA (or empty pLLB vector) was mixed with 10 uCi ^{35}S -labeled Cys + Met (Express ^{35}S Protein Labeling Mix, Perkin Elmer) and 40 uL TnT master mix in a 50 uL total volume. The mixture was incubated 90 minutes in a 30° C water bath then stored at -20° C until use. To verify equal expression of the NS1 proteins and expression of the F2F3-FLAG fragment, an aliquot of each TnT reaction was resuspended in 3X SDS buffer, then subject to SDS-PAGE electrophoresis and autoradiography. To assess NS1 binding to F2F3-FLAG, equal volumes (10 uL of TnT reaction) of each radiolabeled NS1(or empty pLLB vector TnT reaction) were incubated with 10 uL of radiolabeled F2F3-FLAG in the presence of Protein G Dynabeads (Invitrogen, Burlington, Ontario) pre-incubated with rabbit α -FLAG M2 (Sigma) antibody in NP-40 lysis buffer. The pulldown assay was performed as per the manufacturer's protocol. 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 uL 1xSDS buffer and heated at 100° C for 1 minute before the proteins were separated by SDS-PAGE electrophoresis and detected by autoradiography. For quantification of band intensity, each gel was exposed to a PhosphorScreen (GE Health Sciences) that was then read on a Typhoon Image Analyzer (GE Health Sciences). Relative band intensity was quantified using Imagequant software (GE Health Sciences) for a total of three independent experiments, and calculated as the average $\pm$ standard deviation of the % value relative to HK-wt NS1. The absence of non-specific binding of the NS1 protein (wt or mutant) to the α -FLAG M2-conjugated beads was shown by pull-down in the absence of F2F3-FLAG protein (34).

2.13 Densitometry analysis

With the exception of the CPSF binding and the pulse chase assays, band density of radiolabeled or Western blot detected proteins was quantified by densitometry analysis using the UN-SCAN-IT software (Scientific Software Solutions). In the case of Western blot analysis, NS1 band density was normalized to band density of loading control (actin, tubulin, or H3, respectively). To determine the percentage of NS1 localized to the nucleus, whole cell, cytoplasmic, and nuclear fractions of infected cells were probed for NS1, histone H3, and tubulin (n=2-3). To account for loading control as well as recovery of nuclear pellet, the NS1 band of the nuclear fraction was normalized in respect to the ratio of the histone H3 band density in the nuclear and in the whole cell lysate, and the NS1 band of the cytoplasmic fraction was normalized in respect to the ratio of the tubulin band density in the cytoplasmic and in the whole cell lysate. Following normalization, the NS1 band density of the cytoplasmic fraction was divided by the sum of NS1 band densities from cytoplasmic and nuclear fractions. This value represented the % of NS1 localized to the cytoplasm.

2.14 Statistical Analysis

Statistical significance was measured using the student t-test (Microsoft Excel, 2007) or paired student t-test (GraphPad Prism 5) using the parameters of equal variance (unless otherwise indicated) and two-tailed significance with a probability of <0.05 considered as statistically significant. Values were calculated as means $\pm$ standard deviation (SD), unless otherwise noted as means $\pm$ standard error (SE).

3. Results

3.1 Sequence analysis of NS genome segments for mouse-adapted viruses

The human influenza A virus (IAV) isolate A/Hong Kong/1/1968 (H3N2) (HK-wt) was serially passaged in the mouse lung to select virulent variants (11, 34, 71, 140, 141). Upon sequencing the full viral genome, each resulting mouse-adapted (MA) variant was found to possess mutations in several viral genes, where 18 MA isolates had acquired 11 NS1 gene mutations occurring as single or double point mutations (141) (Table 1). Upon IAV mouse adaptation, evolution of the NS1 gene involved instances of positive selection as evidenced by increased prevalence of mutations in virus populations as well as the independent selection of the same mutation, or site of mutation, among independently passaged populations. Parallel evolution was demonstrated at position 106, where two independently passaged populations selected different amino acid mutations at the same site (M106V and M106I), and convergent evolution was demonstrated at position 125, where two independently passaged populations selected the same NS1 mutation (D125G). Three of the eleven NS1 mutations also induced mutations in the nuclear export protein (NEP) gene (Table 1), one of which occurred in the nuclear export signal (S23P), suggesting the mutation may affect NEP cellular distribution. Upon mapping the mutations to the secondary structure and binding domains of the NS1 protein, I found the majority of the mutations were located in the effector domain, in regions responsible for binding to cellular factors involved in post-transcriptional processing and translation (Figure 1).

3.2 Adaptive phenotype of NS mutant MA viruses

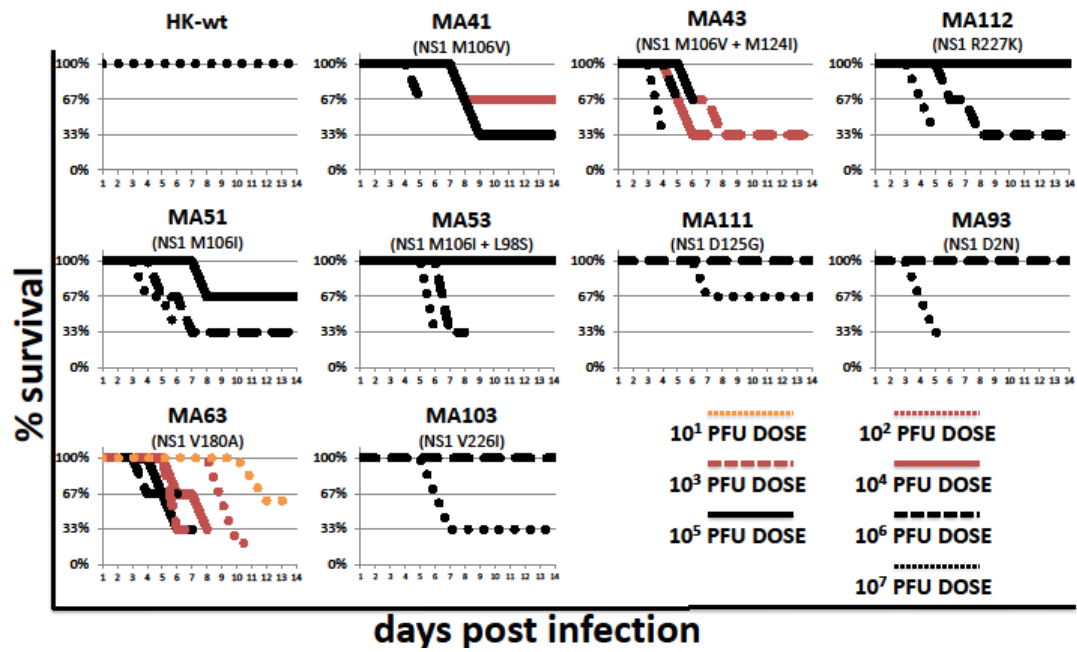
3.2.1 Virulence of NS mutant MA viruses in CD-1 mice

To assess the virulence of NS mutant MA viruses in the mouse, CD-1 mice were intranasally inoculated with representative NS mutant MA viruses (10^1 to 10^7 PFU (plaque forming units)). Survival, body weight loss, and body temperature were monitored over a 14 day period (Figure 2a-b). Whereas a 10^7 PFU dose of the HK-wt virus did not cause death or disease in the CD-1 mouse, inoculation with NS mutant MA viruses induced killing that correlated with reduced body weight (Figure 2) and body temperature (data not shown). MA63 induced the greatest degree of virulence in the mouse; inoculation with 10^1 PFU dose was sufficient to induce 33% mortality in the mouse and inoculation with 10^2 PFU dose resulted in 100% mortality (Figure 2a). All NS mutant MA viruses assayed, with the exception of MA111 (D125G), induced 100% mortality upon inoculation with 10^7 PFU, where MA111 induced 33% mortality. Upon calculation of MLD_{50} by the Karber-Spearman method (113), I found 9 of the 10 assayed NS mutant MA viruses demonstrated decreased MLD_{50} compared to HK-wt ($MLD_{50} > 10^{7.5}$ PFU), with values ranging from $10^{1.1}$ to $10^{6.5}$ PFU (geometric mean = $10^{4.5}$ PFU) (Figure 3a). Figure 3b illustrates the increased virulence that resulted in a reduced survival response in mice infected with 10^6 or 10^7 PFU of the NS mutant MA viruses, demonstrating 100% mortality for all but MA111 relative to HK-wt.

3.2.2 Interferon sensitivity of NS mutant MA viruses

Given NS1 is an interferon antagonist, I hypothesized the increased virulence of NS mutant MA viruses in the mouse may be attributed to enhanced resistance to the type I

A



B

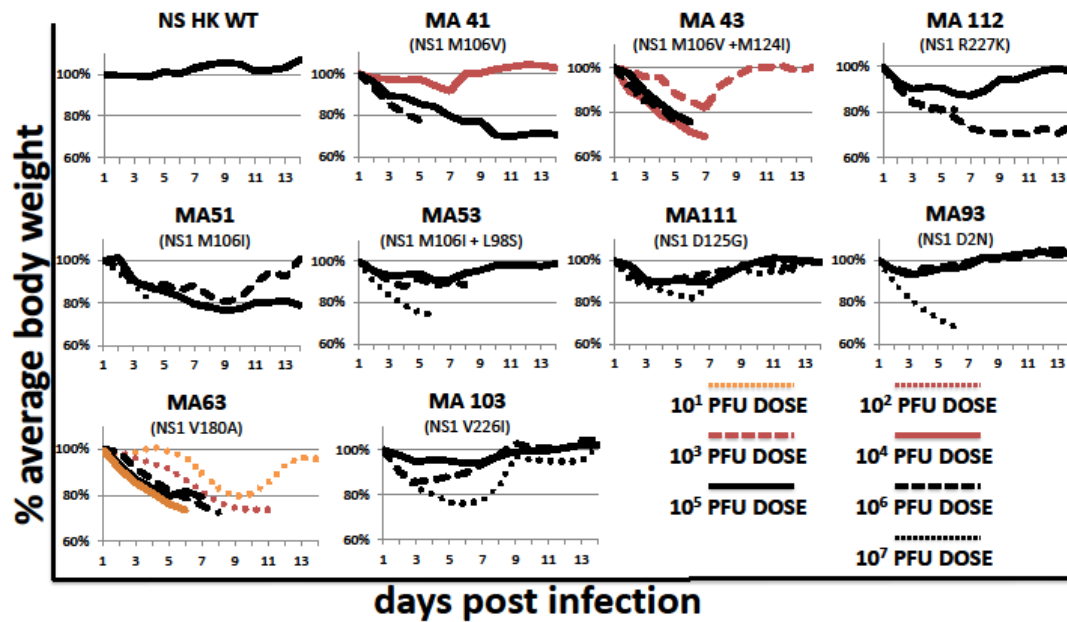


Figure 2. MA viruses with NS1 mutations increase virulence in CD-1 mice. Groups of 3-4 female CD-1 were infected intranasally with 1×10^1 to 1×10^7 PFU dose of A/Hong Kong/1/1968 (H3N2) or mouse-adapted Hong Kong viruses. Survival (A) and body weight loss (B) were monitored for 14 days. (34).

A

Virus	NS1 aa (nt) mutation	NEP aa (nt) mutation	MLD ₅₀ ¹	Genbank no.
HK-wt	-	-	>7.7	CY044265
MA20	V23A (GTA→GCA)	-	2.9	CY044273
MA20C	F103L (TTC→CTC)	-	2.6	CY045720
MA41	M106V (ATG→GTG)	-	4.5	HM641216
MA43	M106V (ATG→GTG) + M124I (ATG→ATA)	-	4.5	CY033125
MA51	M106I (ATG→ATT)	-	5.5	HM641215
MA53	M106I (ATG→ATT) + L98S (TTG→TCG)	-	5.5	CY044281
MA63	V180A (GTC→GCC)	S23P (TCC→CCC)	1.1	CY045736
MA93	D2N (GAT→AAT)	D2N (GAT→AAT) + E108K (GAA→AAA)	6.5	CY033533
MA103	V226I (GTT→ATT)	-	n/a	JN896299
MA111	D125G (GAT→GGT)	-	>7.5	CY033541
MA112	R227K (AGG→AAG)	G70S (GGT→AGT)	5.8	CY033549

¹MLD₅₀ measured as log₁₀ pfu dose required to kill 50% of CD-1 mice

B

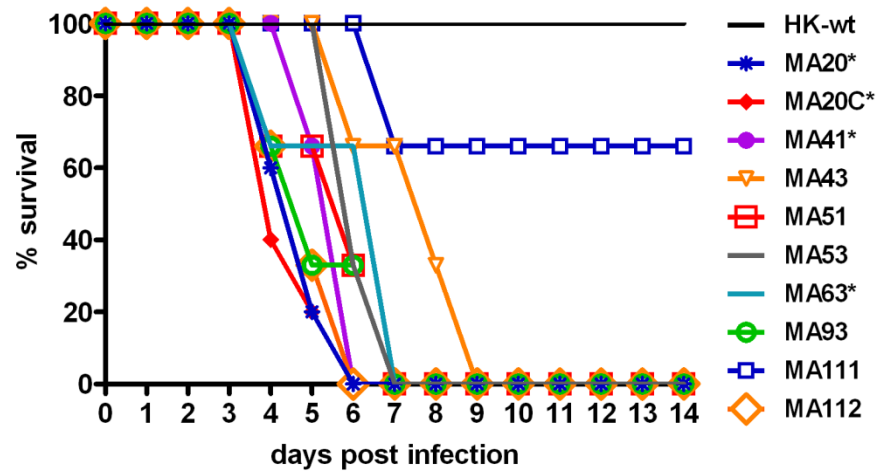


Figure 3. MA viruses with NS mutations are associated with decreased survival in the mouse. (A) Table of MA viruses indicating mutations in the NS gene segment at the protein (aa) and mRNA (nt) level as well as MLD₅₀ value in CD-1 mice. (B) Kill curve of MA viruses with NS mutations. CD-1 mice were intranasally infected with 1 x 10⁷ PFU dose (*1 x 10⁶ PFU dose) and survival was monitored for 14 days. Figure adapted from (34).

interferon (IFN) response. To assess this hypothesis, a set of NS mutant MA viruses (the first mutants to be isolated) were used to infect mouse kidney epithelium (M1) cells at MOI=2 that were either untreated or pre-treated 24 hours with mouse IFN- β (200 U/mL) and viral titres were quantified by plaque assay of supernatants collected at 24 hours post infection (hpi) (Figure 4a). To assess the extent of IFN antagonism for the NS mutant MA viruses in respect to virus replication, IFN sensitivity was calculated by dividing the viral yield in untreated cells by the viral yield in cells pre-treated with IFN- β . The HK-wt virus demonstrated a 14-fold reduction in virus titre upon IFN- β pre-treatment, whereas all assayed NS mutant MA viruses induced only a ≤ 4 -fold reduction, demonstrating enhanced resistance to IFN compared to HK-wt (Figure 4b). Increased IFN resistance demonstrated by NS mutant MA viruses suggests the NS1 mutations are contributing to enhanced IFN antagonism that may function to increase virulence in the mouse; however given each MA virus had mutations in additional viral genes, the extent of this contribution by the individual NS1 mutations was unknown.

To determine the role of MA NS1 genes in virulence and adaptive phenotypes, recombinant viruses were generated, where gene segments 1-7 possessed the HK-wt sequence, and gene segment 8 sequences were derived from NS mutant MA viruses (rHK NS mutants). In addition, I also combined the rHK viral backbone with the HK NS1 L98S mutant, which was a NS1 mutation found only in combination with NS1 mutation M106I (MA53, Table 1). The NS gene segments of rescued viruses were sequenced to confirm that they were free of unwanted mutations.

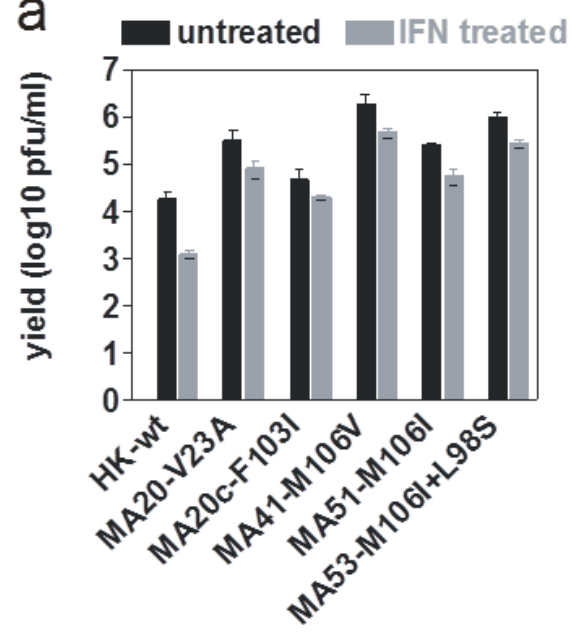
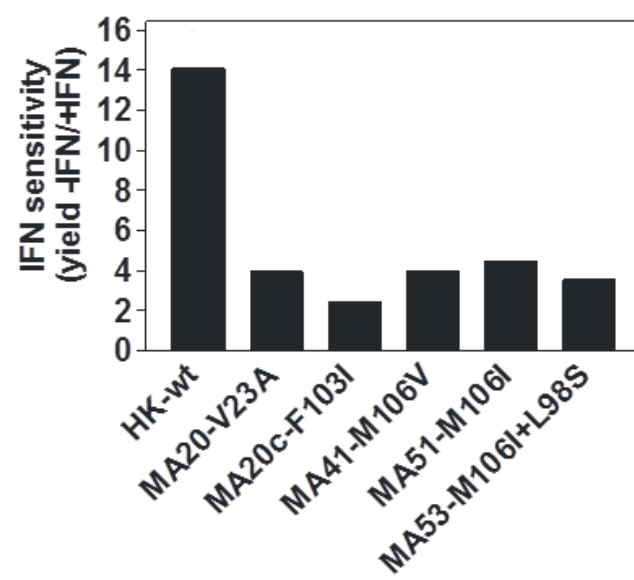
a**b**

Figure 4. Interferon sensitivity of MA viruses with NS1 mutations upon infection of mouse M1 cells. Mouse M1 cells were either left untreated (-IFN) or pre-treated for 24 hours with IFN- β (200 U/mL MEM) (+IFN) then infected at MOI = 2 with a panel of mouse-adapted viruses with the indicated NS1 mutations. Virus titre was assayed 24 hpi by plaque assay on MDCK monolayers. IFN sensitivity was quantified by dividing virus yield (-IFN) by yield (+IFN). This experiment was performed by Shuai Wang, previous student in Earl Brown's lab.

3.3 NS1 mutations selected upon mouse adaptation enhance virulence of the parental HK virus in the mouse

3.3.1 Mortality and morbidity

To assess the contribution of MA NS1 mutations on virulence, groups of 5 to 6 CD-1 mice were infected intranasally with 5×10^6 PFU of each rHK NS mutant as well as the rHK-wt virus. Where infection with rHK-wt did not cause mortality, 7 of the 12 NS1 mutants resulted in reduced % of survival, with the NS1 D2N mutant inducing the highest extent of mortality (83%; $MLD_{50} = 10^{6.2}$ PFU) (Figure 5a). Infection with NS1 mutant M106I + L98S resulted in 40% mortality, while single NS1 mutants L98S and M106I, as well as M106V, D125G and V180A induced 20% mortality (Figure 5a). Although several of the NS1 mutants did not induce mortality in the mouse at this dose, the majority of the NS1 mutations did increase body weight loss, another indicator of disease severity. Infection with the rHK-wt virus induced a maximum average body weight loss of 5%, while all NS1 mutants with the exception of V226I and R227K induced significantly more body weight loss than rHK-wt from days 1-6 post infection (pi) (paired t-test; $p < 0.05$ to $p < 0.001$) (Figure 5b). NS1 mutant M106I induced the greatest weight loss, followed by NS1 mutants D2N and M106I + L98S, indicating body weight loss is associated with mortality (Figure 5).

Given that NS1 mutants D2N and M106I + L98S induced the greatest extent of mortality in the mouse (Figure 5a), additional aspects of virulence including lung pathology, virus spread, and virus growth in the lung were assessed for recombinant viruses that differed due to the expression of these mutations, as well as the single mutations M106I and L98S, relative to the parental rHK-wt virus.

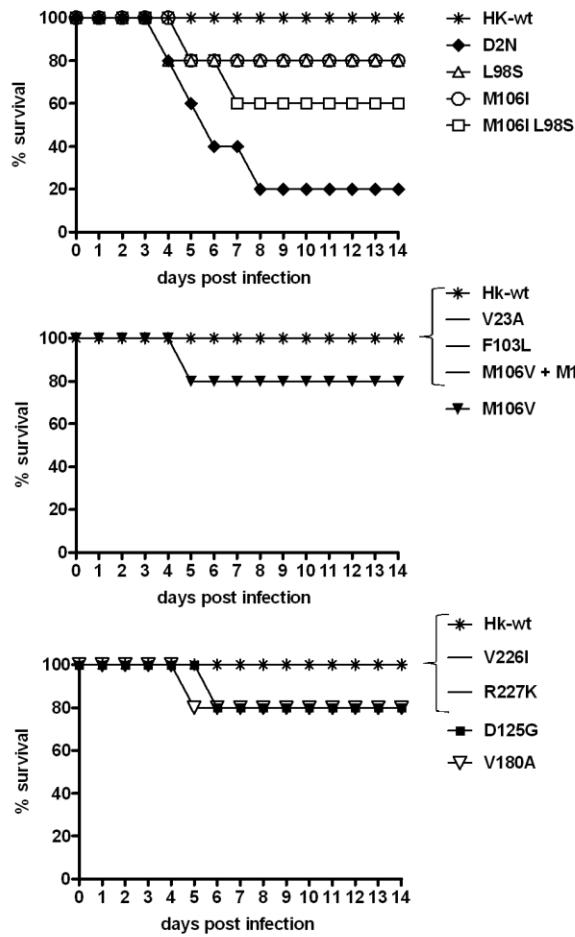
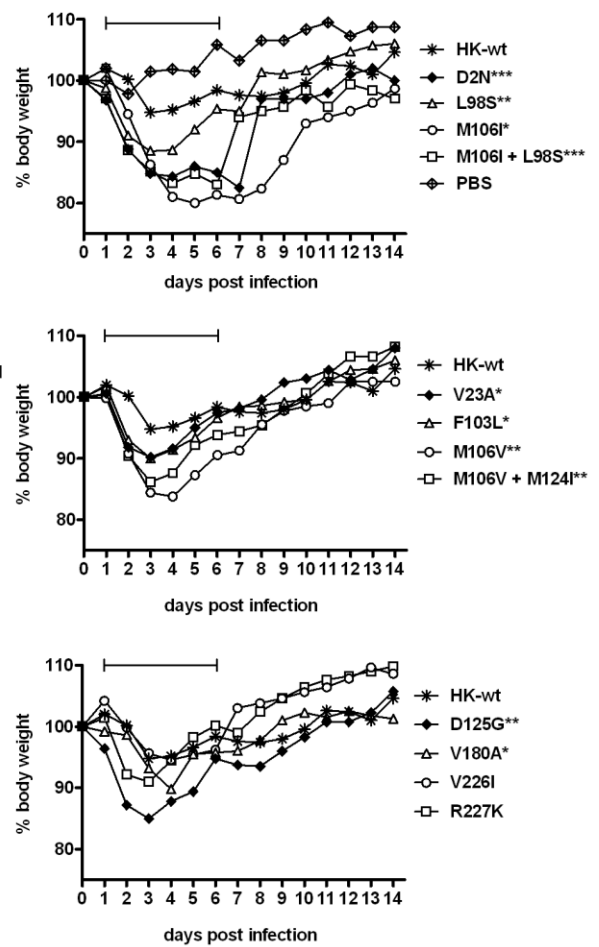
A**B**

Figure 5. MA NS1 mutations increase virulence in CD-1 mice. Groups of 5 to 6 female CD-1 mice were intranasally infected with 5×10^6 PFU of rHK NS MA or rHK-wt viruses (ϕ rHK NS1 M106V + M124I was inoculated at 2.5×10^6 PFU due to insufficient viral stock titre). Survival (A) and body weight loss (B) were monitored for 14 days pi. Percent body weight is expressed as the mean value of 5 mice (or total mice surviving) (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; two-tailed paired t-test for days 1-6 pi) (34).

3.3.2 Lung pathology and virus spread

To assess lung pathology and virus spread for a panel of NS1 mutant and rHK-wt viruses, mice were inoculated with 1×10^5 PFU dose then frozen lung sections were collected at days 2 and 6 pi and processed to determine lung pathology (H & E stain) or virus spread (immunofluorescence (IF) staining of HK virus antigen) (see methods for further details). IF staining of lungs infected with rHK-wt showed virus antigen present in focal regions in the bronchi at day 2 pi but not at day 6 pi (Figure 6). However, NS1 mutants D2N, M106I, L98S, and M106I + L98S all induced greater infection of bronchiolar tissue at day 2 and in addition all mutants but M106I increased virus spread to alveolar regions of the mouse lung. Unlike rHK-wt, virus antigen was still detected in the mouse lung at day 6 pi for NS1 mutants M106I and M106I + L98S in both alveolar and bronchiole regions (Figure 6, white arrows). Lung pathology associated with rHK-wt infection was mild, where at day 2 pi, H & E staining was similar to mock-infected (PBS) lungs. In contrast, NS1 mutants L98S and M106I + L98S induced lung pathology at day 2 pi, including alveolar thickening and polymorphonuclear inflammatory cell recruitment. At day 6 pi, rHK-wt infection induced increased inflammatory cell infiltration to bronchiole regions of the lung (Figure 6), however the extent of this pathology increased and was extended to alveolar regions for NS1 mutants D2N, M106I, and M106I + L98S (Figure 6).

3.3.3 Virus growth kinetics in the mouse lung for virulent MA NS1 mutants

To determine the effect of NS1 mutants D2N, M106I + L98S, M106I, and L98S on virus growth in the mouse lung, CD-1 mice were intranasally infected with 5×10^3 PFU, and

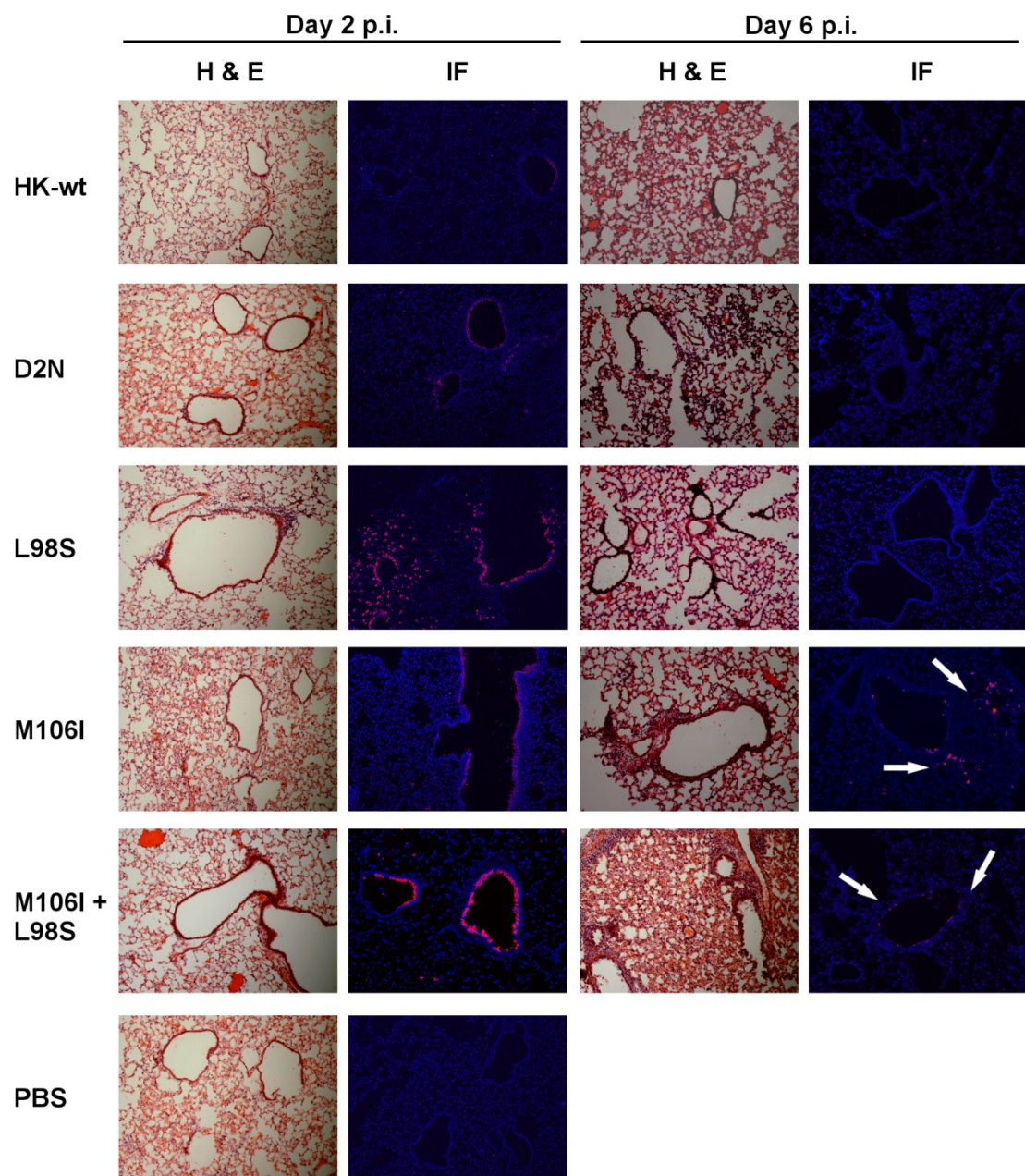


Figure 6. MA NS1 mutations increase pathology and virus spread in the mouse lung.

Groups of 4 female CD-1 mice were infected with 1×10^5 PFU of selected rHK NS mutant or rHK-wt viruses and two mice were culled at both days 2 and 6 pi to collect the lung. IF: virus was detected by immunofluorescence; frozen lung sections were stained with anti-HK primary antibody, Cy3- conjugated secondary antibody (red) and Hoeschst (blue). Images were taken using a 20X objective lens. White arrows indicate foci of staining at 6 dpi. H&E: lung pathology was assessed by H&E stain. Images were taken using a 10X objective lens (34).

lungs were collected at days 1, 3, 5, and 7 pi to determine viral titre (n=3 per virus per time point). NS1 mutants D2N, M106I + L98S and the single mutant L98S but not M106I all significantly enhanced viral growth in the lung but to different extents and times pi (Figure 7). The differences in yield between rHK-wt and NS1 mutant M106I was not statistically significant throughout the time course (Figure 7). However, the M106I mutation was observed to synergistically increase the replicative ability conferred by the L98S mutation, where expression of M106I + L98S significantly increased lung replication by 24 and 8 fold at days 1 and 3 pi, respective to rHK-wt titres (Figure 7). The NS1 mutant that induced the highest mortality in the mouse upon high dose infection, D2N (Figure 5a), also induced a viral growth curve distinct from rHK-wt and other assayed NS1 mutants. At day 1, D2N virus titre exceeded rHK-wt titre by 57 fold ($p < 0.0001$), however at days 3 and 5 pi, virus titre was below rHK-wt titre, and undetectable at day 7 pi (Figure 7). Overall, NS1 mutants D2N, L98S, and M106I + L98S significantly increased viral replication in the mouse lung compared to rHK-wt, and the extent of replication enhancement was consistent with the corresponding increases in virulence.

3.3.4 IFN- β production in the mouse lung

To determine the role of MA NS1 mutations on IFN- β production in the mouse lung, a mouse IFN- β ELISA assay was performed on homogenized lung extracts collected 1 and 3 days pi following intranasal infection with a moderately high dosage of each NS1 mutant

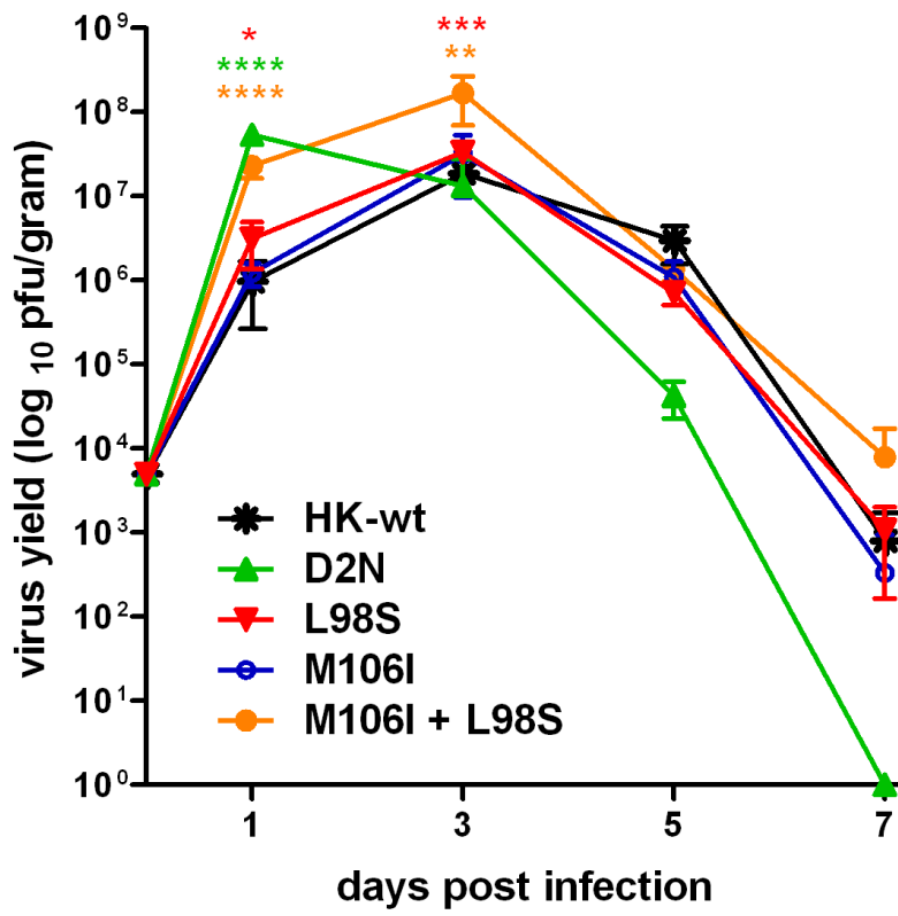


Figure 7. MA NS1 mutations enhance virus replication in the mouse lung. Groups of 12 female CD-1 mice were infected intranasally with 5×10^3 PFU dose with select rHK NS mutants and rHK-wt virus. At days 1, 3, 5, and 7 pi, 3 sets of lungs were collected, homogenized, and virus titre was quantified by plaque assay on MDCK cell monolayers. Data represents the mean titre $\pm$ SD. All data points contain error bars, however in some cases the error bars are less visible due to small SD. Statistical analysis was calculated by student's two-tailed t-test compared to rHK-wt titre at the respective time point (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$).

virus (1×10^5 PFU dose). Following 1 day of infection, rHK-wt induced 200 pg/g IFN- β , while all NS1 mutants except D2N and M106I + L98S induced significantly less IFN- β in the mouse lung, where the value was below the limit of detection for the majority of mutants (Figure 8, upper left panel). NS1 mutant M106I + L98S induced IFN- β levels similar to that of rHK-wt 1 dpi, while D2N was the only mutant that induced significantly more IFN- β ($p < 0.01$). Lungs collected 3 days pi from mice infected with NS1 mutants D2N, L98S, M106I + L98S, and V180A demonstrated significantly increased IFN- β levels compared to rHK-wt, while five NS1 mutants were again observed to limit IFN- β production below the limit of detection (Figure 8, lower left panel), indicating the majority of NS1 mutations selected upon mouse adaptation were capable of reducing the amount of IFN- β produced in the mouse lung.

3.3.5. Viral yield in the mouse lung

Given that several NS1 mutants displayed pronounced differences in IFN- β production in the mouse lung compared to rHK-wt, I hypothesized viral replication in the lung may be limited by NS1 mutations, leading to the decreased activation of antiviral responses in the mouse lung as described in the previous section. To this end, the previously collected lung extracts were assayed for virus titre by plaque assay on MDCK cell monolayers. Compared to rHK-wt, all NS1 mutants but L98S, F103L, M106I, and M106V + M124I resulted in enhanced yield at day 1 and/or day 3 pi (Figure 8, right panel). At 1 day pi, viral titre associated with NS1 mutants D2N, V23A and D125G exceeded rHK-wt titre by > 10 fold. In contrast, viral titre was significantly attenuated for NS1 mutant M106V + M124I at both time points, and for NS1 mutant V23A at day 3 pi, in respect to rHK-wt values (Figure 8, right panel). NS1 mutants L98S, F103L, and M106I grew

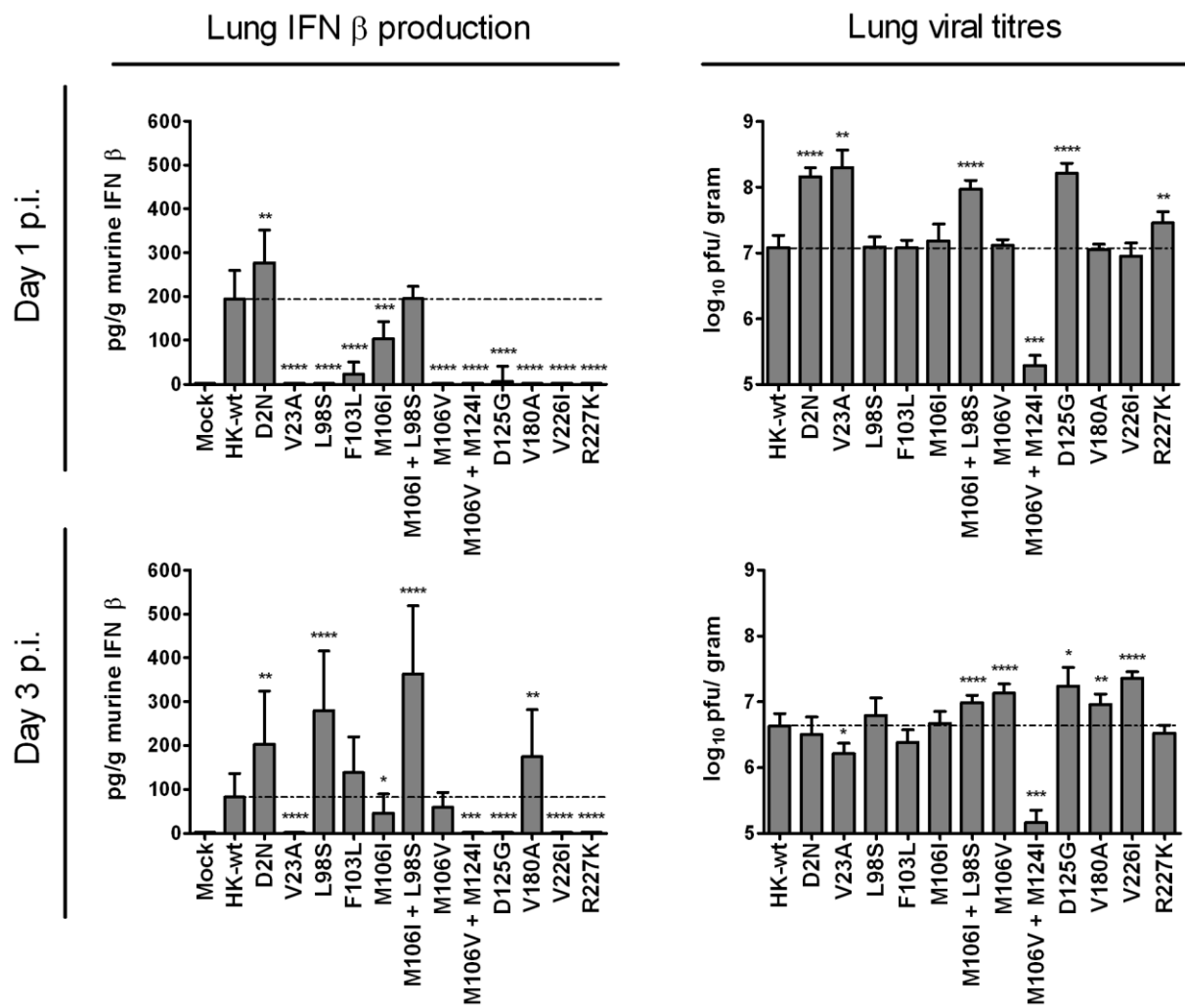


Figure 8. MA NS1 mutations affect IFN- β production and viral yield in the mouse lung.

Groups of 6-12 female CD-1 mice were infected intranasally with 1×10^5 PFU of rHK NS mutants or rHK-wt or mock infected with PBS. Lungs were collected 1 and 3 days pi, homogenized, and quantified for mouse IFN- β concentration by ELISA (left panel), and for viral titre by plaque assay on MDCK cell monolayers (right panel). Data represent the means $\pm$ SD (*p<0.05, **p<0.01, ***p<0.001, ****p<0.0001; two-tailed student's t-test) (34).

to titres similar to rHK-wt at both days 1 and days 3 and therefore these mutations did not enhance virus replication in the mouse lung. NS1 mutants M106V, V180A and V226I grew to a significantly higher titre ($p < 0.0001$, $p < 0.05$, $p < 0.0001$ respectively) at day 3 but not day 1 post infection, indicating these mutations were associated with a delayed enhancement in viral replication (Figure 8). In contrast, although surpassing rHK-wt virus titre at day 1 ($p < 0.0001$, $p < 0.01$, respectively), NS1 mutants D2N and R227K exhibited a titre similar to rHK-wt at day 3, indicating these mutations induced an early enhancement in virus replication. These results indicate that the ability of the majority of NS mutants to reduce IFN- β production was associated with enhanced viral replication.

3.4 Adaptive phenotype of MA NS mutants in vitro

3.4.1 Plaque morphology

Upon virus rescue in 293-T cells, rHK NS mutants were propagated by two serial passages in SPF embryonated chicken eggs. All viruses grew to a high titre ($> 10^7$ PFU/mL) as determined by plaque assay on MDCK monolayers, with the exception of M106V + M124I, (5×10^6 PFU/mL). The HK-wt virus formed plaques of 2.3 mm average diameter. The rHK NS1 mutants possessing M106V, M106V + M124I, and V226I mutations significantly decreased plaque size compared to rHK-wt ($p < 0.05$, $p < 0.001$, $p < 0.01$, respectively). NS1 mutations L98S, M106I, and M106I + L98S also decreased plaque size however the differences compared to rHK-wt were not statistically significant (Figure 9). The small plaque forming viruses were thus attenuated for growth in MDCK cells indicating host dependent effects for some NS1 mutations. Regardless, all NS1 mutants were capable of forming quantifiable plaques on MDCK cell monolayers.

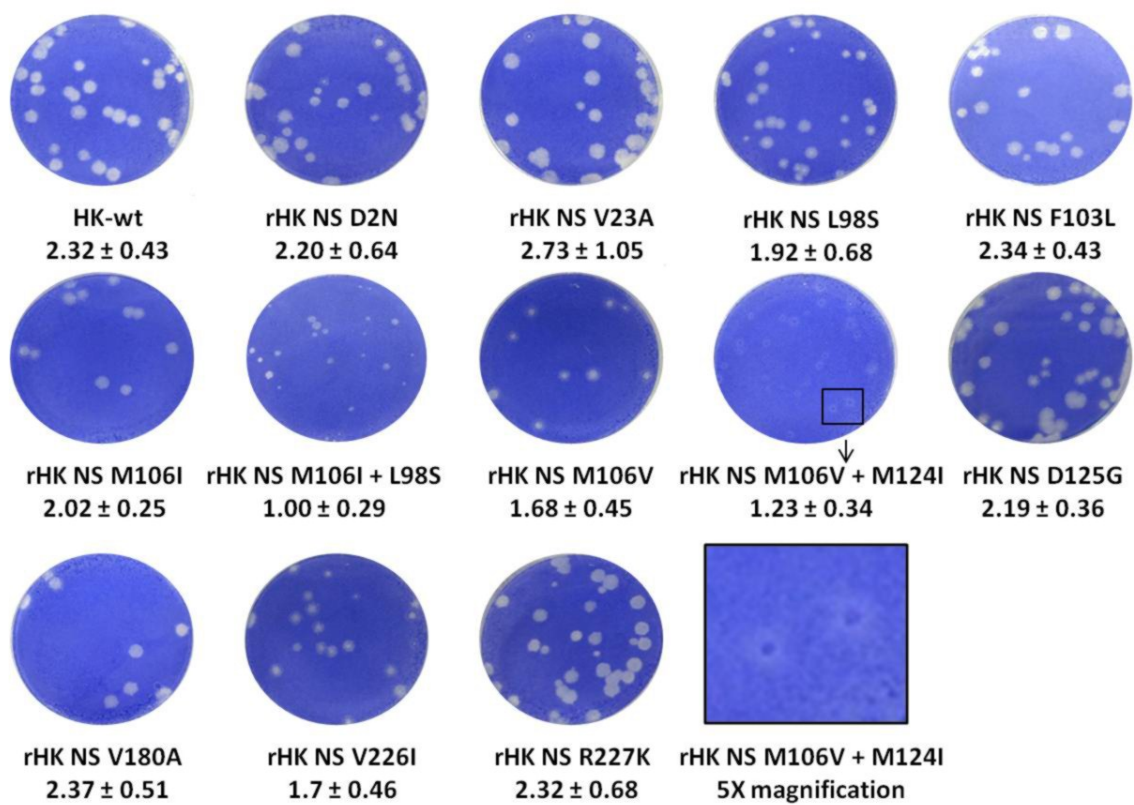


Figure 9. MA NS1 mutations plaque on MDCK cell monolayers. MDCK cells were infected in parallel with rHK NS mutants or rHK-wt virus at varying ten-fold dilutions to produce 10-50 plaques per well. The average plaque size (mm) is indicated below the NS1 mutation, $\pm$ SD. A boxed region within the plaque assay for the NS1 mutant M106V + M124I was enlarged by 5X to illustrate the small but quantifiable plaques resulting from this mutation.

3.4.2 MA NS mutants affect NS1 binding affinity to CPSF30-F2F3

Several MA NS1 mutations mapped to the NS1 binding domain for cellular post-transcriptional processing factor CPSF30 (cleavage and polyadenylation specificity factor; 30 KDa subunit) F2F3 domain (Figure 1). Moreover, previous publications had identified a role for NS1: CPSF30 interaction in interferon antagonism (78, 124). Therefore I hypothesized that the NS1 mutants with decreased IFN- β production may demonstrate increased binding to CPSF30. To assess this hypothesis, mutant and HK-wt NS gene segments as well as FLAG-tagged CPSF-F2F3 (F2F3-FLAG) fragment were expressed in vitro by coupled transcription and translation in rabbit reticulocyte lysates in the presence of ^{35}S -labeled Met and Cys (T7 Quick-Coupled Transcription and Translation Kit, Promega). Radiolabeled proteins were subject to SDS-PAGE electrophoresis then detected by autoradiography (Figure 10a). In an anti-FLAG antibody pulldown assay (n=3), HK-wt and mutant NS proteins were mixed with FLAG-F2F3 protein and precipitated using anti-FLAG M2 antibody (Sigma) and Protein G Dynabeads (Invitrogen). Bound proteins were separated by SDS-PAGE and detected by autoradiography (Figure 10b) and quantified by phosphorscreen signal amplification and ImageQuant software (GE) (Figure 10c).

The majority of NS1 mutants either partially or fully suppressed NS1-F2F3 binding. The NS1 mutants D2N and V226I bound F2F3-FLAG with comparable affinity to HK-wt NS1, while NS1 mutants V23A and R227K were found to increase binding affinity by 2-fold ($p < 0.05$ and $p < 0.01$, two-tailed student's t-test). NS1 mutants L98S and F103L bound at detectable but significantly decreased levels than HK-wt NS1 ($p < 0.0001$, $p < 0.00001$) (Figure 10c).

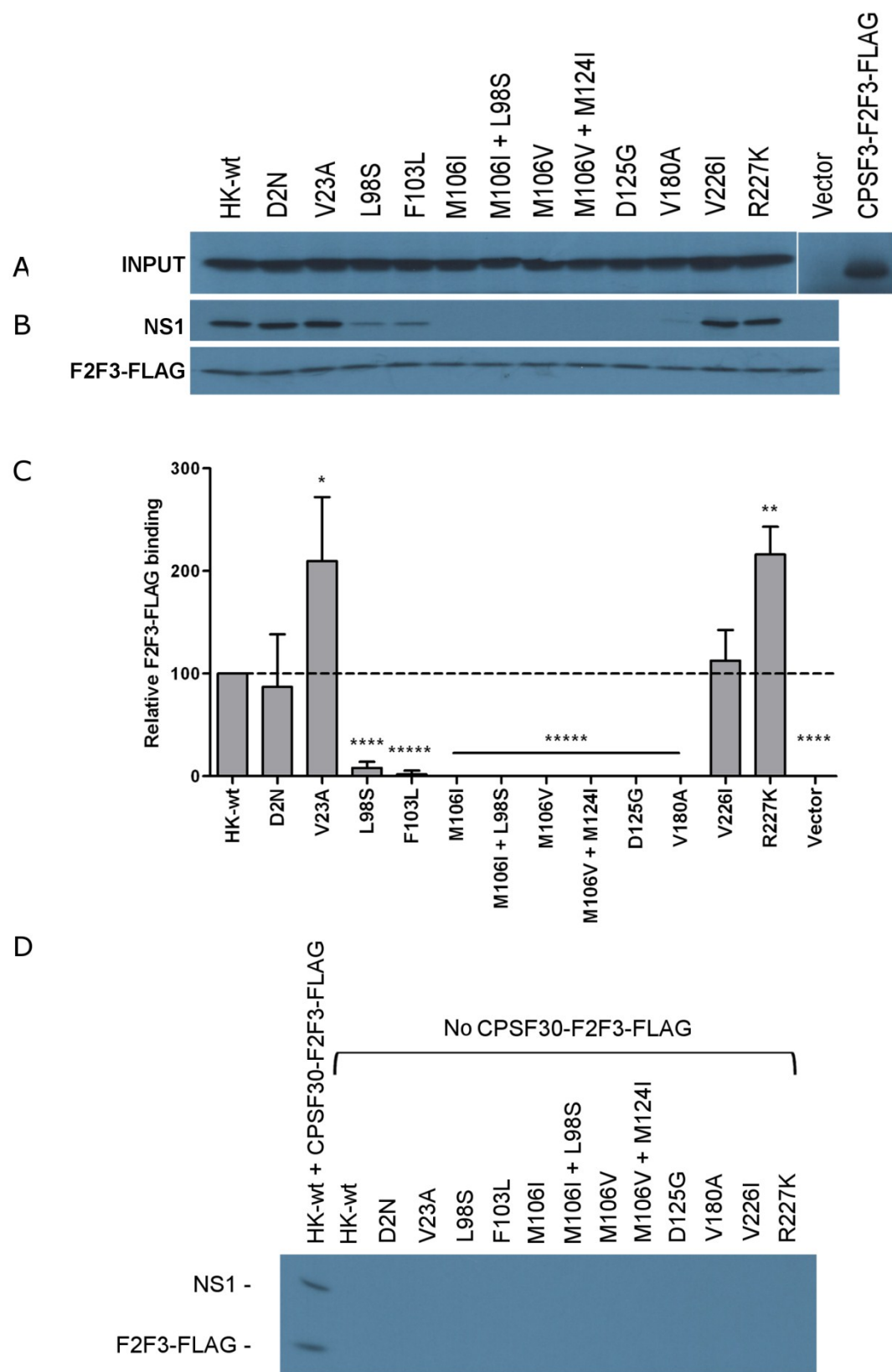


Figure 10. MA NS1 mutations affect binding affinity to CPSF30-F2F3. (A) NS1 proteins as well as the CPSF30-F2F3-FLAG construct were expressed by in vitro coupled transcription and translation of expression plasmids or empty vector in the presence of ^{35}S labeled Cys and Met residues. Expression levels were evaluated by SDS gel electrophoresis and autoradiography. (B) Radiolabeled mutant or HK-wt 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 (Invitrogen) then SDS gel electrophoresis and autoradiography. A representative pull-down of 3 independent replicates is shown. (C) Pull-down SDS gels were subject to phosphorscreen signal amplification; band intensity was then quantified by GE Imagequant software. Data represents the means $\pm$ SD (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; two-tailed student's t-test) (D) NS1 mutants do not induce non-specific affinity to the α -FLAG M2 antibody. Anti-flag pull-down was performed in the absence of CPSF30-F2F3-FLAG to ensure NS1 mutant proteins were not cross reacting with the α -FLAG M2 antibody (34).

NS1 mutants M106I, M106I + L98S, M106V, M106V + M124I, D125G, and V180A demonstrated undetectable binding to F2F3 in any of the three independently conducted anti-FLAG F2F3 pull down experiments ($p < 0.00001$, Figure 10c). To verify the anti-FLAG antibody was specific to the F2F3-FLAG protein, a control pull-down was performed in the absence of F2F3-FLAG protein, which showed a lack of non-specific binding for all NS1 mutants (Figure 10d). The data indicate that altered NS1:CPSF30-F2F3 binding affinity is a common property for NS1 mutations selected upon mouse adaptation, where two mutants demonstrated enhanced affinity and the majority demonstrated a marked decrease in binding affinity. These data also demonstrated a lack of correlation between the ability of a given NS1 protein to bind CPSF30-F2F3 and to limit IFN- β production.

3.4.3 Effect of MA NS1 mutations on IFN- β production in infected mouse M1 cells

Given that the majority of MA NS1 mutants suppressed IFN- β production in the mouse lung in vitro, the effect of MA NS1 mutations on IFN- β production in mouse cells in vitro was also assessed. Briefly, mouse kidney epithelial (M1) cell monolayers were infected in triplicate with rHK-wt or rHK NS mutants at a high multiplicity of infection (MOI = 2) and cell supernatants collected at 24 hpi were subject to mouse IFN- β ELISA assays. Cells were also infected with the VSV mutant M Δ 51 (a VSV mutant with a deletion in the M protein at residue 51 which abrogates VSV-mediated type I IFN suppression (170)) to serve as a positive control for IFN- β production. Cells infected with rHK-wt induced low but detectable levels of IFN- β (5.4 pg/mL) (Figure 11). However, NS1 mutants D2N, V23A, M106V + M124I,

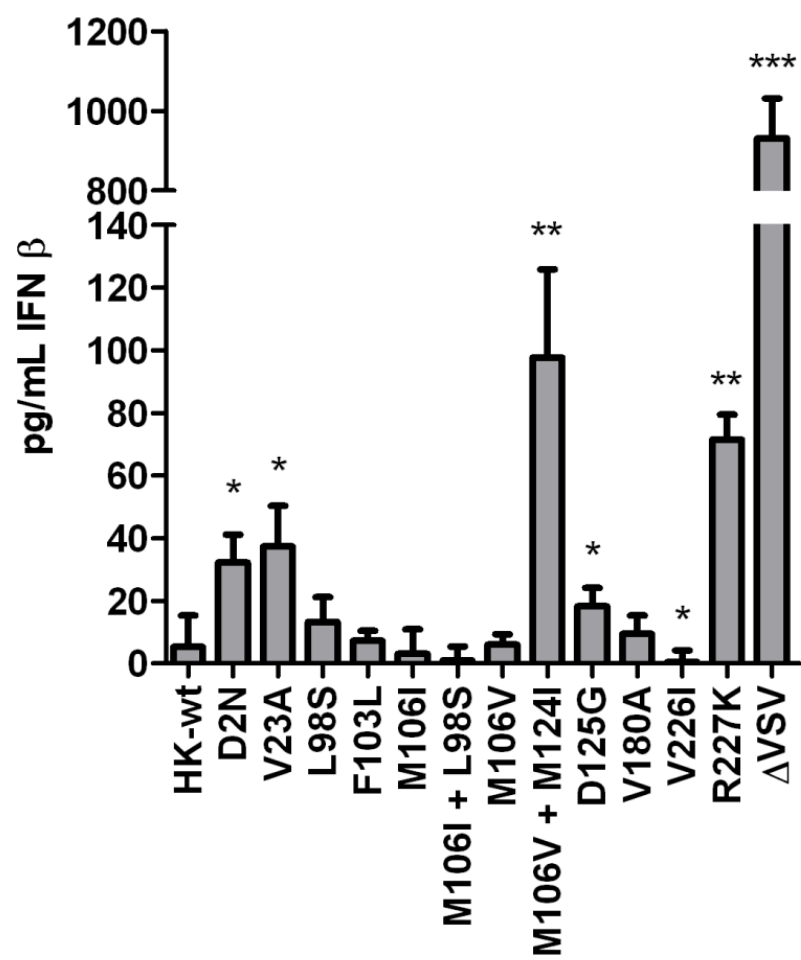


Figure 11. MA NS1 mutations affect IFN- β production in mouse cells in vitro. Mouse M1 cells were infected (MOI = 2) with rHK NS mutants, rHK-wt, or VSV M Δ 51 (Δ VSV), a VSV mutant incapable of suppressing type I IFN production (170). Cell supernatant was collected 24 hpi then assayed for IFN- β concentration using a commercial ELISA. Data represents the means $\pm$ SD (*p<0.05, **p<0.01, ***p<0.001; two-tailed student's t-test).

D125G, and R227K induced significantly more IFN- β production (Figure 11). NS1 mutant M106V + M124I induced the highest level of IFN- β production, surpassing rHK-wt levels by >18 fold. In contrast, NS1 mutant V226I produced significantly less IFN- β than rHK-wt ($p < 0.05$) (Figure 11). These results indicated that numerous NS1 mutations selected upon mouse adaptation alter IFN- β production in infected mouse cells in vitro and the effect of NS1 mutants on IFN production in vitro does not grasp the effect in vivo in mouse lungs indicating that different factors control IFN production between these 2 systems (see discussion for further analysis).

3.4.4 Effect of MA NS1 mutations on virus replication in vitro

To assess the effect of MA NS1 mutations on viral growth in mouse M1 cells, confluent monolayers were infected with rHK-wt or rHK NS mutants at a low multiplicity of infection (MOI = 0.02) and the viral titre of cell supernatants were quantified at 12, 24, 48, and 72 hpi. Numerous NS1 mutants (D2N, V23A, L98S, M106I, M106I + L98S, F103L, M106V + M124I, and D125G) significantly enhanced viral yield throughout the course of infection compared to rHK-wt ($p < 0.05$, paired, t test) (Figure 12, left panel). The highest level of virus growth was demonstrated by NS1 mutant M106I + L98S; where at both 48 and 72 hpi virus titre exceeded rHK-wt titre by > 100 fold ($p < 0.0001$ and $p < 0.001$, respectively). NS1 mutants M106V and R227K significantly enhanced viral growth at early times compared to rHK-wt (12-24 hpi; $p < 0.01$ and $p < 0.05$, respectively; paired t-test) while NS1 mutants V180A and V226I resulted in viral growth curves that were not significantly different from rHK-wt (Figure 12, left panel). Maximum yield was reached at 72 hpi, where NS1 mutants D2N,

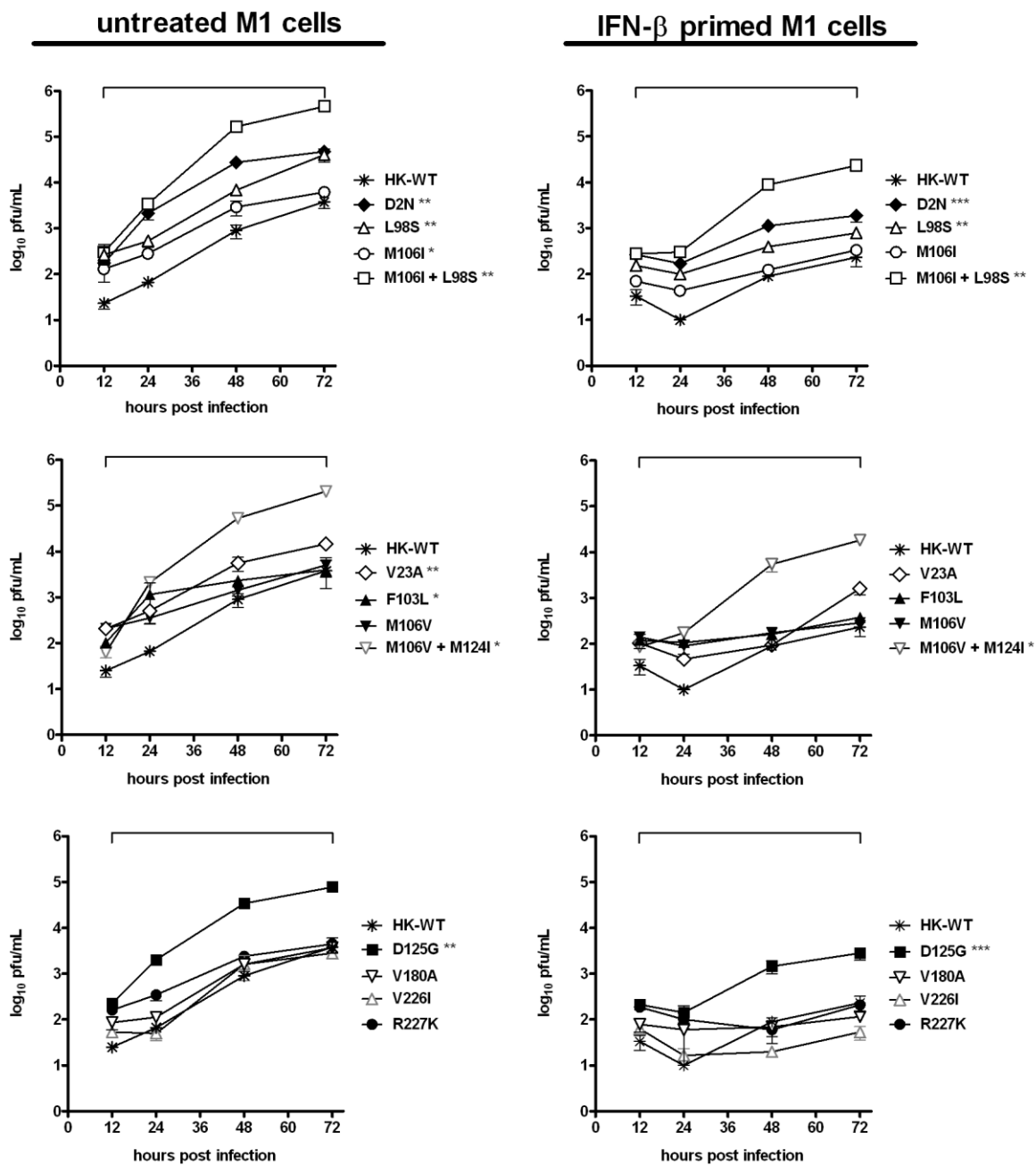


Figure 12. MA NS1 mutations enhance virus growth in untreated and IFN- β primed M1 cells. Mouse M1 cells were untreated (left panel) or pre-treated with mouse IFN- β (1000 U/mL) for 24 hours (right panel), then infected in triplicate at an MOI of 0.02 with rHK NS mutants or rHK-wt virus, and supernatant collected 12, 24, 48, and 72 hpi was quantified for viral titre by plaque assay on MDCK cells. Data represent the means $\pm$ SD (* p <0.05, ** p <0.01, *** p <0.001; two-tailed student's paired t-test for 12-72 hpi, indicated by horizontal bracket above time range) (34).

V23A, L98S, M106I + L98S, M106V + M124I, and D125G demonstrated significantly increased maximum titre compared to rHK-wt ($p < 0.05$). These results indicate that the majority of NS1 mutations (all except V180A and V226I) selected upon HK-wt virus passage in the mouse were adaptive in vitro, and increased viral replication in mouse cells by up to 100 fold (Figure 12, left panel).

3.4.5 Effect of MA NS1 mutations on virus replication in vitro in IFN- β primed cells

Given that the NS1 protein is an interferon antagonist, I hypothesized NS1 mutations selected upon mouse adaptation would demonstrate increased resistance to IFN- β during in vitro virus replication. To this end, viral growth was assessed as per the previous protocol, however prior to infection the cells were primed with 1000 U/mL recombinant mouse IFN- β for 24h to induce an antiviral state. Compared to viral yield in untreated cells, rHK-wt viral yield at 72 hpi was reduced by 16 fold upon IFN- β pre-treatment (Figure 12). Similar to growth enhancement seen in untreated cells, numerous NS1 mutants (D2N, L98S, M106I + L98S, M106V + M124I, and D125G) significantly enhanced viral growth throughout the course of infection, or at early times in infection (12-24 hpi; V23A, F103L, M106I, M106V, and R227K) in IFN- β primed cells when compared to rHK-wt (Figure 12, right panel). NS1 mutants M106I + L98S, M106V + M124I, and D125G produced the greatest viral yield in IFN- β primed cells, and exceeded rHK-wt maximum yield obtained at 72 hpi by 100, 78, and 12 fold, respectively. These data indicate that the majority of NS1 mutants selected upon mouse adaptation (all except V180A and V226I) enhanced viral growth in both naïve and IFN- β primed mouse cells in vitro, indicating the

virus replicative advantage conferred by the NS1 mutations is independent of IFN- β production in vitro.

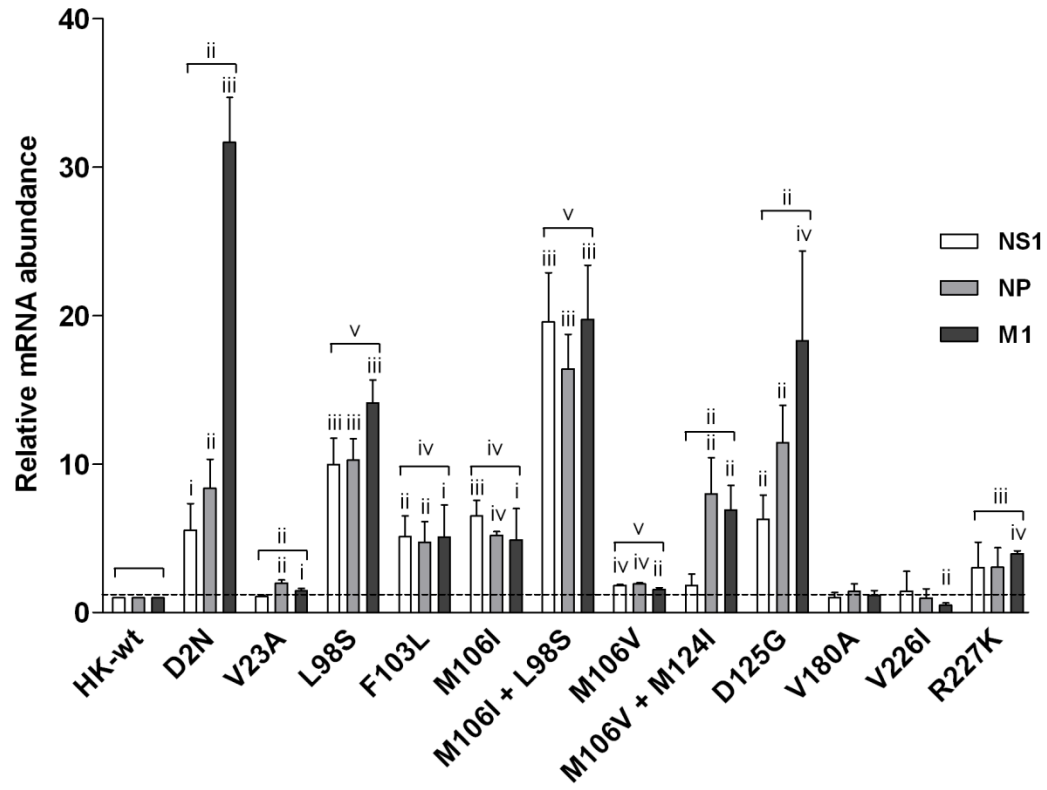
3.4.6 MA NS1 mutants increase viral transcription in mouse cells in vitro

To determine whether enhanced in vitro viral replication that was associated with the majority of the NS1 mutants was due to increased viral transcription, confluent monolayers of M1 cells were infected in triplicate with rHK-wt or rHK NS mutants (MOI= 2) and total RNA was extracted from the cells at 8 hpi for real-time quantitative RT-PCR analysis of viral mRNA and viral RNA (vRNA) encoding NS1, NP (a representative early gene transcript), and M1 (a representative late gene viral transcript) genes.

3.4.6.1 The majority of MA NS1 mutants increased viral mRNA accumulation

Compared to rHK-wt viral mRNA production, the relative abundance of NS1, NP, and M1 mRNA was significantly higher for all NS1 mutants with the exception of mutants V180A and V226I ($p < 0.01$, paired t-test; Figure 13a). NS1 mutants D2N, L98S, M106I + L98S, and D125G all exceeded rHK-wt levels by >10 fold. All assayed viral mRNAs were significantly up-regulated for NS1 mutants D2N, L98S, F103L, M106I, M106I + L98S, M106V, and D125G (Figure 13a) In addition, several NS1 mutants also affected the ratio of mRNA (NS1 to NP and/or M1 mRNA transcript abundance), indicating that MA NS1 mutations affect the relative regulation of gene expression. NS1 mutant L98S demonstrated a significantly greater amount of the late gene M1 mRNA compared to NS1 mRNA ($p < 0.05$), and levels of M1 and NP mRNA were both significantly higher than NS1 mRNA for NS1 mutants M106V + M124I and D125G ($p < 0.05$, NP $>$ NS1 mRNA; $p < 0.01$, M1 $>$ NS1 mRNA) (Figure 13a).

A



B

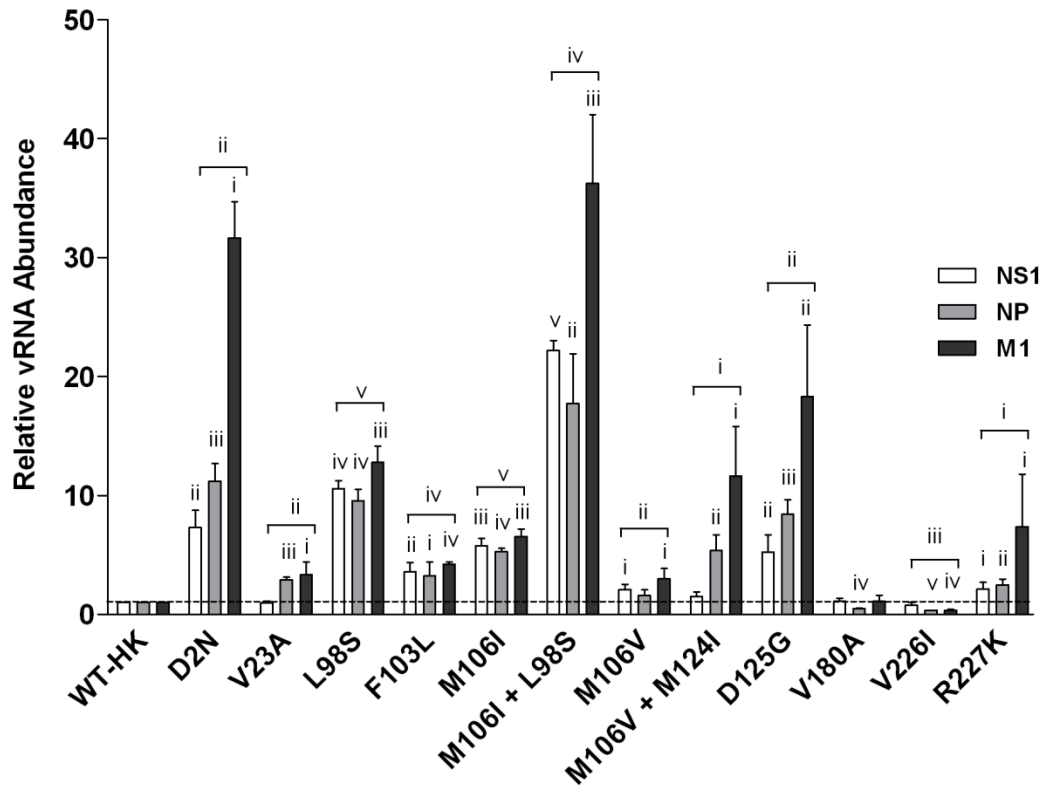


Figure 13. MA NS1 mutations enhance transcription of viral mRNA and replication of genomic viral RNA in vitro. Mouse M1 cells were infected in triplicate at an MOI of 2 with rHK NS mutants or rHK-wt virus, and total RNA isolated at 8 hpi was reverse transcribed using primers specific for viral mRNA (A) and genomic viral RNA (B). Real-time PCR (qPCR) was performed to quantify NP, M1 and NS1 viral transcript levels. Results were normalized by β -actin levels, and presented as values relative to rHK-wt transcript levels. Data represent the means $\pm$ SD (two-tailed student's paired t-test) for NP, NS1 and M1 transcript relative levels (indicated by bracket) or two-tailed student's t-test for individual mRNA relative values (i $p < 0.05$; ii $p < 0.01$; iii $p < 0.001$; iv $p < 0.0001$; v $p < 0.00001$).

3.4.6.2 The majority of MA NS1 mutants increased viral RNA accumulation

Similar to mRNA abundance, all NS1 mutants with the exception of V180A and V226I significantly increased the relative abundance of NS1, NP, and M1 vRNA ($p < 0.05$, paired t-test; Figure 13b). Compared to the fold difference of mRNA relative to rHK-wt levels, relative abundance of vRNA for the three tested transcripts were strikingly similar. The only exceptions were NS1 mutants M106I + L98S and M106V + M124I, where increased levels of M1 vRNA were observed compared to NS1 vRNA (36.2 -fold versus 19.7-fold; $p < 0.01$; and 11.6-fold versus 6.9-fold; $p > 0.05$). These data demonstrate NS1 mutations selected upon IAV adaptation to the mouse increase viral transcript abundance in mouse cells in a gene-specific mechanism shared between vRNA and mRNA production.

3.4.6.3 MA NS1 mutants increase viral polymerase activity

To further support the findings of NS1-mediated increases in RNA synthesis, Jihui Ping, a previous post-doctoral fellow in the Brown Lab, performed a luciferase mini-genome assay to determine the effect of mutant NS gene segments on viral RNA polymerase activity. RNA polymerase activity was measured in HEK 293-T cells as luciferase activity expressed from an influenza firefly luciferase minigenome plasmid expressed by human RNA polymerase I transcription of luciferase flanked by the NP gene non-coding regions (141) in the presence of HK-wt PB2, PB1, PA, NP and specified HK-wt or mutant NS genome segments (see methods for further details). Virus-dependent transcription of firefly luciferase was standardized to renilla luciferase expression levels, which were under control of the host RNA polymerase II dependent SV40 promoter.

Polymerase activity was increased in the presence of NS genes with NS1 mutants F103L, M106I, M106V, D125G, and V180A compared to the activity associated with WT NS gene segment by > 2.5 fold ($p < 0.01$) (Figure 14a). NS1 mutants M106V + M124I and M106I + L98S demonstrated the highest increases in viral polymerase activity (>4.5 fold; $p < 0.01$) (Figure 14a), which indicated enhanced activity due to the second mutation in both instances. Renilla expression did not significantly differ for any of the NS mutants compared to HK-wt (Figure 14b), indicating differences in relative luciferase activity were not due to host transcription suppression.

During the experiment, we found that expression of the HK-wt NS gene segment decreased viral polymerase activity when compared to the empty vector control ($p < 0.01$) (Figure 14a), which suggests the NS1 and/or NEP genes negatively regulated viral polymerase activity. By using pLLB vector plasmids that express the NS gene segment, as well plasmids that express the NS1 or NEP genes alone, we found that expression of the NS gene segment inhibited viral polymerase activity due to both NS1 and NEP gene expression (Figure 14c). Moreover, expression of NS1 alone was shown to significantly reduce renilla luciferase activity, however upon co-expression with NEP in the NS gene segment, renilla luciferase activity was rescued to levels of the vector control (Figure 14c). Further analysis is required to determine the mechanism by which NS1 and NEP regulate polymerase activity. Overall, this data shows that NS1 mutations selected upon mouse adaptation increase viral gene expression at the level of transcription both in the luciferase mini-genome assay and in infected mouse cells, and that several NS1 mutants (L98S, M106I + L98S, M106V + M124I and D125) also differentially affected transcriptional regulation among different viral genes.

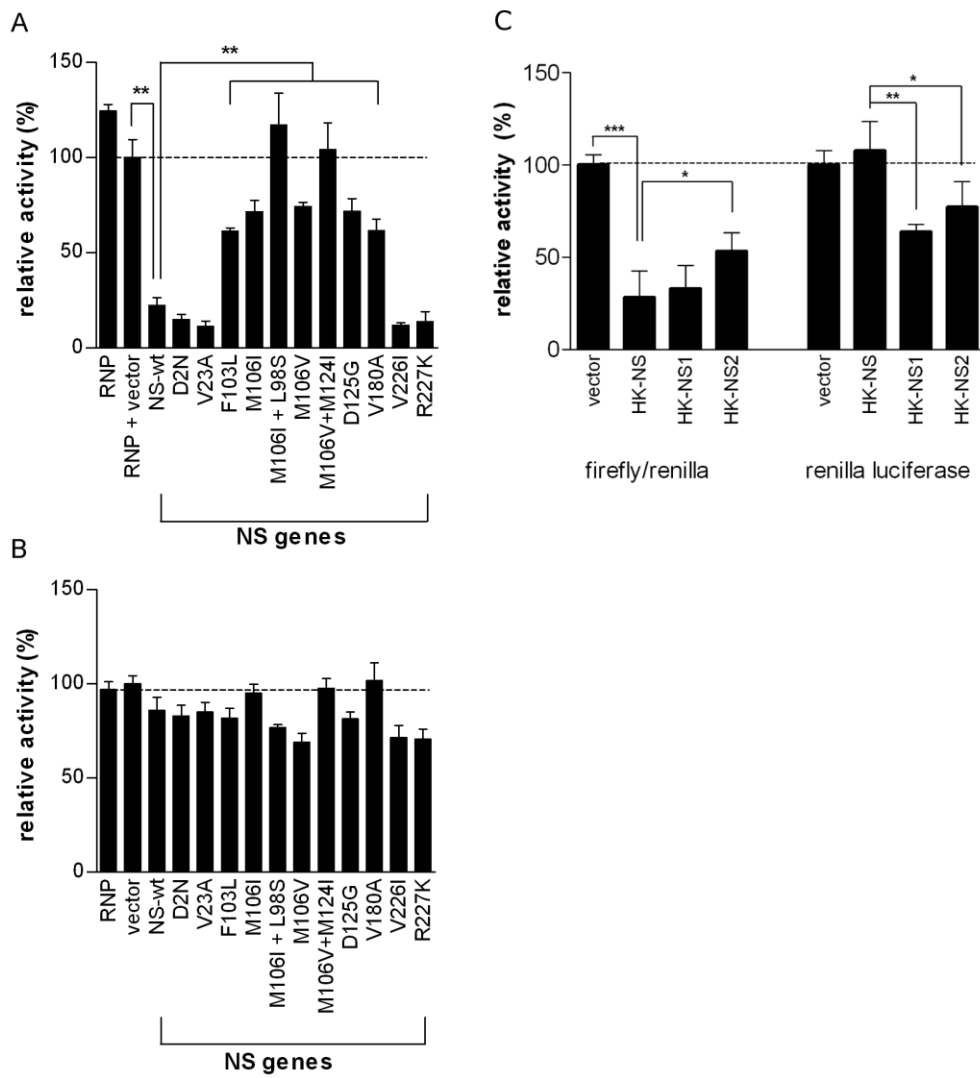


Figure 14. RNA polymerase mini-genome assay. Viral RNA polymerase activity was measured in HEK 293-T cells expressing mutant or HK-wt NS genes as well as HK-wt PB2, PB1, PA, NP, and firefly luciferase driven by the NP promoter. (A) Relative polymerase activity was calculated as the ratio of firefly luciferase to renilla luciferase (under SV40 promoter control) luminescence and presented as means $\pm$ SD for three experiments (** $p < 0.01$, student's t test). (B) NS mutants do not affect host gene expression. Renilla luciferase activity in cells transfected with HK RNP is not affected by NS mutant expression ($p > 0.05$, student's t-test, compared to HK wt NS expression). (C) NS genes alter host gene expression. Relative polymerase activity as well as corresponding renilla luciferase activities of HEK 293-T cells expressing HK-wt NS (NS1 + NS2/NEP proteins) as well as individual NS1 or NS2/NEP proteins, or empty vector control, in combination with HK-wt RNP. Polymerase activity is presented as means $\pm$ SD for 4 experiments as well as the corresponding renilla luciferase activities (* $p < 0.05$, ** $p < 0.01$ *** $p < 0.001$, student's t test) (34).

3.4.7 MA NS1 mutants increase viral protein synthesis in vitro, in untreated and IFN- β primed mouse M1 cells

The NS1 protein functions both to antagonize IFN-mediated antiviral responses as well as to enhance viral protein synthesis. I hypothesized that a factor contributing to the replicative advantage of NS1 mutants in IFN- β primed mouse cells (Figure 12, right panel) may be increased IFN-resistant viral protein synthesis. To test this hypothesis, I determined the level of M1 and NS1 viral protein synthesis by pulse labelling mouse M1 cell monolayers with ^{35}S -labeled Cys and Met residues following infection (MOI=2). The experiment was also performed in parallel on M1 cells that had been IFN- β primed (see methods).

Beginning at 8 hpi, untreated cells infected with rHK-wt produced detectable M1 and NS1 proteins, and viral protein production was markedly reduced in IFN- β primed cells. In contrast, the double mutant M106I + L98S, as well as the single mutant L98S (data not shown), induced robust protein expression starting at 4 hpi regardless of IFN- β pre-treatment (Figure 15a). However, as the remaining NS1 mutants did not demonstrate increased viral protein synthesis relative to rHK-wt prior to 8 hpi (data not shown), quantification of M1 and NS1 protein bands was performed by densitometry for both cell treatment conditions (n=2) (Figure 15 a-d). Compared to rHK-wt infection in untreated cells, viral protein levels were significantly increased for NS1 mutants V23A, L98S, M106I + L98S, M106V + M124I, D125G, and R227K (Figure 15a-c). NS1 mutants L98S and M106I + L98S exhibited the greatest fold increase in viral protein synthesis at 8 hpi in both untreated and IFN- β primed cells. In untreated cells, NS1 mutant L98S produced > 2.5 fold

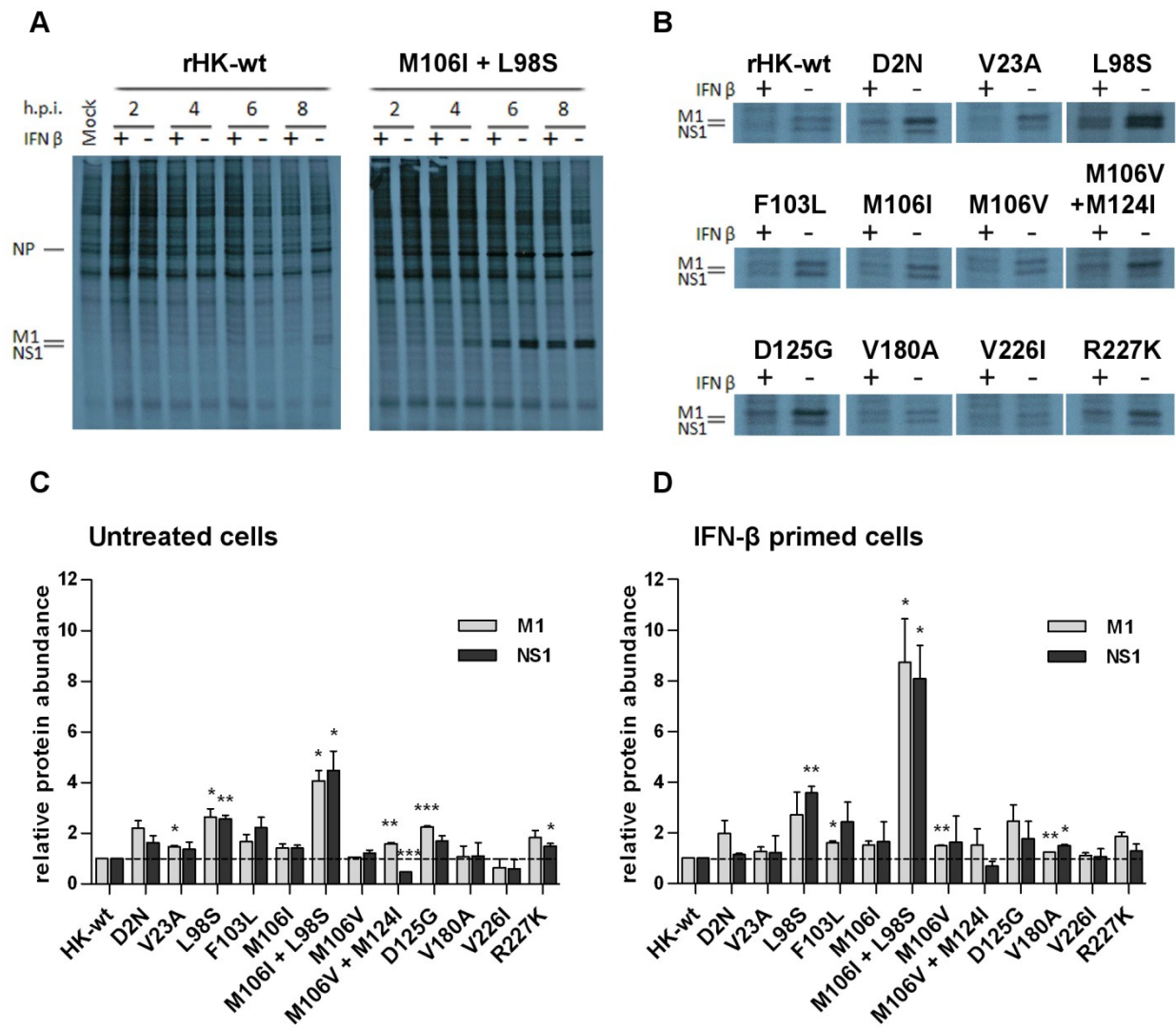


Figure 15. MA NS1 mutations enhance virus protein synthesis in untreated and IFN- β primed mouse cells in vitro. M1 cells were left untreated or were pre-treated with 200 U/mL murine IFN- β for 24 hours, then infected with rHK NS MA or HK-wt virus at an MOI of 2. At 2, 4, 6, and 8 hpi the cells were pulsed with ^{35}S labeled Cys and Met residues for one hour then lysate was collected in SDS buffer. Autoradiography of collected samples are shown for (A) full time courses of HK-wt and rHK NS M106I + L98S and (B) 8 hour time points of all NS mutants. (+) and (–) symbols indicate whether cells were primed with IFN- β . NP, M1 and NS1 protein positions are indicated, and were verified by western blot (data not shown). Data shown are representative of two independent experiments, which were each subject to densitometry analysis. Data represent the means $\pm$ SE (* p <0.05, ** p <0.01, *** p <0.001; two-tailed student's t-test) for NS1 and M1 protein band density relative to rHK-wt in untreated cells (C) and in IFN- β primed cells (D) (34).

more M1 and NS1 protein, and mutant M106I + L98S produced > 4 fold more M1 and NS1 protein (Figure 15c). In IFN- β primed cells, NS1 protein levels were > 2.5 fold higher for NS1 mutant L98S, and both M1 and NS1 levels were > 8 fold higher than rHK-wt for mutant M106I + L98S (Figure 15d). All other mutants, with the exception of V23A and V226I, demonstrated a trend of increased viral protein synthesis in IFN- β primed cells, which was significantly greater than rHK-wt for NS1 mutants F103L, M106V, and V180A (Figure 15d). This data strongly suggests increased IFN-resistant viral protein synthesis in mouse cells in vitro is a phenotype shared by NS1 mutations selected upon mouse adaptation

3.4.8 MA NS1 mutants with altered M1:NS1 protein production ratios do not demonstrate altered viral protein stability

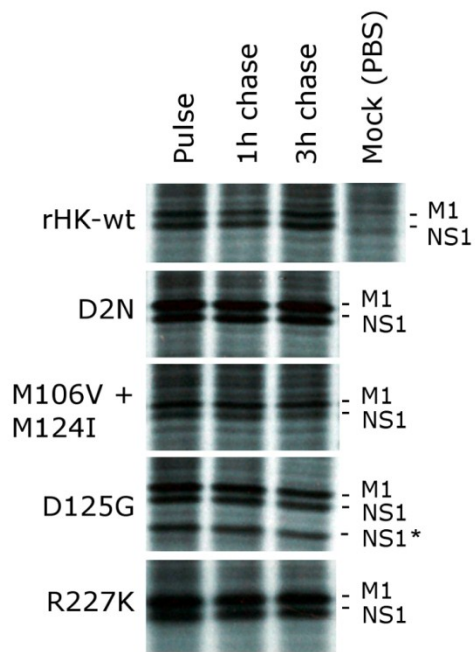
When analyzing the effect of NS1 mutations on viral protein production in vitro in mouse M1 cells, I observed that the ratio of M1 protein production to NS1 protein production was affected for NS1 mutant M106V + M124I. Unlike rHK-wt infection, which produced M1 and NS1 protein bands of similar intensity, infection with NS1 mutant M106V + M124I produced significantly more M1 protein than NS1 protein ($p < 0.01$) (Figures 15b-c). This trend was also observed for NS1 mutants D2N, D125G, and R227K (Figures 15b-c), although the difference was not statistically significant. To determine whether this observation was due to altered NS1 protein stability, I performed a ^{35}S pulse-chase experiment. Briefly, M1 cells were infected with rHK-wt, and NS1 mutants M106V + M124I, D2N, D125G, and R227K. At 8 hpi the cells were pulsed with ^{35}S -labeled Cys and Met, then at 9 hpi, the cells were either collected (pulse samples) or were chased for 1 or 3 hours with

complete MEM (n=3). Samples were run on SDS gel then subject to autoradiography and phosphorscreen signal amplification (Figure 16a). Compared to rHK-wt virus protein stability, the NS1 mutations tested showed no effect, as measured as the ratio of M1 to NS1 protein levels in each condition (Figure 16b). An additional protein band of 20 kDa was detected with NS1 polyclonal antibody in cells infected with the NS1 mutant D125G (See section 3.4.11 for further comment). When taken together, these observations indicate that several NS1 mutations alter the regulation of viral gene expression in a gene-dependent manner (Figure 15b-c, Figure 16). The observation that the relative increases in RNA synthesis (Figure 13) were greater than the relative increases in protein synthesis (Figure 15) suggests that restrictions in protein synthesis are limiting gene expression in mouse cells in vitro.

3.4.9 Effect of NS1 mutants on virus growth, viral protein production, and resistance to human type I IFN in human lung carcinoma (A549) cells

The majority of NS1 mutants selected upon adaptation to the mouse increased in vitro virus replication and virus protein synthesis in untreated and IFN-primed mouse cells (Figure 12, Figure 15). However, it was not known whether this phenotype was host range restricted. Since the HK virus is a human viral strain, I hypothesized these phenotypes may extend to human cell infection. To this end, virus replication and viral protein synthesis were assessed as per the previous protocol, in A549 cells, a human type II lung carcinoma cell line.

A



B

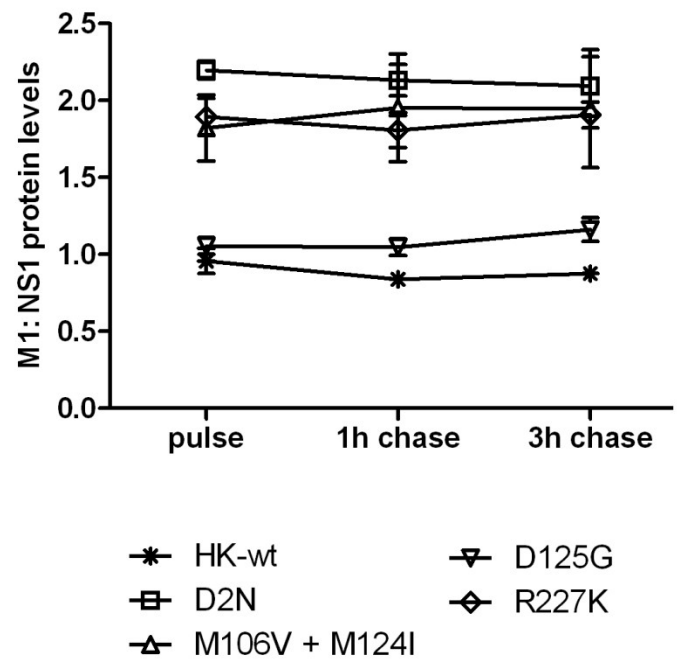


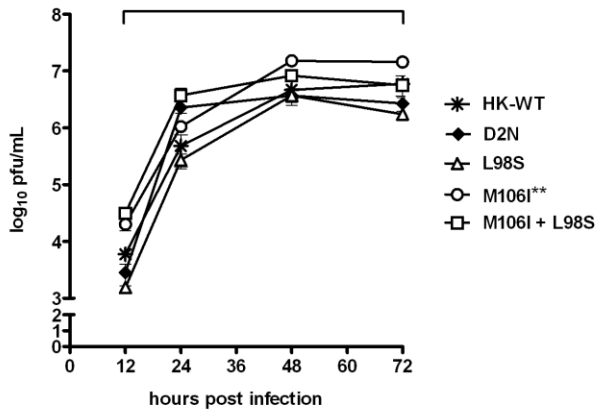
Figure 16. NS1 mutants with altered M1: NS1 protein production ratio do not demonstrate altered viral protein stability. M1 cells were infected with specified rHK NS1 mutants or HK-wt virus at an MOI of 2. At 8 hpi the cells were pulsed with ³⁵S labeled Met and Cys residues for one hour. For pulse samples, the lysate was then collected in SDS buffer. For chase samples, the pulse medium was replaced with serum-free MEM then cell lysate was collected 1 or 3 hours later (n=3). (A) Autoradiography of collected samples, indicating bands corresponding to M1 or NS1 protein, as verified by Western blot, (B) protein bands were quantified by PhosphorScreen signal amplification, then band intensity quantification using GE Imagequant software. Data represent the means ± SD of the ratio of M1 to NS1 protein levels. Statistical analysis was performed, however no virus induced a statistically significant (p>0.05, student's t test) difference in the ratio of M1:N1 from pulse to chase (34).

3.4.9.1 Several NS1 mutants enhance virus replication in untreated human A549 cells

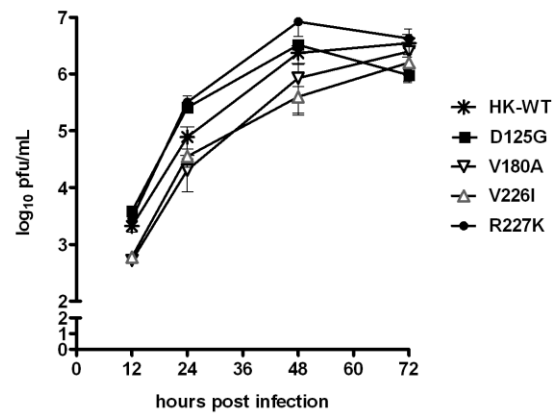
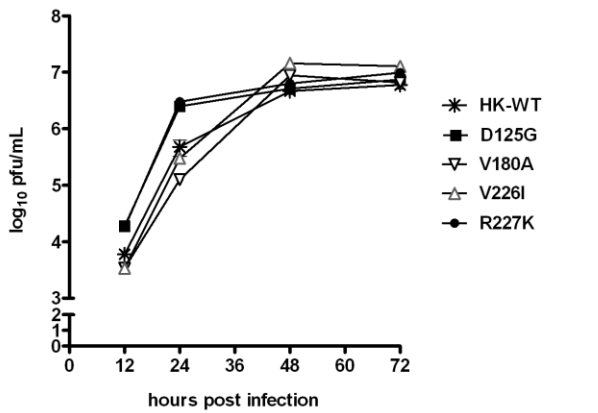
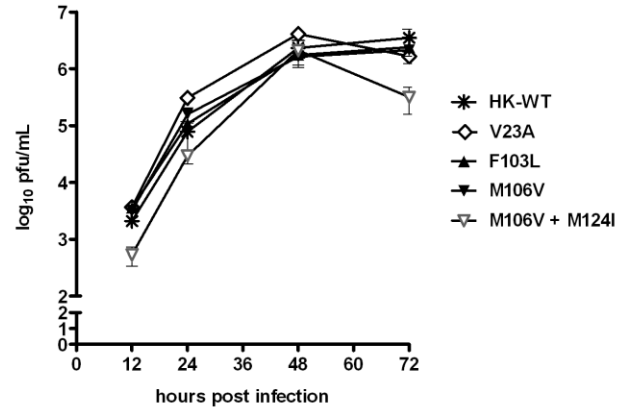
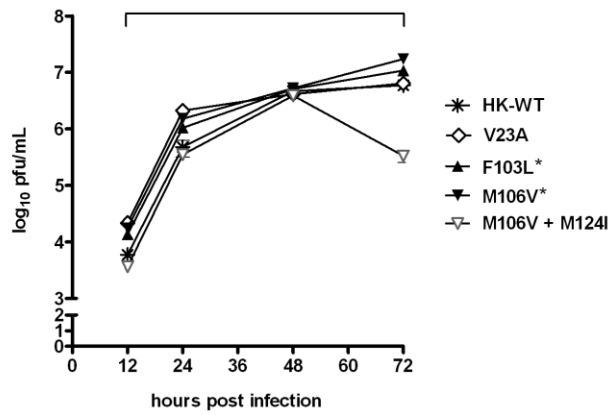
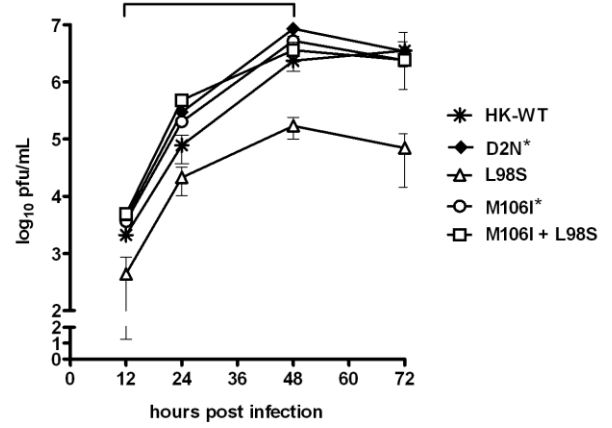
To assess the effect of MA NS1 mutations on virus replication in human cells, confluent monolayers of human A549 cells were infected with rHK NS mutant and rHK-wt viruses (MOI = 0.02; n=3). Unlike in mouse M1 cells, rHK-wt virus replication reached high titres, attaining a maximum yield of 5.9×10^6 PFU/mL at 72 hpi (Figure 17a, left panel). The majority of NS1 mutant viruses demonstrated virus growth that was statistically indistinct from rHK-wt when assessed by two-tailed paired student's t-test, with the exception of NS1 mutants M106I ($p < 0.01$), F103L ($p < 0.05$), and M106V ($p < 0.05$), where virus growth from 12-72 hpi surpassed rHK-wt levels (Figure 17a, left panel). Most NS1 mutants did reach statistically higher virus titres at early times in infection (12 and/or 24 hpi; Figure 17b). NS1 mutant M106V reached the highest virus titre (1.73×10^7 PFU/mL; 72 hpi), which was 2.9 fold greater than rHK-wt virus maximum titre ($p < 0.05$). All NS1 mutants surpassed rHK-wt virus titre for at least one time point following infection ($p < 0.05$) with the exception of NS1 mutants L98S and M106V + M124I (Figure 17b). However, several NS1 mutants were unable to initiate virus replication at early times to the same level as rHK-wt. Virus titre at 12 hpi was significantly attenuated for NS1 mutants D2N, L98S, M106V + M124I, V180A and V226I (Figure 17b). This data indicates that although several NS1 mutations selected upon human IAV mouse adaptation confer a replicative advantage in vitro in human cells, this advantage was not observed at all times following infection and was not a universal phenotype associated with MA NS1 mutants.

A

untreated A549 cells



IFN primed A549 cells



B

	Untreated cells			
NS1 mutation	12h	24h	48h	72h
D2N	*	**	-	-
L98S	**	-	-	*
M106I	**	*	***	**
M106I + L98S	**	*	*	-
V23A	***	*	-	-
F103L	*	*	-	*
M106V	***	**	-	*
M106V + M124I	*	-	-	*
D125G	**	**	-	-
V180A	*	-	**	-
V226I	*	-	**	*
R227K	**	***	-	*

Legend
p value > WT
p value < WT
* p<0.05
** p<0.01
*** p<0.001
**** p<0.0001

	IFN primed cells			
NS1 mutation	12h	24h	48h	72h
D2N	**	**	*	-
L98S	**	-	**	*
M106I	-	*	*	-
M106I + L98S	*	***	-	-
V23A	*	***	-	-
F103L	***	-	-	-
M106V	***	-	-	-
M106V + M124I	***	-	-	*
D125G	*	**	-	*
V180A	***	-	-	-
V226I	***	-	*	-
R227K	-	*	-	-

Figure 17. MA NS1 mutations enhance virus growth in untreated and in IFN- α primed human A549 cells in vitro. Human A549 cells were untreated (left panel) or pre-treated with human IFN- α 2b (1000 U/mL) for 24 hours (right panel), then infected in triplicate at an MOI of 0.02 with rHK NS mutants or rHK-wt virus, and supernatant collected 12, 24, 48, and 72 hpi was quantified for viral titre by plaque assay on MDCK cells. (A) Data represent the mean viral titre $\pm$ SD (*p<0.05, **p<0.01, ***p<0.001; two-tailed student's paired t-test for time range indicated by horizontal bracket above data). (B) Individual time point p-value of rHK NS mutant viral titre versus rHK-wt viral titre.

3.4.9.2 Several NS1 mutants increased virus growth in IFN-primed human A549 cells

To assess the effect of NS1 mutations on resistance to the antiviral response in infected human cells, A549 cells were pre-treated for 24 hours with human IFN- α 2b (1000 U/mL) then infected as per the previous protocol. The rHK-wt virus yield was reduced by 2.7 fold by IFN- α pre-treatment at 72 hpi, where viral yield reached a maximum of 3×10^6 PFU/mL in IFN-primed cells, and 8.2×10^6 PFU/mL in untreated cells (Figure 17a). IFN sensitivity, estimated by the fold difference in rHK-wt virus maximum yield due to IFN pre-treatment, was much less than that observed in infected mouse cells (2.7 fold versus 16 fold reduction; Figures 12 and 17). While no NS1 mutant significantly increased virus growth throughout the course of infection in IFN-primed A549 cells, NS1 mutants D2N and M106I did increase virus growth from 12-48 hpi ($p < 0.05$) (Figure 17a, right panel). The majority of NS1 mutants demonstrated significantly increased titres at early time points following infection (12 and/or 24 hpi; $p < 0.05$ to $p < 0.001$) (Figure 17b). However, only NS1 mutants D2N and M106I demonstrated a statistically significant increase in virus titre at 48 hpi ($p < 0.05$) and no NS1 mutant significantly increased viral titre at 72 hpi compared to rHK-wt. These data demonstrate that the majority of NS1 mutants increase IFN-resistant viral growth in IFN-primed human cells at early times in infection, while NS1 mutants D2N and M106I exhibited a more sustained replicative advantage.

3.4.9.3 MA NS1 mutations convergent with HPAI H5N1 virus enhance viral protein

synthesis in A549 cells at early times following infection

NS1 mutations V23A, F103L, and M106I were also found in highly pathogenic avian influenza (HPAI) H5N1 strains such as A/Hong Kong/156/1997 (HK H5N1), which was fatal in humans (50). Given that all three mutations localize within binding domains for cellular factors involved in translation (PABPI and eIF4GI, Figure 1), and all three evolved in the HK H5N1 virus lineage, culminating in fatal human infection, I hypothesized that these NS1 mutants may enhance viral protein synthesis in human cells. To this end, I infected confluent monolayers of A549 cells with rHK-WT and NS1 mutants V23A, F103L, M106I, as well as the double mutant F103L + M106I (MOI=2; n=2). Following infection, the cells were pulse labelled with ³⁵S-labeled Cys and Met residues for one hour at 2, 4, 6 and 8 hpi, then the cell lysate was collected and subject to SDS PAGE electrophoresis and autoradiography (Figure 18a). Densitometry was performed on the bands corresponding to the NS1 and M1 proteins for each time point (Figure 18b-c). While the rate of viral protein synthesis rate was similar to that of rHK-wt for later time points, NS1 mutations co-selected in mouse-adaptation as well as the natural evolution of the HK H5N1 virus (V23A, F103L, M106I, F103L + M106I) significantly increased both NS1 and M1 viral protein synthesis at the 4 hour time point (p<0.05, Figure 18b). Therefore, following human A549 cell infection, rHK viruses expressing MA/HK H5N1 NS1 mutations synthesize viral protein at an enhanced rate seen early in infection.

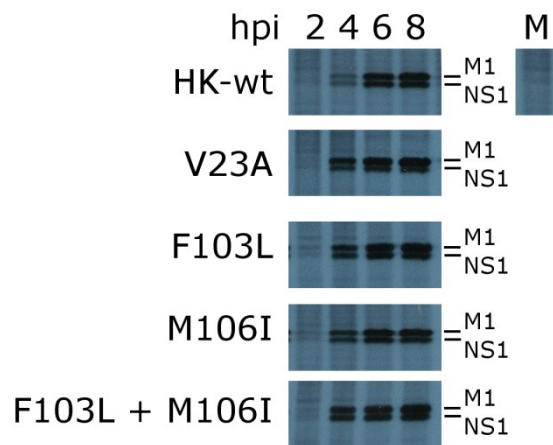
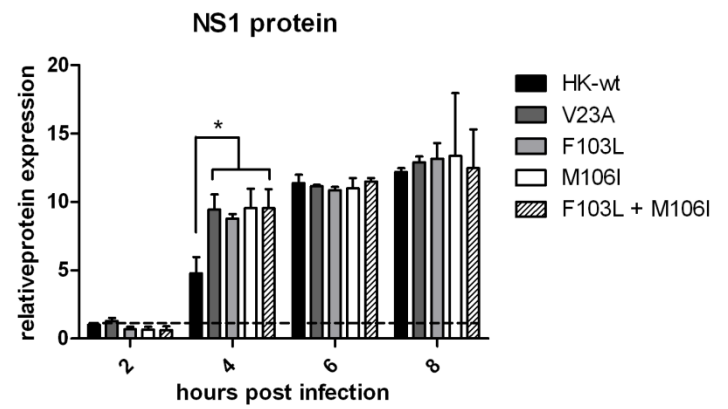
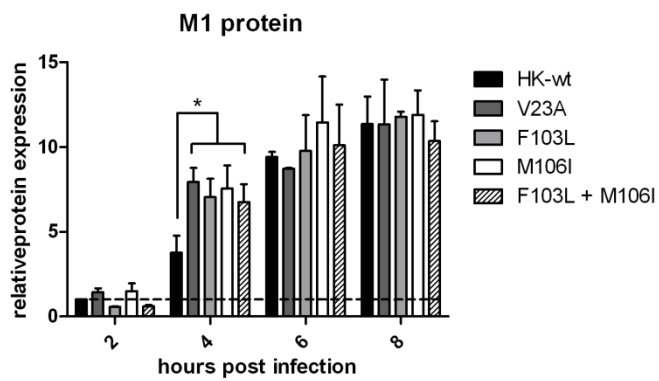
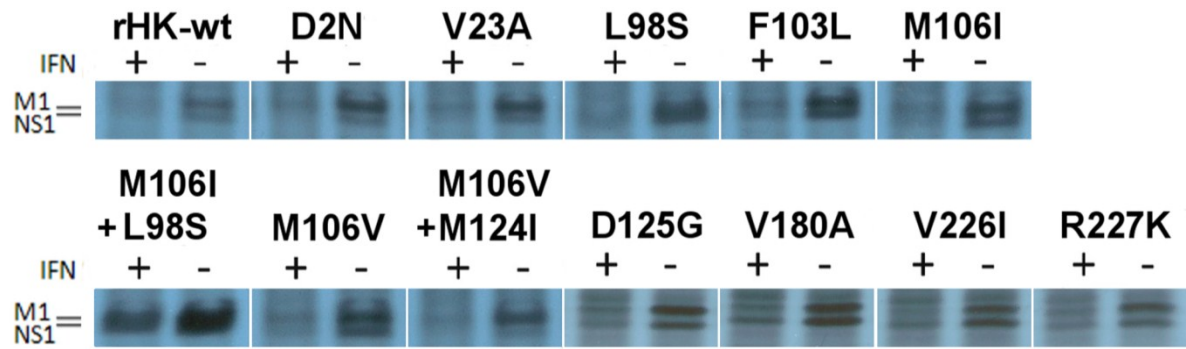
A**B****C**

Figure 18. MA NS1 mutations shared with A/Hong Kong/156/1997 (H5N1) enhance viral protein synthesis in human A549 cells in vitro. A549 cells were infected with specified rHK NS MA or HK-wt viruses or with PBS (M) at an MOI of 2. At 2, 4, 6, and 8 hpi the cells were pulsed with ³⁵S labeled Cys and Met residues for one hour then lysate was collected in SDS buffer. (A) Autoradiography of collected samples are shown. Data shown are representative of two independent experiments, which were each subject to densitometry analysis. Data represent the means $\pm$ SE (*p<0.05; two-tailed student's t-test) for NS1 (B) and M1 (C) protein band density relative to rHK-wt virus protein production at 2 hpi.

3.4.9.4 MA NS1 mutations increase viral protein synthesis in untreated and IFN pre-treated A549 cells

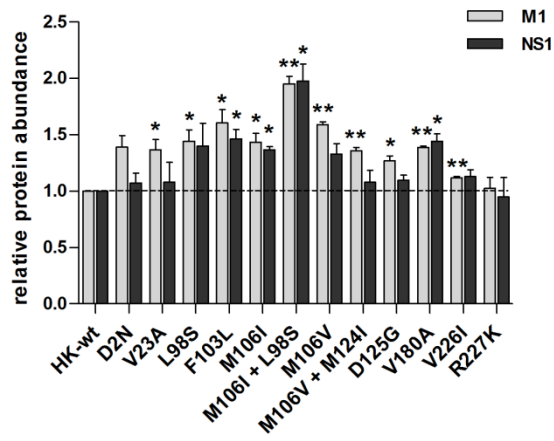
Since viral protein synthesis was enhanced at 4 hours following infection in A549 cells for MA NS1 mutations convergent with HK H5N1, other MA NS1 mutations may also share this phenotype. Moreover, given that MA NS1 mutants increased viral protein synthesis irrespective of IFN pre-treatment in mouse cells, enhanced viral protein synthesis in human A549 cells may also be type I IFN-resistant. To this end, A549 cells were left untreated or primed with human IFN- α 2b (1000 U/mL, 24h) then infected with rHK NS mutants or rHK WT as per the previous protocol. Cell lysate collected at 4 hpi was subject to SDS PAGE electrophoresis and autoradiography (Figure 19a). Densitometry was performed on the bands corresponding to the NS1 and M1 protein for each time point (Figure 19b). In untreated A549 cells, all NS1 mutants induced significantly more NS1 and/or M1 protein production ($p < 0.05$ to $p < 0.01$) with the exception of NS1 mutant R227K. In cells primed with human IFN- α 2b, NS1 mutants M106I + L98S, D125G, V180A and V226I exceeded rHK-wt viral protein synthesis for both M1 and NS1 protein or M1 protein alone ($p < 0.05$ to $p < 0.01$). Although the difference in viral protein synthesis was not significant, the remainder of the NS1 mutants did enhance viral protein synthesis in IFN-primed cells compared to rHK-wt (Figure 19b). Similar to viral protein production of NS1 mutants in M1 cells, NS1 mutant M106I + L98S produced the highest levels of viral protein in both untreated cells and cells primed with human IFN- α 2b (>2 fold compared to rHK-wt levels) however it is important to note that the fold enhancement was far less than that observed

A



B

Untreated A549 cells



IFN primed A549 cells

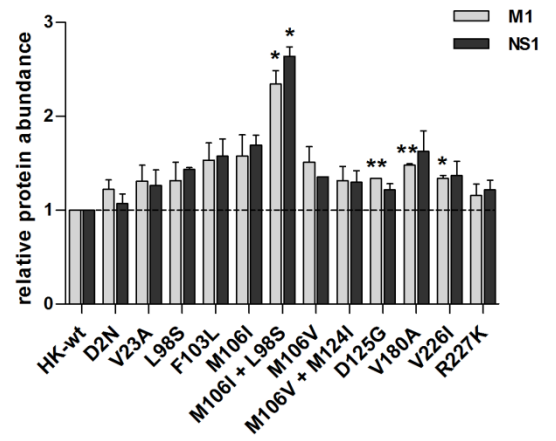


Figure 19. MA NS1 mutations enhance virus protein synthesis in untreated and IFN- α primed human cells in vitro. A549 cells were left untreated or were pre-treated with 1000 U/mL human IFN- α 2b for 24 hours, then infected with rHK NS MA or HK-wt virus at an MOI of 2. At 4 hpi the cells were pulsed with ^{35}S -labeled Cys and Met residues for one hour then lysate was collected in SDS buffer. (A) Autoradiography of collected samples are shown. (+) and (–) symbols indicate whether cells were pre-treated with IFN. M1 and NS1 protein positions are indicated, and were verified by Western blot (data not shown). Data shown are representative of two independent experiments, which were each subject to densitometry analysis. (B) Data represent the means $\pm$ SE (* p <0.05, ** p <0.01; two-tailed student's t-test) for NS1 and M1 protein band density relative to rHK-wt in untreated and IFN-primed cells.

in mouse cells (>4 fold in untreated M1 cells, >8 fold in IFN primed M1 cells, Figure 15c).

These observations indicate that the majority of NS1 mutants enhance viral protein synthesis in human A549 cells, albeit at a reduced level than that observed in mouse cells.

3.4.10 Effect of NS1 mutants on virus growth and viral protein production in canine kidney cells

3.3.10.1 The majority of NS1 mutants do not enhance virus replication in MDCK cells

I also assessed viral growth and viral protein production on Madin Darby canine kidney (MDCK) cells, a cell line highly permissive to IAV infection. To determine whether NS1 mutants selected upon IAV mouse adaptation conferred increased viral replication in MDCK cells, cell monolayers were infected and viral titres were quantified as per the previous protocol. Since canine type I IFN is not commercially available, the experiment was only performed in untreated cells. The rHK-wt virus grew to high titres, where maximum titre of 1.2×10^8 PFU/mL was reached at 48 hpi (Figure 20). Virus was quantified at 72 hpi, however since virus replication was found to plateau at 48 hpi, the 72h time points were excluded from the analysis. The difference in virus growth between rHK-wt and NS1 mutants was much smaller in MDCK cells compared to M1 and A549 cells; here, the NS1 mutant with the greatest fold increase in maximum viral titre, V23A (2.43×10^8 PFU/mL), surpassed rHK-wt titre by only 2 fold (Figure 20). NS1 mutants V23A as well as F103L significantly increased virus replication between 24 and 48 hpi compared to rHK-wt levels, while NS1 mutants M106I, M106V, M106V + M124I and R227K were significantly attenuated compared to rHK-wt ($p < 0.05$, Figure 20). Viral titre of several NS1 mutants (D2N, F103L, M106I + L98S, and D125G) surpassed rHK-wt titre early in infection, at 12 hpi

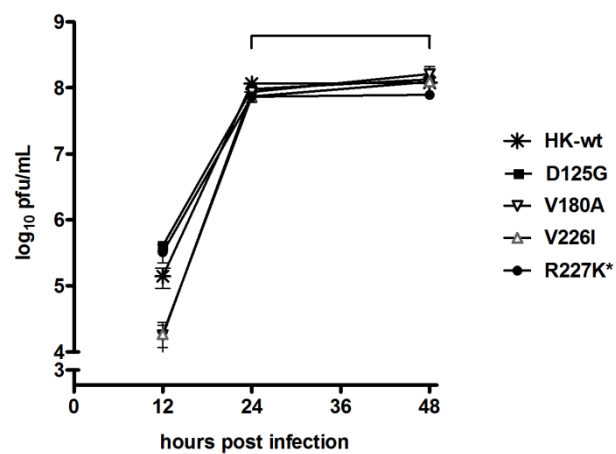
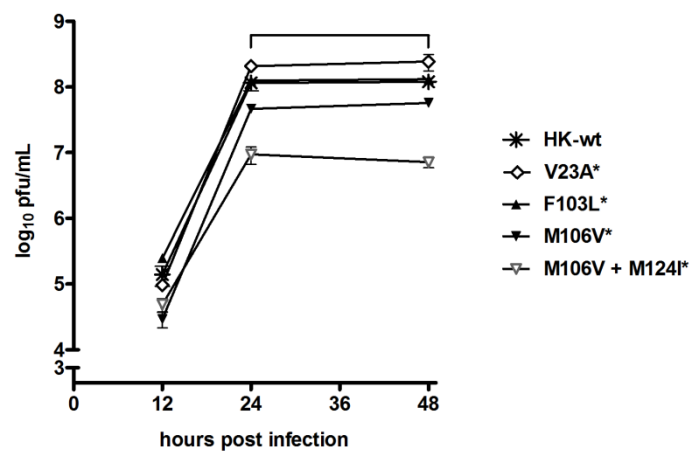
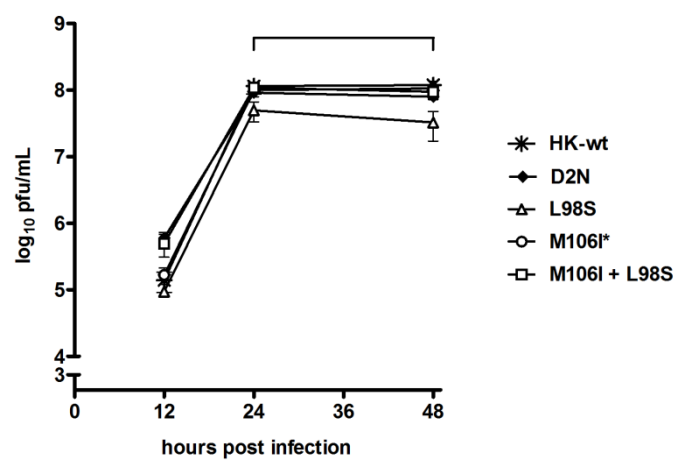


Figure 20. MA NS1 mutations enhance virus growth in MDCK cells in vitro. MDCK cells were infected in triplicate at an MOI of 0.02 with rHK NS mutants or rHK-wt virus, and supernatant collected 12, 24, and 48 hpi was quantified for viral titre by plaque assay on MDCK cells. (A) Data represent the means $\pm$ SD (* $p < 0.05$; two-tailed student's paired t-test for 24-48 hpi, indicated by horizontal bracket above time range).

($p < 0.05$), however for all but F103L, titres were equal to or less than rHK-wt at later time points (Figure 20). From these results I conclude that the viral replication enhancement associated with MA NS1 mutants is host specific. The most extreme phenotype was demonstrated upon mouse M1 cell infection, where the majority of NS1 mutants enhanced viral replication, with some exceeding rHK-wt titres by up to 100 fold (Figure 12). Virus replication in human A549 cells was enhanced for several NS1 mutants; however the greatest enhancement only reached 2.9 fold (Figure 17a). In canine MDCK cells, the majority of NS1 mutants replicated at a similar or lesser extent than rHK-wt, with the greatest fold enhancement reaching only 2 fold. This may be due to the highly permissive nature of MDCKs, where the rHK-wt virus reached titres that exceeded 10^8 PFU/mL.

3.4.10.2 Specific NS1 mutants increased viral protein synthesis in infected MDCK cells

I next assessed virus protein production in MDCK cells, using ^{35}S pulse labeling and autoradiography as per the previous protocol, collecting samples at 2, 4, 6 and 8 hpi (Figure 21). The experiment was first performed prior to rescue of rHK NS L98S; following virus rescue I repeated the pulse labeling experiment for NS mutants L98S and M106I + L98S as well as rHK-wt for an internal control (Figure 21, right panel). The rate of rHK-wt viral protein production was detectable starting at 4 hpi, and increased through the time course. Several NS1 mutants were able to induce viral protein synthesis earlier than the rHK-wt virus. NS1 mutants D2N, D125G, and R227K produced visible bands corresponding to M1 and NS1 proteins at 2 hpi, and all NS1 mutants assayed except F103L, V180A, V226I and R227K produced more viral protein at 4 hpi compared to rHK-wt (Figure 21). However,

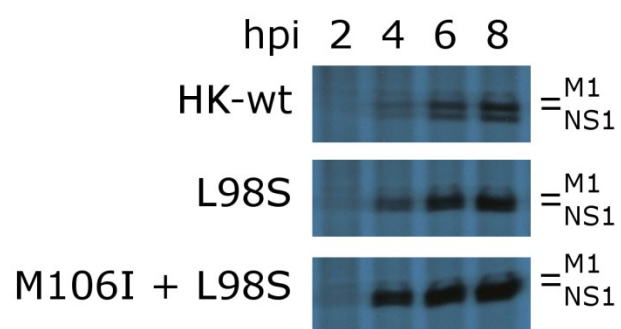
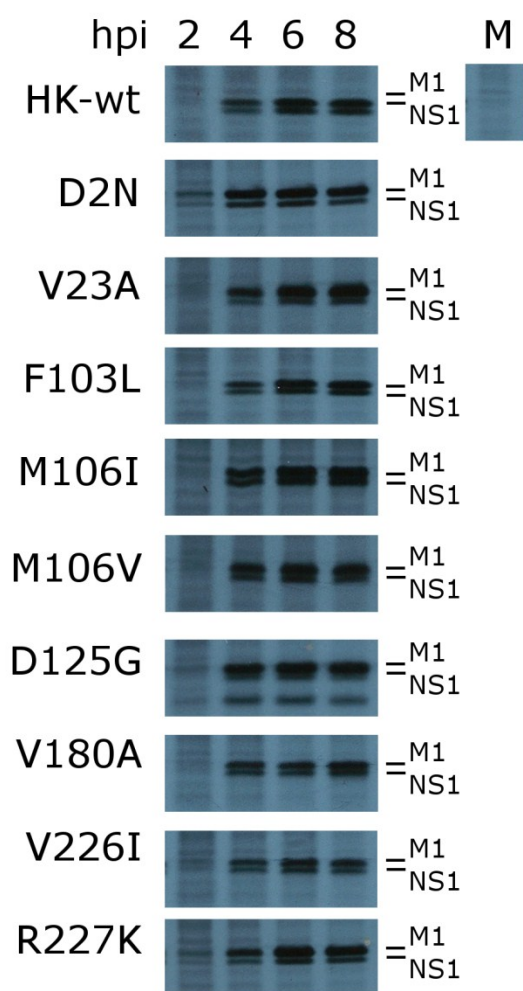


Figure 21. MA NS1 mutations enhance virus protein synthesis in canine cells in vitro. MDCK cells were infected with rHK NS MA or HK-wt virus at an MOI of 2. At 2, 4, 6, and 8 hpi the cells were pulsed with ^{35}S -labeled Cys and Met residues for one hour then lysate was collected in SDS buffer and subject to SDS gel electrophoresis and autoradiography. M1 and NS1 protein positions are indicated, and were verified by western blot (data not shown).

similar to viral protein produced in A549 cells, the majority of NS1 mutants produced similar levels of viral protein at later time points. Only NS1 mutants V23A, M106I, L98S and M106I + L98S demonstrated enhanced viral protein synthesis at 6 and 8 hpi. NS1 mutant M106I + L98S induced the greatest enhancement of viral protein synthesis in MDCK cells. This data shows that several NS1 mutations are capable of enhancing viral protein synthesis in multiple cell lines derived from varying host species, however enhanced viral protein synthesis does not universally correlate with enhanced viral replication.

3.4.11 A novel virus protein is produced due to the NS1 D125G mutation: The NS3 Protein

Mouse-adapted strains MA102 and MA111 both selected the NS1 D125G mutation, which was associated with increased virulence, viral replication, and inhibition of IFN- β production in the mouse lung. Moreover, this mutation obliterated NS1 binding affinity to the cellular factor CPSF30 F2F3 domain and was associated with increased growth in mouse and human cell lines, and enhanced viral gene expression at both transcriptional and translational levels in mouse cells, and in human and canine cells at the level of viral protein translation. During pulse labeling experiments, I observed that the rHK NS D125G virus produced an additional viral protein band of approximately 20 kDa, and this observation was consistent among all three cell lines tested (Figure 22a). Moreover, the 20 kDa band was also detected for the MA111 virus by pulse labelling infected A549 cells (data not shown).

Mohammed Selman, a fellow researcher in the Brown Lab, undertook the research project of characterizing the phenotype of the NS1 mutations located within the PKR binding domain, D125G and M124I, as well as the double mutant M124I + D125G. He

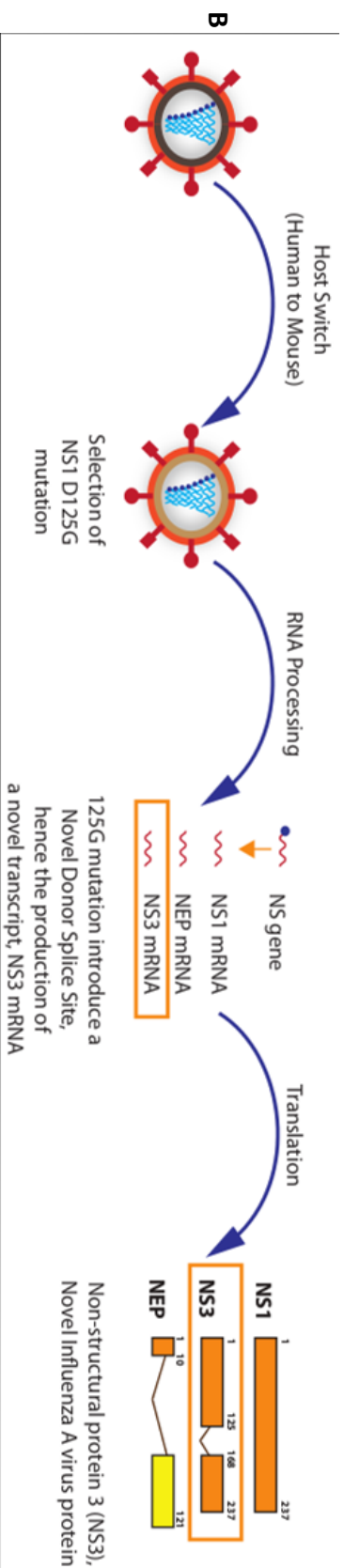
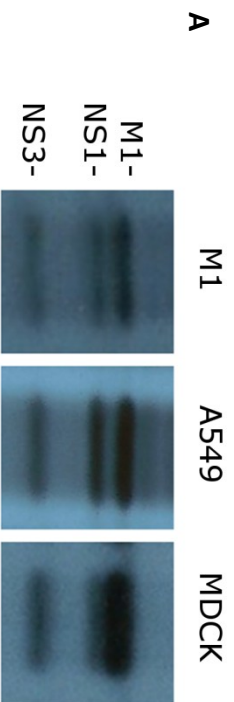


Figure 22. The NS1 D125G mutation induces expression of a novel virus protein. (A) Upon infection of human (A549), mouse (M1), or canine (MDCK) cells, the rHK NS1 D125G mutant induces expression of a novel protein ~20 KDa in size. Representative autoradiography images of pulse labeled infected cells are shown. (B) Mutation at D125G introduces a novel splice site in the NS gene segment, leading to the expression of the NS3 protein.

initially observed that the additional mutation of M124I increased the production of the 20 kDa band (data not shown). Mohammed then determined the amino acid sequence of the 20 kDa protein by liquid chromatography and tandem mass spectrometry of peptide fragments resulting from HK-wt and M124I + D125G NS1 proteins. Following peptide sequencing, he identified the 20kD band as a novel IAV protein, NS3, similar to NS1 but with an internal deletion of 43 amino acids between residues 125 and 168. mRNA analysis of transcripts resulting from HK-wt and D125G/M124I + D125G NS gene segments showed the D125G mutation produced an additional transcript corresponding to the predicted length of the NS3 protein (unpublished results). Mohammed then analyzed the RNA sequences of the WT, D125G and M124I + D125G NS genes using an algorithm to predict potential human splice sites (185). The HK-wt and M124I NS1 sequences showed one predicted donor and acceptor splice site at position 30 and 503 corresponding to the NEP splice sites. However, the D125G and double mutant showed an additional predicted donor splice site at position 373 corresponding to position 1 of codon 125 at the protein level (unpublished results). The A to G mutation forms a requisite GU donor splice site at the 5' end of the intron. Therefore NS3 is produced by a splicing event involving the novel donor splice site at position 373 and the NEP acceptor splice site at position 503 (Figure 22b).

Although we know NS3 is expressed upon NS1 D125G mutation, our lab is still undertaking experiments to determine the functional nature of the NS3 protein. Is the protein expressed in other IAV isolates sharing the D125G mutation? Does the NS3 protein dimerize, or interact with the NS1 protein, or known NS1 binding partners, including RNA? Does NS3, like NS1 interact with all other IAV proteins and nucleic acids, and localize in both

the nucleus and cytosol? In addition, D125G mutants that are unable to splice to form NS3 transcripts (GGT and GGC) are currently being phenotypically characterized. Thus at this point, the extent that the D125G NS1 mutation contributes to the rHK NS D125G virus phenotype is unknown, as it has yet to be assessed in the absence of NS3 expression.

3.4.12 NS1 mutants alter cellular localization of the NS1 protein in infected M1 cells

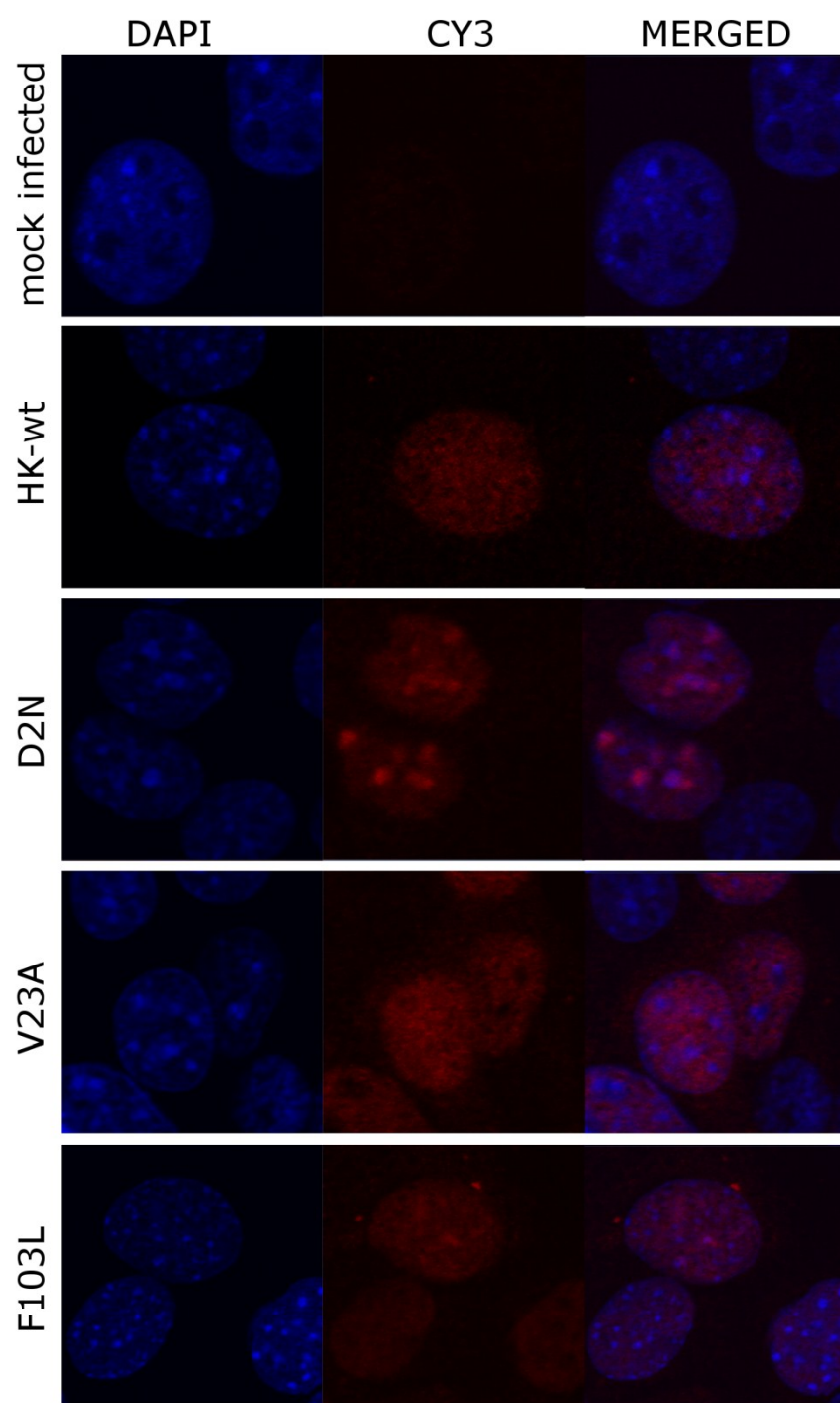
During infection, the NS1 protein functions in both the nucleus and in the cytoplasm of the host cell. Like all IAV proteins, NS1 is synthesized in the cytoplasm by host translational machinery. The protein localizes to the nucleus by two nuclear localization signals (NLS), which are targeted by the cellular protein importin α (Reviewed in (54)). Several studies have shown that NS1 also localizes to the cytoplasm at later times following infection (56, 89, 90). The protein does contain one nuclear export signal (NES); however there has been little research on the mechanism by which NS1 translocates from the nucleus to the cytoplasm. Nonetheless, it is also unknown whether host adaptation affects cellular distribution of the NS1 protein. I hypothesize that upon adaptation to the mouse, mutant NS1 proteins may have altered cellular distribution compared to the rHK-wt virus. To assess this hypothesis, I performed confocal microscopy on infected M1 cells to determine the extent of NS1 co-localization with the nucleus. I also performed Western blots on infected M1 cell nuclear and cytosolic fractions to assess NS1 subcellular localization. As these experiments followed the discovery of the NS3 protein, I also included the rHK NS viruses expressing NS1 mutations M124I and D125G + M124I in the experiments investigating the cellular localization of the NS1 protein.

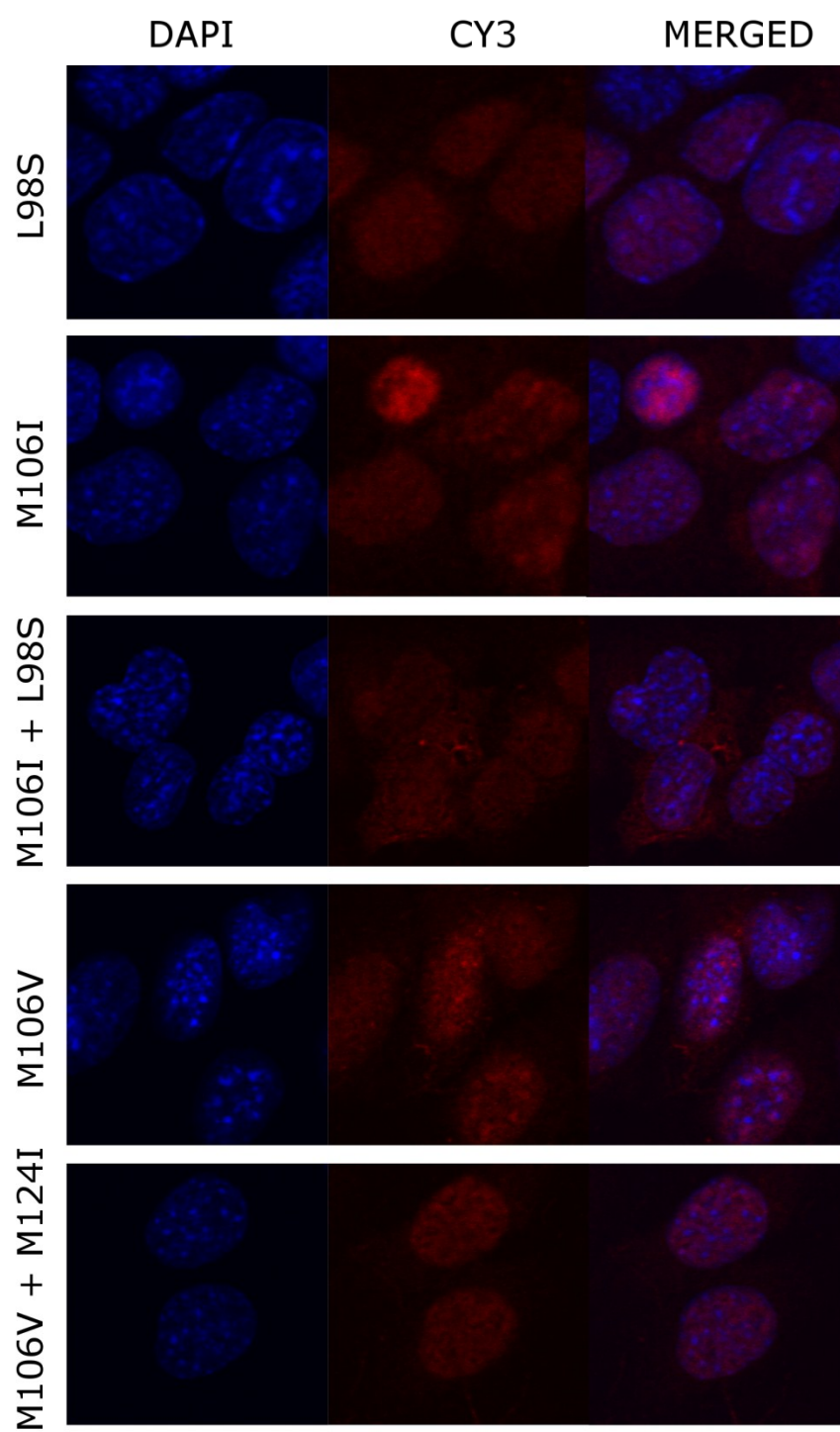
3.4.12.1 Confocal microscopy

To analyze NS1 localization in mouse M1 cells, cells were infected with rHK NS mutant viruses (MOI=3) then fixed and stained at 16 hpi using anti-NS1 polyclonal antibody, a Cy3- conjugated secondary antibody, and DAPI to detect the nucleus (see methods). Images were taken (n=5) using a 63X oil immersion objective lens (Zeiss LSM 510 META/AxioVert 200 Confocal Microscope).

3.4.12.1.1 Select NS1 mutants increase cytoplasmic staining at 16 hpi

NS1 expressed from the rHK-wt virus predominantly co-localized with the nucleus in M1 cells (Figure 23a). The majority of the NS1 mutants exhibited a similar phenotype, however mutants M106I + L98S, D125G, and D125G + M124I, and to a lesser extent F103L + M106I, M106I, V23A, and V226I, increased NS1 staining in the cytoplasm (Figure 23). To quantify the cellular distribution of NS1, confocal images were assessed for the percentage of NS1 positive cells with: 1) NS1 exclusively in the nucleus, 2) NS1 in both the nucleus and cytoplasm, and 3) NS1 exclusively in the cytoplasm. Only NS1 mutants V23A, M106I + L98S and D125G + M124I induced a percentage of cells with NS1 found exclusively in the cytoplasm (condition 3) (Figure 24). However, as this was not a prevalent phenotype, I then separated the data into two categories: cells with NS1 exclusively in the nucleus (condition 1), and cells with NS1 in the cytoplasm (conditions 2 and 3 combined) (Figure 25). Compared to rHK-wt, where only 6% of positive cells stained for cytoplasmic NS1, NS1 staining in the cytoplasm was much more frequently observed for NS1 mutants M106I (23% ($p<0.05$), M106I + L98S (53%, $p<0.001$), D125G (64%, $p<0.001$), D125G + M124I (94%, $p<0.0001$), and V226I (19%, $p<0.05$) (Figure 25).





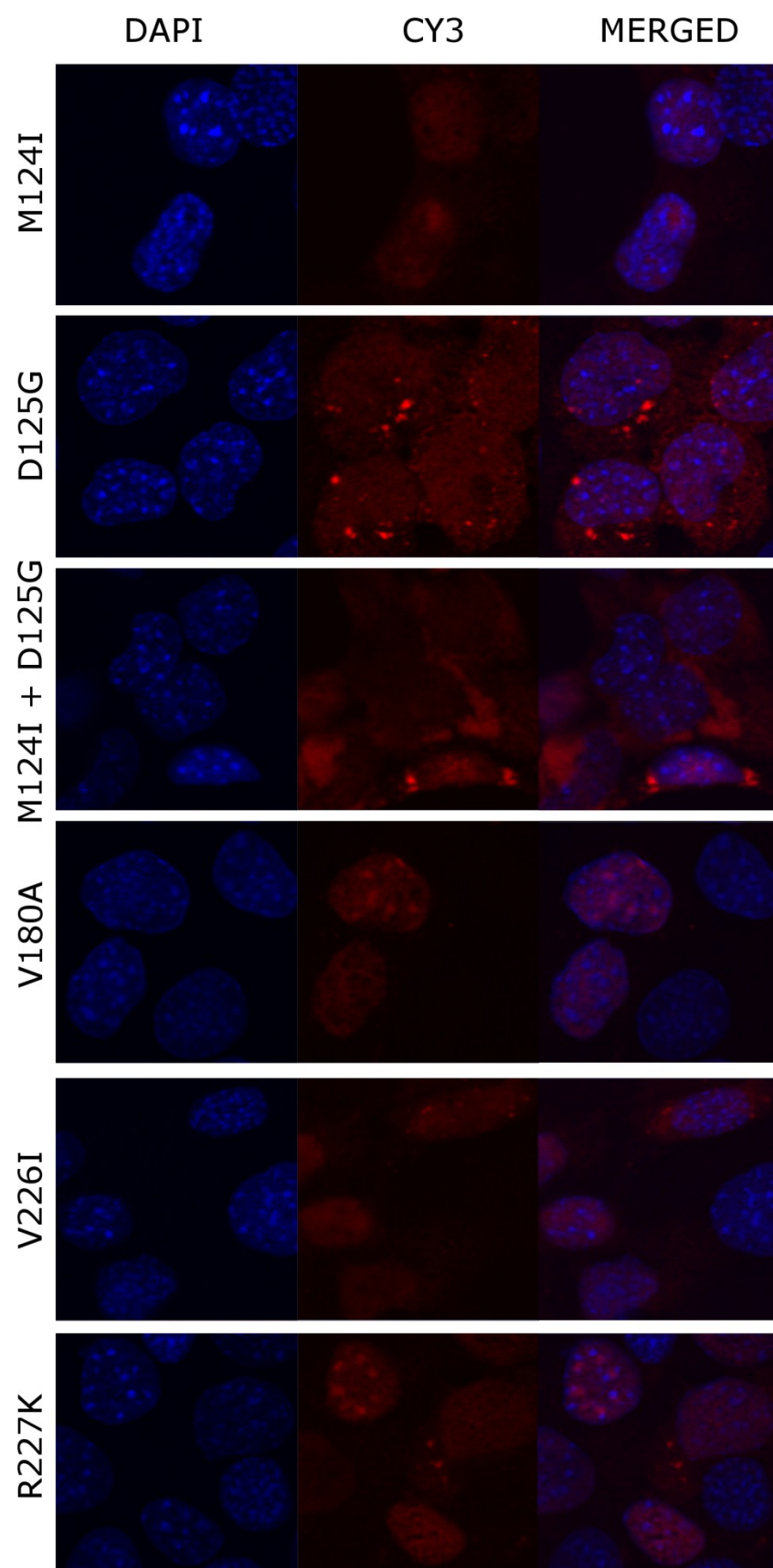


Figure 23. MA NS1 mutants alter subcellular localization of the NS1 protein in infected mouse cells in vitro. Mouse M1 cells were infected at MOI=3 with rHK NS mutant or rHK-wt viruses. Following 16 hpi, cells were fixed and stained using a polyclonal anti-NS1 antibody and Cy3-conjugated secondary as well as DAPI to localize the nucleus. Representative images are shown, taken at 63X using oil immersion.

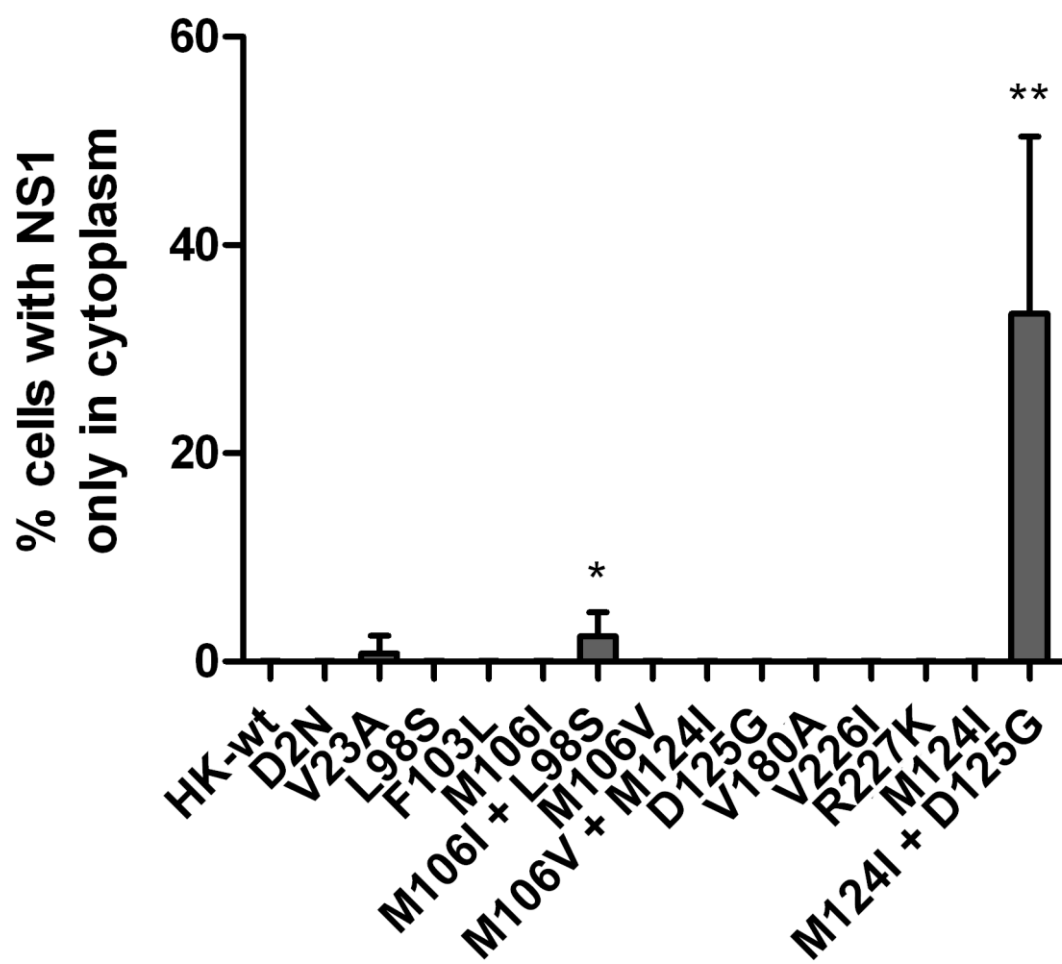


Figure 24. MA NS1 mutants increase frequency of infected mouse cells positive for NS1 in the cytoplasm. Confocal microscopy was performed (Figure 23), and five random images from each subset of infected cells were quantified for the percentage of cells positive for cytoplasmic NS1 (but negative for nuclear NS1) out of total positive cells per image. Data represent the means $\pm$ SD (* $p < 0.05$, ** $p < 0.01$; two-tailed student's t-test).

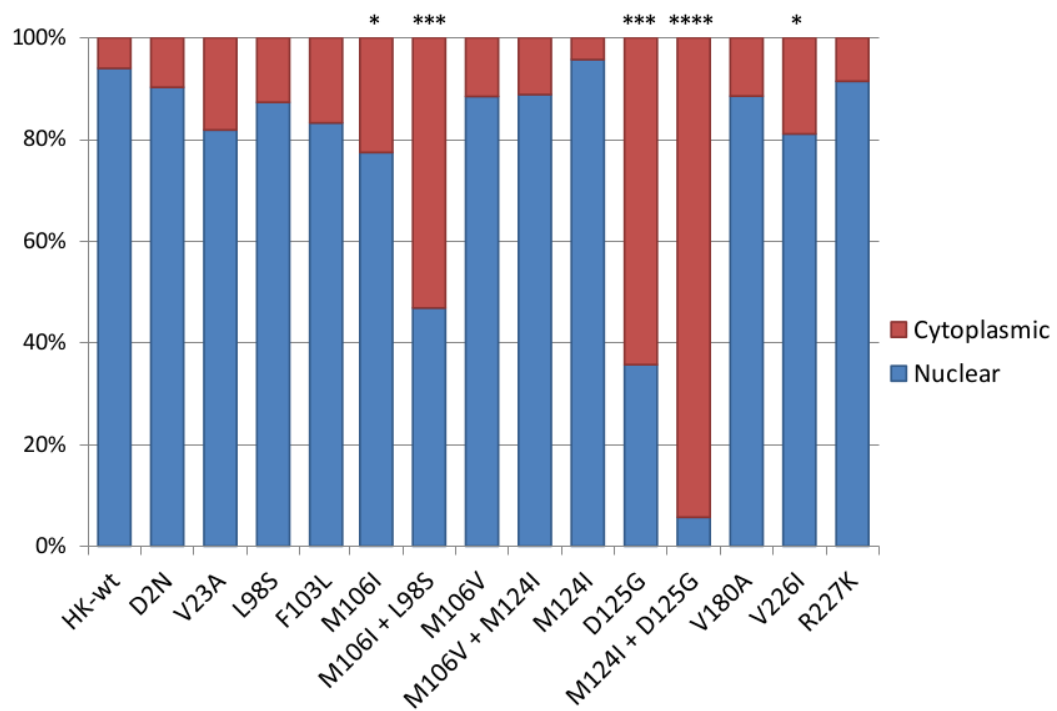
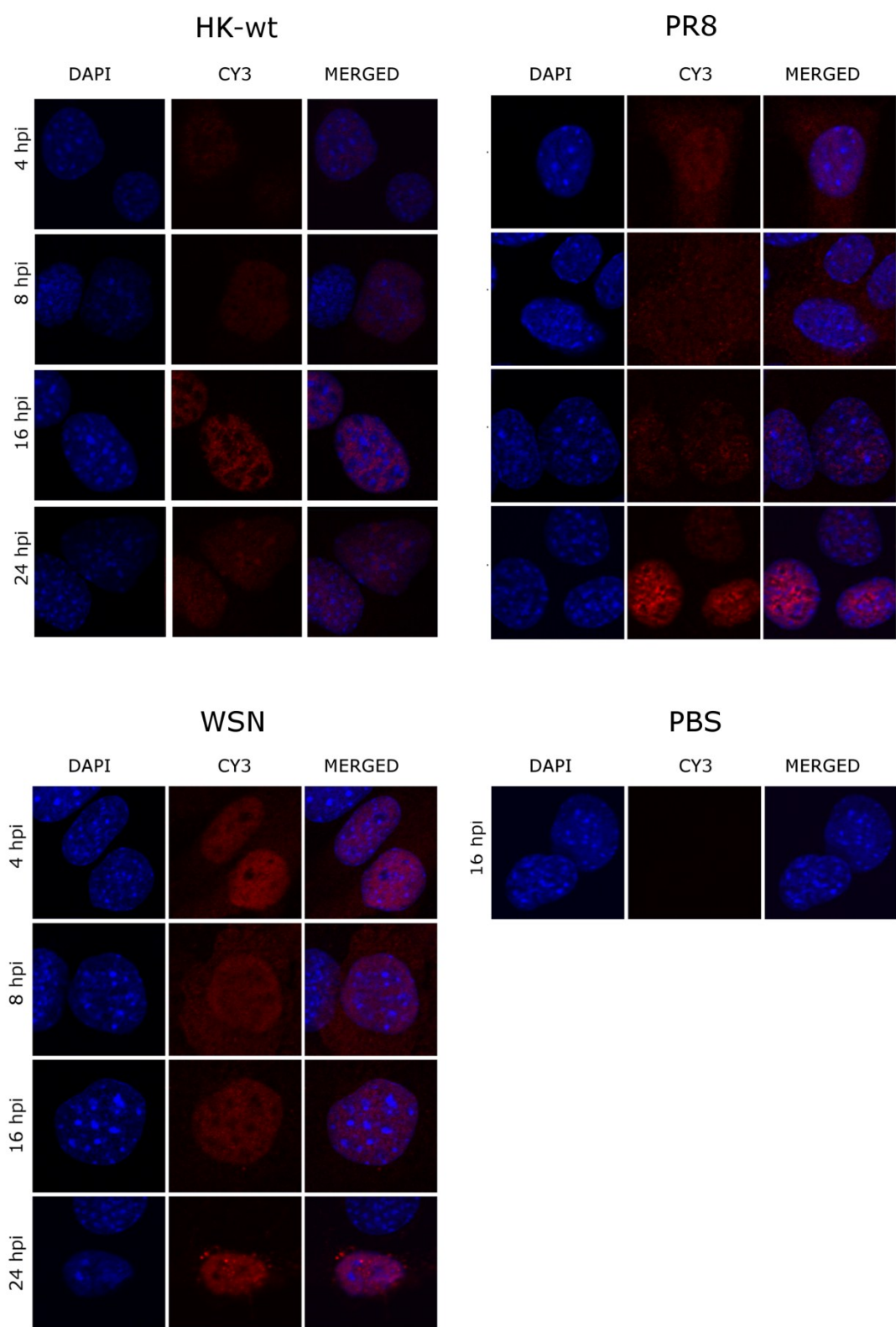


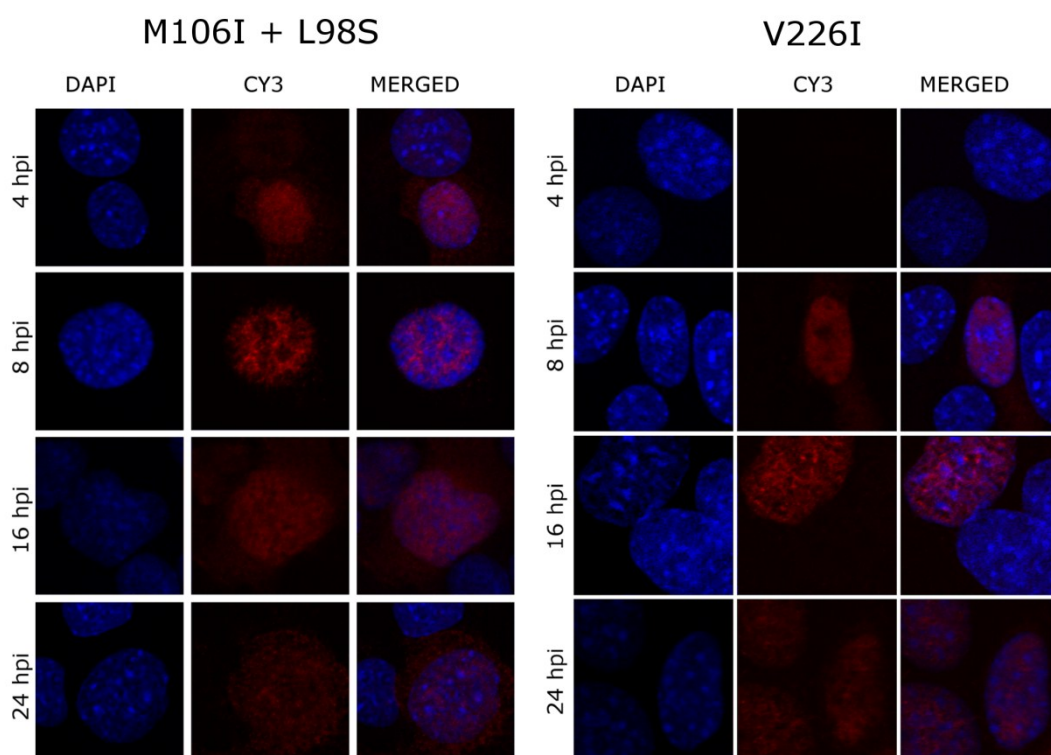
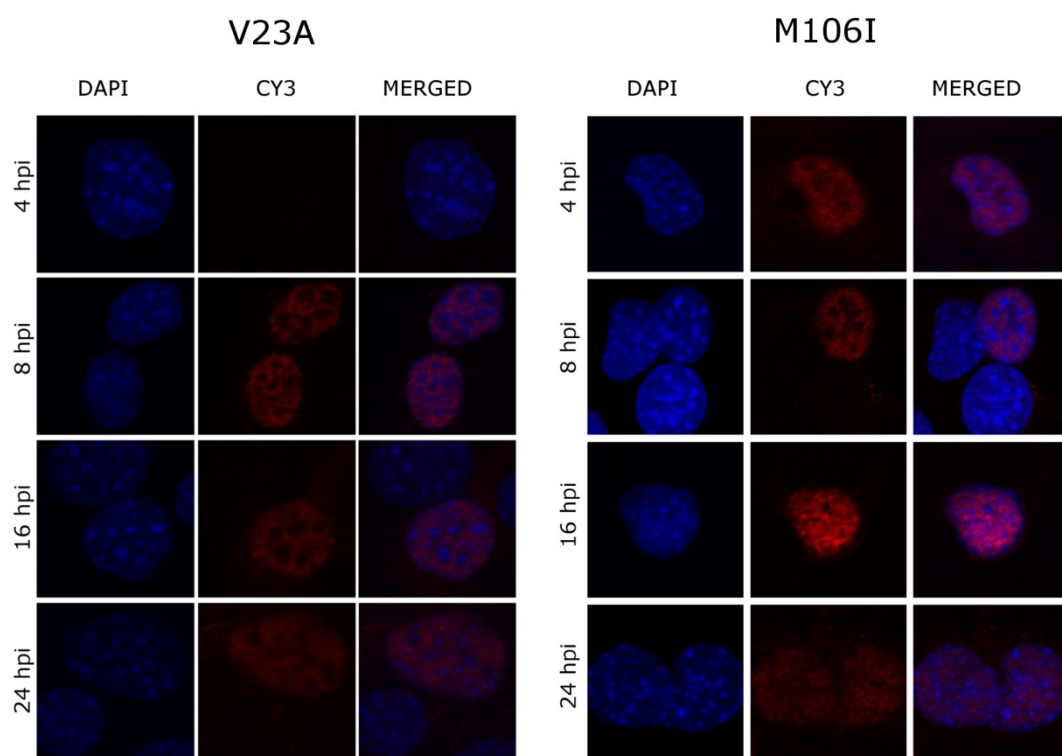
Figure 25. MA NS1 mutations alter the subcellular localization of NS1 in infected mouse cells. Data represent the average percentage of cells with specified NS1 subcellular localization phenotype (red region designates cytoplasmic staining, blue region designates nuclear staining) (* $p < 0.05$, *** $p < 0.001$, **** $p < 0.0001$; two tailed t-test).

3.4.12.1.2 NS1 mutants with cytoplasmic NS1 demonstrate increased staining over time

Given that several NS1 mutants induced a pronounced difference in NS1 localization in infected mouse cells compared to the human HK-wt virus, I next repeated the experiment for a subset of NS1 mutants showing phenotypic divergence from rHK-wt in respect to NS1 subcellular localization. I also included two prototype viruses known to be fully adapted to the mouse, A/WSN/1933 (H1N1) (WSN) and A/PR/8/1934 (H1N1) (PR8) and extended the experimental duration, fixing cells at 4, 8, 16 and 24 hpi. At every time point, NS1 in cells infected with rHK-wt virus demonstrated a predominantly nuclear localization (Figure 26), where an average of only 12.4% of cells was positive for cytoplasmic NS1 (Figure 27a). However, both PR8 and WSN had a dramatically different NS1 localization phenotype (Figure 26). Both PR8 and WSN had a significantly greater percentage of cells with cytoplasmic NS1 at 4h than rHK-wt (33% and 28% versus 9.3%, respectively ($p < 0.01$)) and again at 8h (31.7% ($p < 0.05$) and 84.3% ($p < 0.0001$) versus 8.4%, respectively) However the 16h phenotype was statistically indistinguishable from rHK-wt, and at 24h only WSN showed a statistical significant increase in cytoplasmic NS1 positive cell compared to rHK-wt (29% versus 15%, $p < 0.05$) (Figure 27a).

NS1 mutants M106I + L98S, D125G and D125G + M124I, like the prototype mouse-adapted PR8 and WSN viruses, also demonstrated an increase in cells positive for NS1 in the cytoplasm, although the kinetics of NS1 localization was quite different. Unlike PR8 and WSN, the rHK NS1 mutants showed a temporally progressive increase in cytoplasmic positive NS1 cells, with the greatest percentage at 24 hpi (Figure 27b). Representative cells images for each time point are shown (Figure 26). NS1 mutants V23A, V226I, and M106I





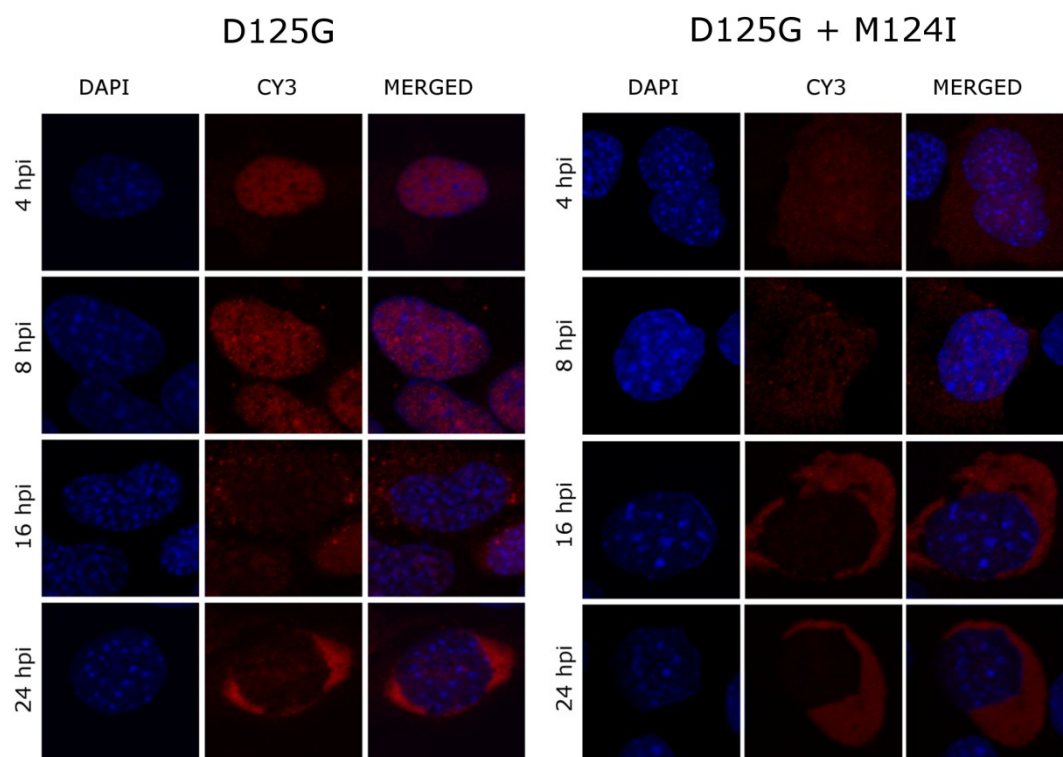
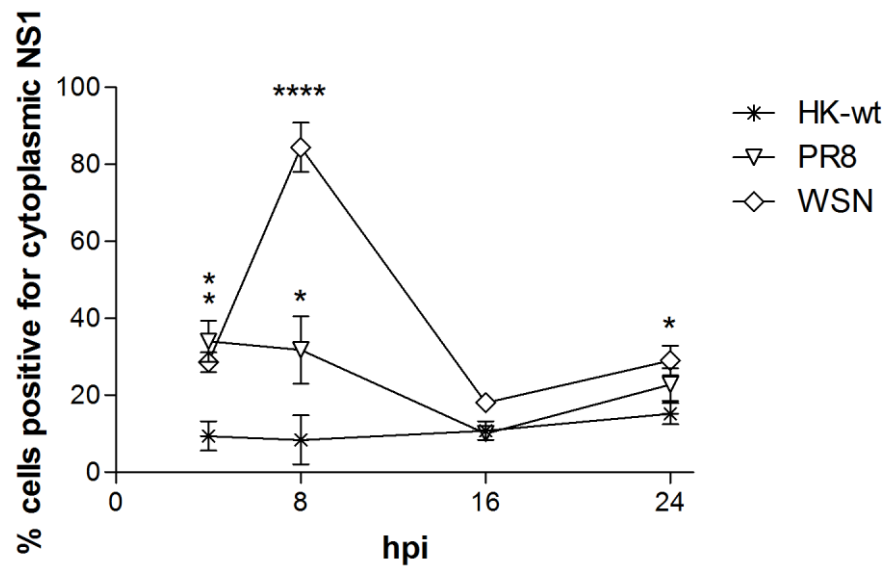


Figure 26. Several MA NS1 mutants increase frequency of subcellular localization of the NS1 protein to the cytoplasm over time in infected mouse cells in vitro. Mouse M1 cells were infected at MOI=3 with rHK NS mutant or rHK-wt viruses. Following 4, 8, 16, and 24 hpi, cells were fixed and stained using a polyclonal anti-NS1 antibody and Cy3-conjugated secondary as well as DAPI to localize the nucleus. Representative images are shown, taken at 63X using oil immersion.

A



B

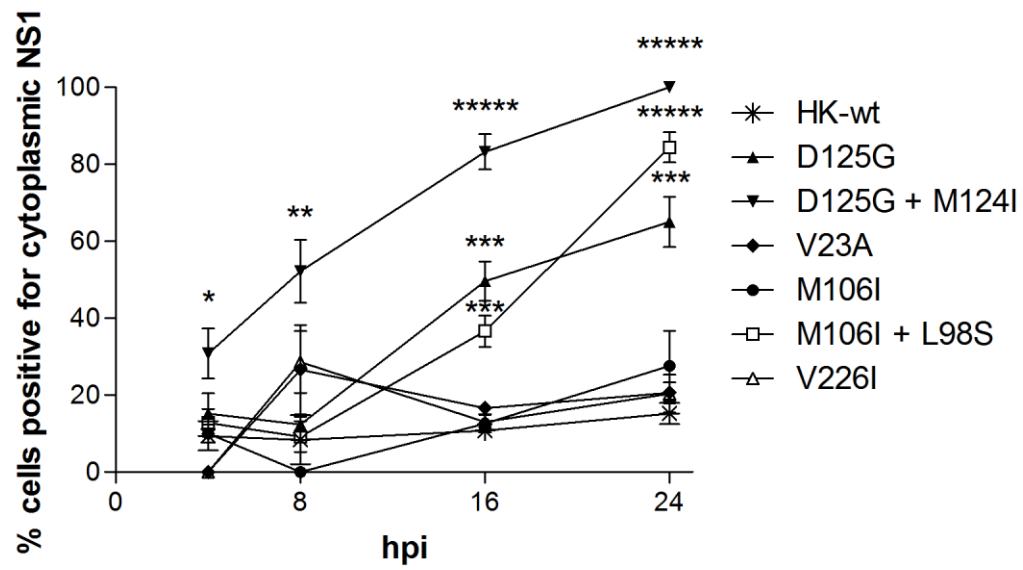


Figure 27. MA NS1 mutants as well as prototype MA IAV strains demonstrate increased prevalence of cytoplasmic NS1 in infected mouse cells in vitro. Confocal microscopy was performed (Figure 26), and five random images from each subset of infected cells were quantified for the percentage of cells positive for cytoplasmic NS1 of total positive cells per image. Data represent the means $\pm$ SD (* $p < 0.05$, ** $p < 0.01$; *** $p < 0.001$, **** $p < 0.0001$, ***** $p < 0.00001$; two-tailed student's t-test).

increased the number of cells positive for cytoplasmic NS1, both at 16 and 24 hours pi, although compared to rHK-wt the differences were not statistically significant (Figure 27b). The most dramatic phenotypes were for NS1 mutants M106I + L98S, D125G, and D125G + M124I, as seen in the previous localization data (Figure 25), where at 24 hpi 85%, 65% or 100% of positive cells stained for cytoplasmic NS1 ($p < 0.00001$, $p < 0.0001$, $p < 0.00001$, respectively). However the polyclonal antibody used to stain for NS1 also reacts with the NS3 protein (Western blot, data not shown). Given that the NS1 mutation D125G induces expression of the NS3 protein through alternative splicing, the signal associated with Cy3 must be considered as the summation of total NS1 and NS3 proteins for NS1 mutants D125G and D125G + M124I. Therefore this experiment does not allow deduction of the proportion of the cytoplasmic Cy3 signal resulting from the NS1 protein for NS1 mutants D125G and D125G + M124I.

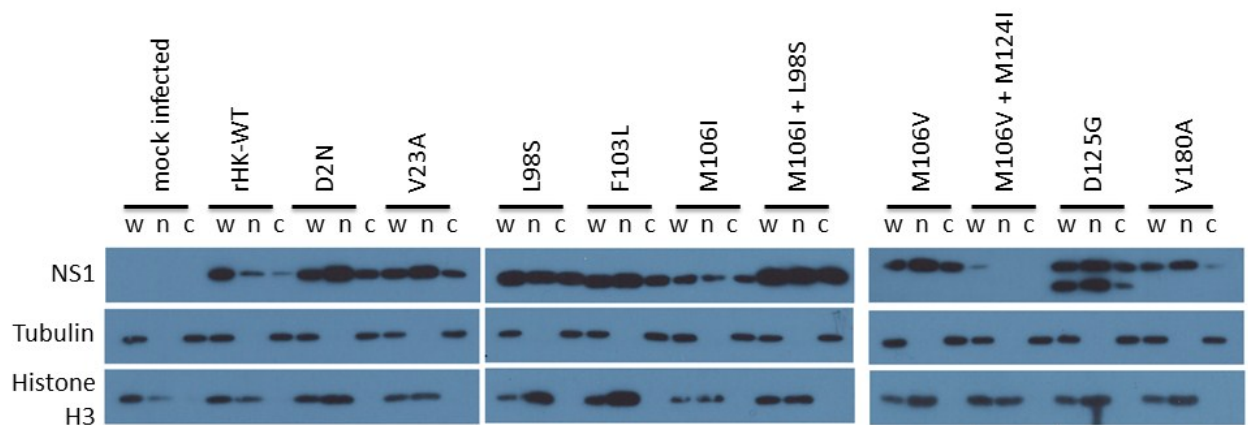
3.4.12.2 Western blot of cytoplasmic and nuclear cell fractions

To quantify the distribution of the NS1 protein in the nuclei and cytoplasm in mouse cells, cytoplasmic and nuclear fractions were assessed for M1 cells infected with rHK NS mutant viruses (MOI=2), rHK-wt, or the prototype mouse-adapted strains PR8 and WSN. Given the double mutant D125G + M124I demonstrated a pronounced cytoplasmic phenotype for NS1 subcellular distribution in M1 cells assessed by confocal microscopy, the double mutant as well as the single mutant M124I were also included. Following 16 hpi, the cells were washed, lysed with 1% NP-40, and subjected to centrifugation to obtain nuclear and cytosolic cellular fractions (n=2-3) as per published protocol (172). In addition, an aliquot was taken prior to fractionation to represent the whole cell lysate. Each fraction was

re-suspended in SDS buffer, separated by SDS PAGE and transferred to PVDF membranes. Western blot analysis was performed to detect NS1, histone H3 (nuclear marker) and tubulin (cytoplasmic marker). Figure 28 shows representative blots of cells infected with each virus (whole cell lysate, nuclear fraction, and cytosolic fraction). Densitometry was performed to quantify relative NS1 abundance in each cellular fraction, which was normalized to the abundance of the tubulin or histone H3 internal standards, respectively.

3.4.12.2.1 NS1 mutants increased the proportion of NS1 localized to the cytoplasm in infected M1 cells

To determine the relative proportion of NS1 localized to the cytoplasm, band densities of cytoplasmic NS1 were divided by the total NS1 protein that comprised the sum of the cytoplasmic and nuclear NS1 band densities. Since the histone H3 band was reduced between the whole cell and nuclear fractions (possibly due to losses from the fractionation procedure), the amount of NS1 protein in the nuclear and cytoplasmic fractions of a cell infected with a given virus was normalized to histone H3 and tubulin levels of the whole cell lysate, respectively. Upon quantification of the percentage of NS1 localized to the cytoplasm, I found that all NS1 mutants with the exception of D2N, V23A, V180A, M124I, and D125G + M124I demonstrated increased NS1 cytoplasmic distribution relative to rHK-wt NS1 (Figure 29). Notably, both WSN and PR8 also increased cytoplasmic NS1 distribution compared to the human rHK-wt virus. These data indicated that an increased proportion of NS1 localized to the cytoplasm is a phenotype common to IAV upon adaption to a novel host.



M106V + M124I
NS1 (longer exposure)

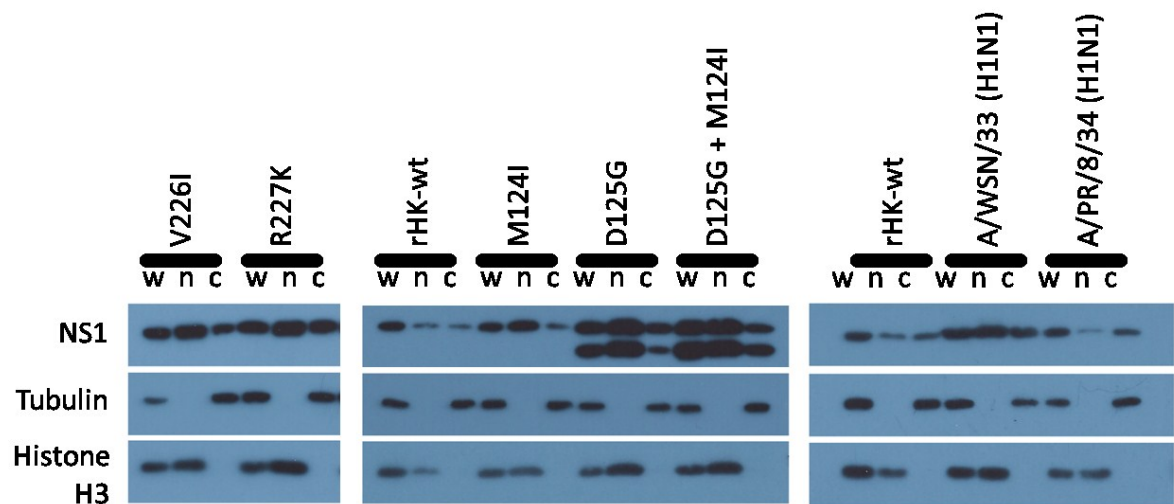


Figure 28. MA NS1 mutations alter subcellular localization of NS1 in infected M1 cells.

Mouse M1 cells were infected (MOI=2) with rHK NS mutants or indicated virus, and at 16 hpi the cells were lysed and differentially centrifuged to obtain whole cell (w), nuclear (n) and cytosolic (c) fractions. Cell fractions were resuspended in SDS buffer then were separated by SDS PAGE electrophoresis, followed by Western blot analysis to detect the NS1 protein as well as loading markers tubulin and histone H3. Above is a representative blot of 2-3 independent experiments.

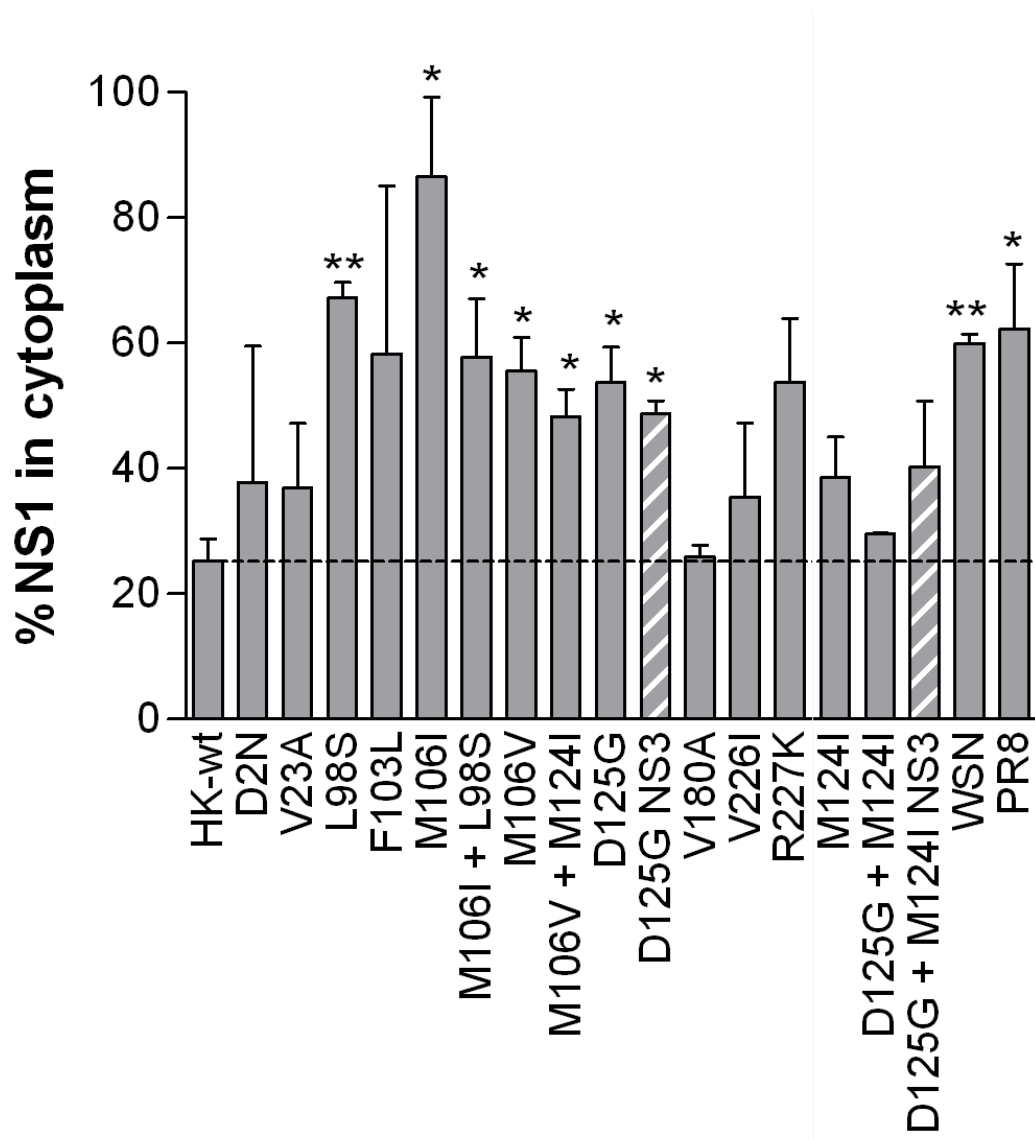


Figure 29. MA NS1 mutants increase percentage of NS1 protein localized to the cytoplasm in infected mouse cells. Western blot data presented in Figure 28 was analysed by densitometry, where the amount of NS1 or NS3 protein in the nuclear and cytoplasmic fractions of a cell infected with a given virus was normalized to histone H3 and tubulin levels of the whole cell lysate, respectively. The percent of NS1 in the cytoplasm was calculated by dividing cytoplasmic NS1 by total NS1 expressed in the cell (nuclear + cytoplasmic NS1). Data represent the means $\pm$ SD (* p <0.05, ** p <0.01; two-tailed student's t-test).

3.3.12.2.2 The majority of NS1 mutants demonstrate increased NS1 accumulation in the whole cell lysate of infected M1 cells

The abundance of NS1 protein in infected M1 cells was determined by SDS gel electrophoresis and Western blot analysis on whole cell lysate samples (Figure 30). Compared to rHK-wt, M1 cells infected with the majority of NS1 mutants demonstrated significantly increased NS1 abundance, where the greatest increase was found for NS1 double mutants D125G + M124I and M106I + L98S (2.0 fold and 1.6 fold increase, respectively) (Figure 30). NS1 mutant M106V + M124I however induced ~50% less NS1 accumulation in M1 cells compared to rHK-wt ($p < 0.01$). For the prototype mouse-adapted viruses, WSN but not PR8 was found to increase NS1 protein abundance in infected M1 cells compared to rHK-wt ($p < 0.01$). These data show that the majority of NS1 mutants selected upon mouse adaptation increase NS1 protein accumulation in mouse cells on in vitro infection.

3.4.12.2.3 The majority of NS1 mutants increase nuclear NS1 in M1 cells

To determine whether the abundance of NS1 in the nucleus of infected M1 cells was altered for NS1 mutants, nuclear fractions of infected M1 cells were subject to SDS PAGE and Western blot analysis ($n=2-3$) (Figure 31). All NS1 mutants but M106V + M124I induced significantly more nuclear NS1 than rHK-wt (4.5 to 17- fold increase; Figure 31). For the mouse-adapted virus strains, only WSN increased nuclear NS1 compared to rHK-wt ($p < 0.05$). These data show that increased nuclear NS1 in infected mouse M1 cells is a

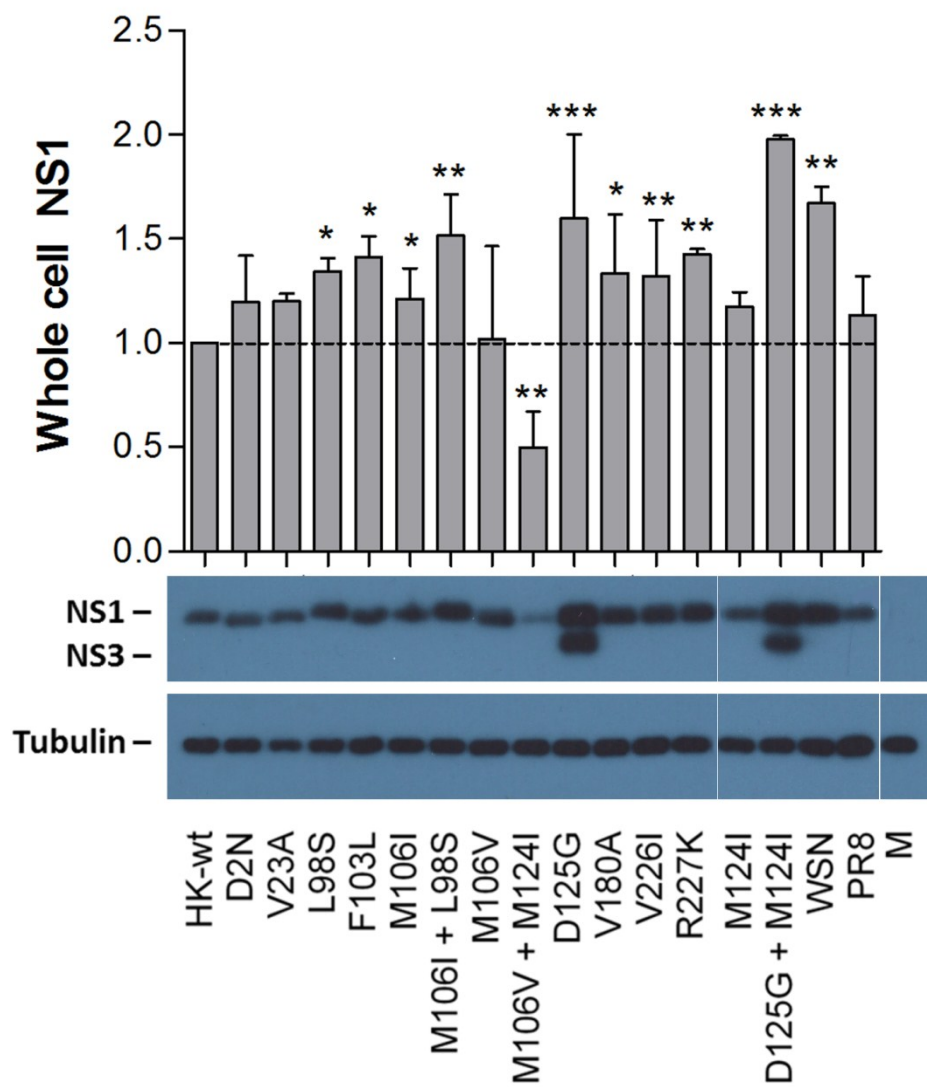


Figure 30. The majority of MA NS1 mutants increase NS1 abundance in the whole cell lysate of infected mouse cells. M1 cells were infected with rHK NS mutants, the specified virus, or mock-infected with PBS. At 16 hpi a sample of the whole cell lysate was obtained, resuspended in SDS buffer and separated by SDS PAGE electrophoresis, followed by Western blot analysis to detect the NS1 protein as well as tubulin to serve as a loading control. Lower panel: a representative blot of 2-3 independent experiments. Upper panel: Densitometry analysis of whole cell NS1 normalized to tubulin value then rHK-wt NS1. Data represent the means $\pm$ SD (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; two-tailed student's t-test).

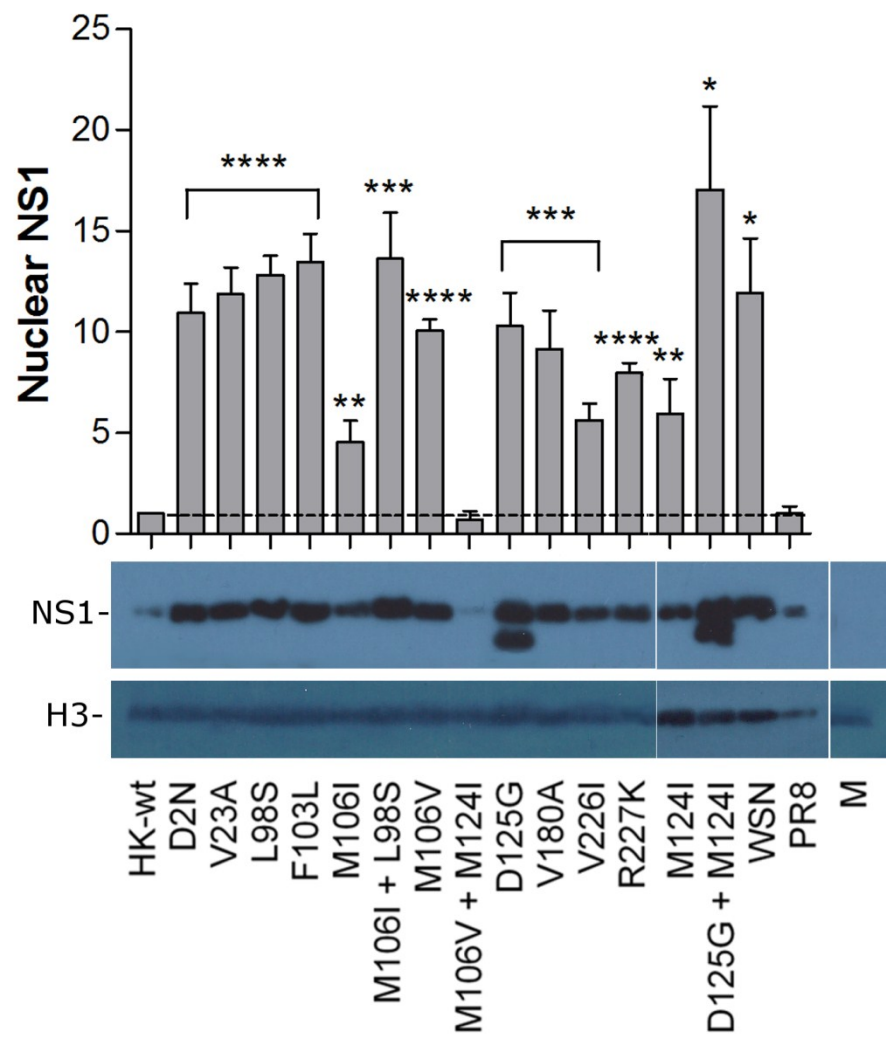


Figure 31. The majority of MA NS1 mutants increase the abundance of nuclear NS1 in infected mouse cells. M1 cells were infected with rHK NS mutants, the specified virus, or mock-infected with PBS. At 16 hpi cells were differentially centrifuged to obtain the nuclear fraction, which was then resuspended in SDS buffer and separated by SDS PAGE electrophoresis, followed by Western blot analysis to detect the NS1 protein as well as histone H3 to serve as a loading control. Lower panel: a representative blot of 2-3 independent experiments. Upper panel: Densitometry analysis of nuclear NS1 normalized to histone H3 value then to rHK-wt NS1. Data represent the means $\pm$ SD (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$; two-tailed student's t-test).

phenotype shared by the majority of MA NS1 mutants, however, like NS1 expression in the whole cell lysate, this phenotype is not shared by all mouse-adapted strains.

3.4.12.2.4 The majority of NS1 mutants increase cytoplasmic NS1 in M1 cells

Cytoplasmic fractions of infected M1 cells were also assessed for NS1 abundance as per the previous protocol. All NS1 mutants induced significantly more cytoplasmic NS1 than HK-wt (1.5 to 8.2 fold), with the exception of M106V + M124I and M124I expressed as a single mutation (Figure 32). NS1 mutant L98S demonstrated the greatest enhancement of cytoplasmic NS1 (8.2 fold increase), followed by the double mutant M106I + L98S (7.8 fold increase). In addition, both PR8 and WSN viruses demonstrated increased cytoplasmic NS1 levels (5.6 and 4.2 fold increase, respectively; $p < 0.05$). These results indicate that NS1 mutants selected upon mouse adaptation increase both cytoplasmic and nuclear NS1 with the exception of NS1 mutants M106V + M124I (decreased in both fractions, consistent with previous pulse labeled protein production data) and M124I (increase in nuclear but not cytoplasmic NS1). Only select NS1 mutants were capable of inducing a greater proportion of NS1 to localize in the cytoplasm compared to rHK-wt (Figure 29); these mutants may be limiting the ability of the NS1 protein to localize to the nucleus, or may be enhancing the nuclear export of NS1 to the cytoplasm.

3.4.13 NS1 mutants alter cellular localization of the NS1 protein in infected A549 cells

3.4.13.1 Confocal microscopy

Several NS1 mutants selected upon mouse adaptation demonstrated increased prevalence of cytoplasmic NS1 in mouse cells. To assess whether this phenotype was

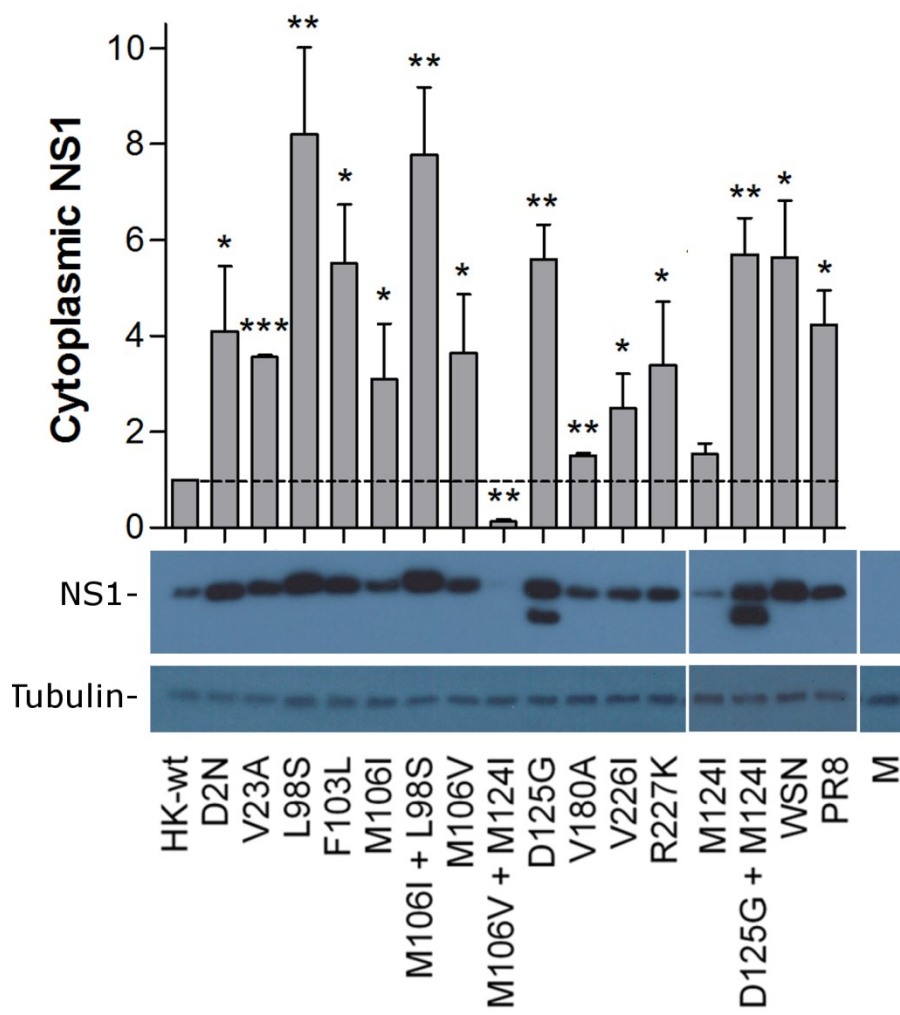


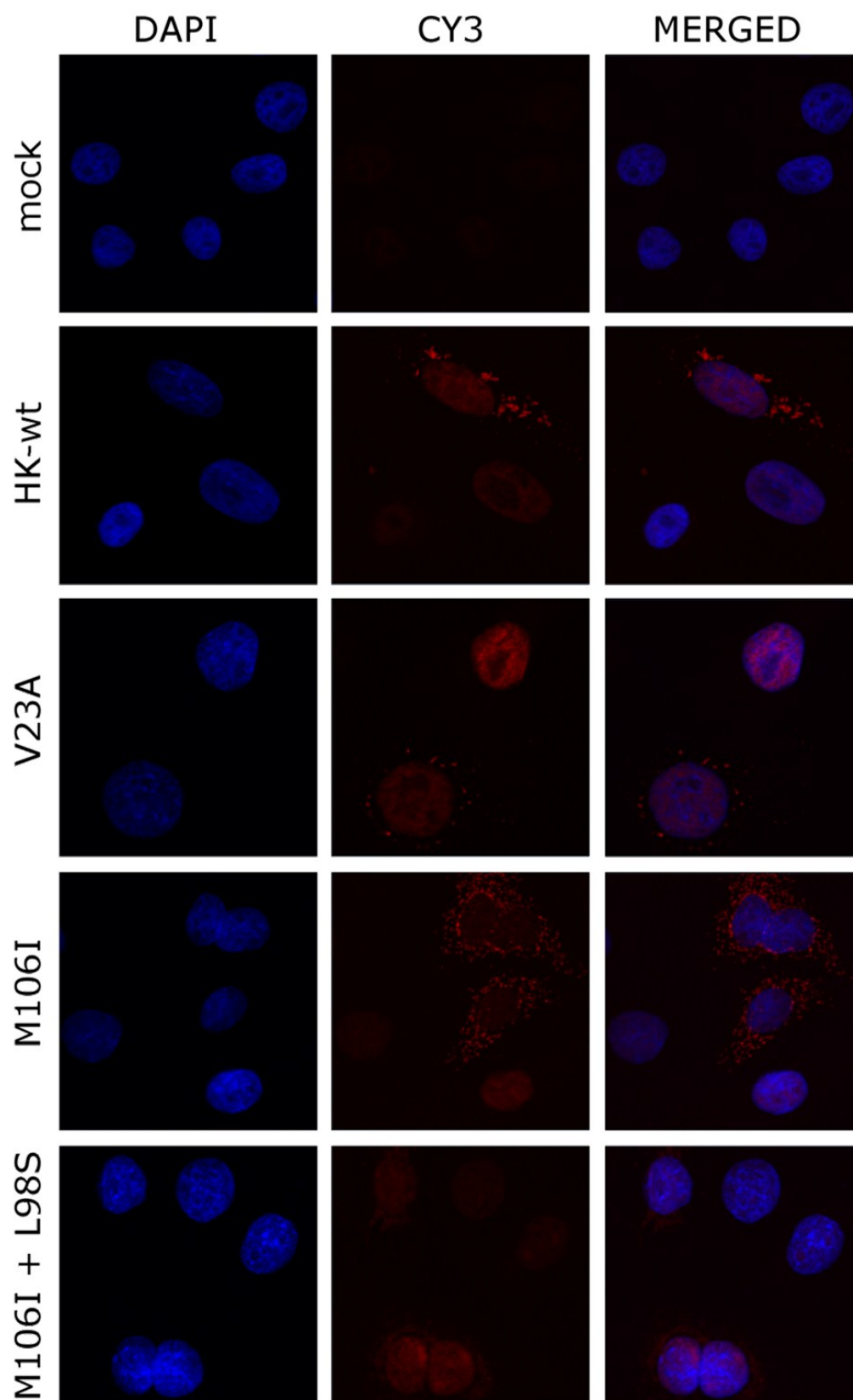
Figure 32. The majority of MA NS1 mutants increase the abundance of cytoplasmic NS1 in infected mouse cells. M1 cells were infected with rHK NS mutants, the specified virus, or mock-infected with PBS. At 16 hpi cells were differentially centrifuged to obtain the cytosolic fraction, which was then re-suspended in SDS buffer and separated by SDS PAGE electrophoresis, followed by Western blot analysis to detect the NS1 protein as well as tubulin to serve as a loading control. Lower panel: a representative blot of 2-3 independent experiments. Upper panel: Densitometry analysis of nuclear NS1 normalized to tubulin value then to rHK-wt NS1. Data represent the means $\pm$ SD (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; two-tailed student's t-test).

specific to the mouse, I repeated the experiment in human A549 cells for rHK-wt, the prototype mouse-adapted strains WSN and PR8, as well as those NS1 mutants that increased the proportion of cells with NS1 staining in the cytoplasm of infected M1 cells.

3.4.13.1.1 All assayed NS1 mutants but D125G + M124I display NS1 subcellular localization similar to rHK-wt in infected A549 cells

NS1 localization in infected A549 cells was assessed at 16 hpi as per the previous protocol (Figures 33a-b). Compared to localization in M1 cells, rHK-wt NS1 showed increased cytoplasmic localization (37% of A549 cells versus 6% of M1 cells) (Figure 34, Figure 25). In contrast, the PR8 virus demonstrated decreased cytoplasmic localization (5% of A549 cells versus 10% of M1 cells), while the WSN virus, similar to rHK-wt, demonstrated increased cytoplasmic localization (48% of A549 cells versus 30% of M1 cells). All surveyed NS1 mutants with the exception of D125G + M124I demonstrated a similar or decreased ratio of cells positive for cytoplasmic NS1 compared to rHK-wt (Figure 34).

Similar to observations in M1 infected cells, the double mutant D125G + M124I induced increased cytoplasmic localization of NS1, where 90% of infected cells were positive for NS1 in the cytoplasm ($p < 0.00001$) (Figure 34). These results indicate that NS1 mutations selected upon adaptation to the mouse increase NS1 cytoplasmic localization by a host-specific mechanism, where the phenotype is evident in mouse but not human cell infection.



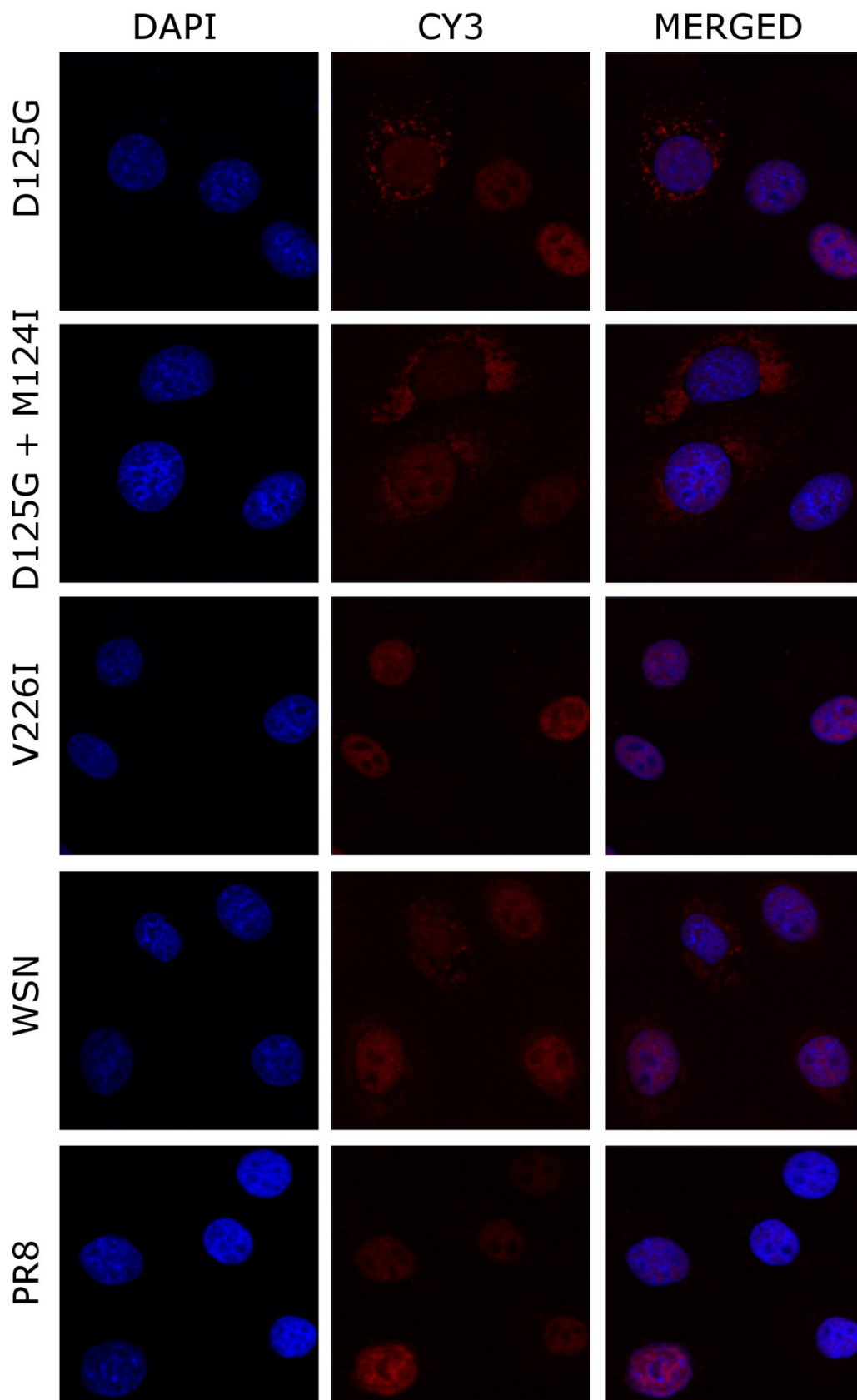


Figure 33. Subcellular localization of MA NS1 proteins in infected human cells. Human A549 cells were infected at MOI=3 with a subset of rHK NS mutant viruses or the rHK-wt virus. Following 16 hpi, cells were fixed and stained using a polyclonal anti-NS1 antibody and Cy3-conjugated secondary as well as DAPI to localize the nucleus. Representative images are shown, taken at 63X using oil immersion.

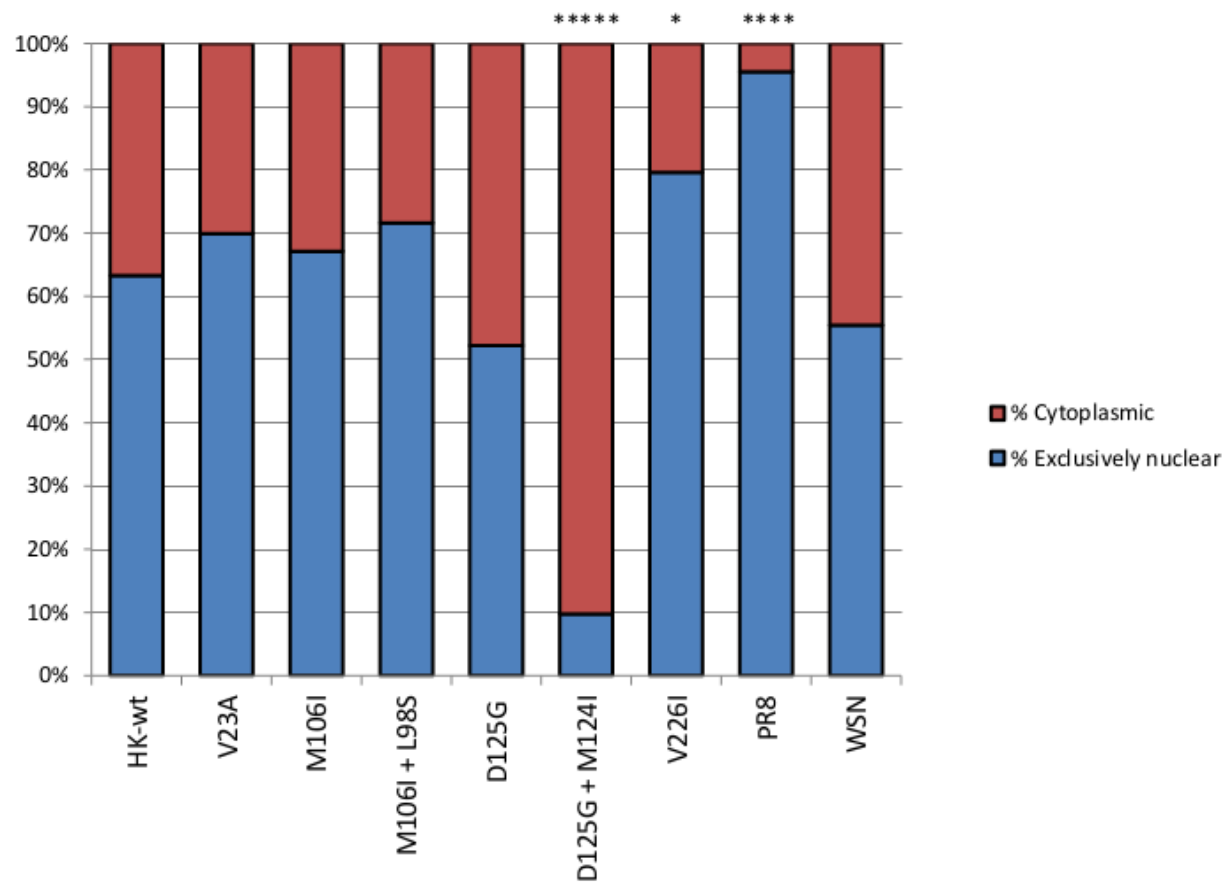


Figure 34. Quantification of subcellular MA NS1 localization in infected human cells. Data represent the average percentage of cells with specified NS1 subcellular localization phenotype (* $p < 0.05$, **** $p < 0.0001$, ***** $p < 0.00001$; two tailed t-test).

3.4.13.1.2 Increased cytoplasmic staining for NS1 mutant D125G + M124I is attributed to the NS1 protein

To determine whether the NS1 protein contributed to the phenotype of D125G + M124I subcellular localization in A549 cells, or whether perhaps NS3 was the predominant source of the cytoplasmic signal, I repeated the experiment using a monoclonal NS1 antibody which does not recognize NS3 (validated by Western blot; data not shown). Similar to the polyclonal antibody capable of recognizing both NS1 and NS3, the NS1 monoclonal antibody produced an abundant signal in the cytoplasm of A549 cells infected with D125G + M124I (Figure 35). This indicates that the NS1 protein of the NS1 D125G + M124I mutant was localizing primarily to the cytoplasm in infected A549 cells.

3.4.13.2 Western blot of cell fractions

To quantitatively assess NS1 subcellular distribution in infected A549 cells at 16 hpi (MOI=2), cellular fractions were obtained and analyzed as per the previous protocol (n=2-3). Figure 36 shows representative blots of A549 cells infected with each virus (whole cell lysate, nuclear fraction, and cytosolic fraction). Densitometry was performed to quantify relative NS1 abundance in each cellular fraction, which was normalized to tubulin or histone H3 abundance, respectively.

3.4.13.2.1 Several NS1 mutants affect NS1 distribution in A549 cells at 16h post infection

To determine whether NS1 mutations affected the cellular localization of the NS1 protein, band densities correlating with cytoplasmic NS1 were divided by the sum of cytoplasmic and nuclear NS1 band densities as per the previous protocol. In A549 cells, 48%

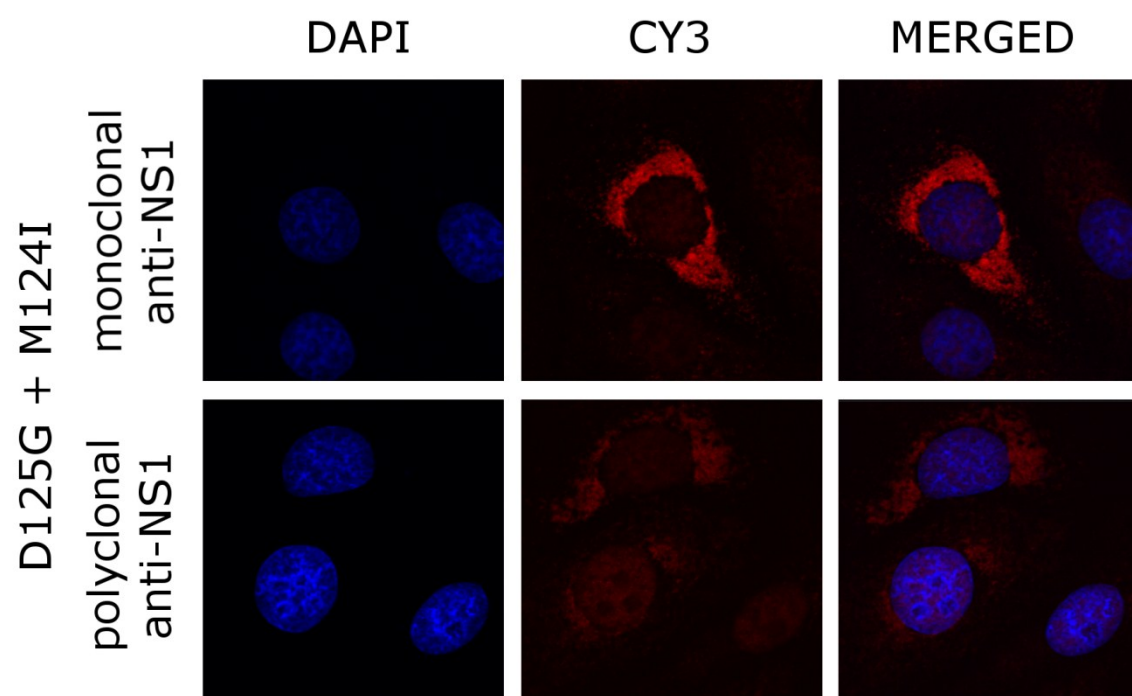


Figure 35. Subcellular localization of the NS1 mutant D125G + M124I is consistent for polyclonal and monoclonal NS1 antibodies. Human A549 cells were infected with the NS1 mutant D125G + M124I then fixed and stained in parallel with either the polyclonal anti-NS1 antibody that cross reacts with the NS3 protein or with the monoclonal anti-NS1 antibody specific to NS1. Cy3-conjugated secondary antibodies as well as DAPI were then applied to each condition. Representative images are shown, taken at 63X using oil immersion.

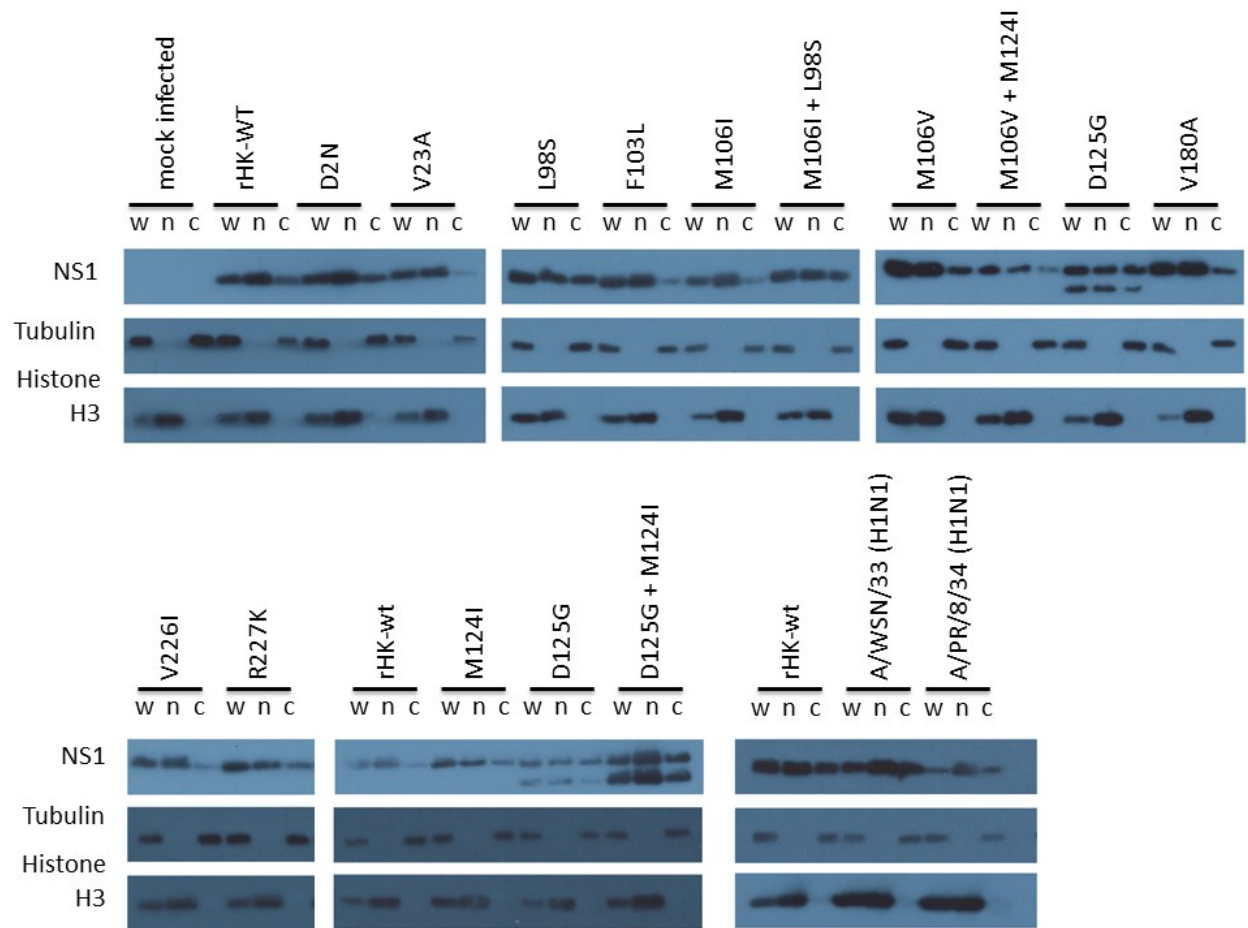


Figure 36. MA NS1 mutations alter subcellular localization of NS1 in infected human cells.

Human A549 cells were infected (MOI=2) with rHK NS mutants or indicated virus, and at 16 hpi the cells were lysed and differentially centrifuged to obtain whole cell (w), nuclear (n) and cytosolic (c) fractions. Cell fractions were resuspended in SDS buffer then were separated by SDS PAGE electrophoresis, followed by Western blot analysis to detect the NS1 protein as well as loading markers tubulin and histone H3. Above is a representative blot of 2-3 independent experiments.

of the rHK-wt NS1 protein was localized in the cytoplasm, and the majority of NS1 mutants as well as the PR8 and WSN mouse-adapted viruses demonstrated similar NS1 localization (Figure 37). NS1 mutants V23A, M106V, V226I, and M124I decreased the percentage of NS1 localized to the cytoplasm compared to rHK-wt levels ($p < 0.01$, $p < 0.05$, $p < 0.01$, $p < 0.05$, respectively). NS1 mutants M106I + L98S and D125G both trended toward increased cytoplasmic localization of NS1 (68% and 65%, respectively) however the difference relative to rHK-wt levels was not statistically significant. The NS3 protein expressed from NS1 mutants D125G as well as D125G + M124I localized to the cytoplasm to an extent similar to rHK-wt NS1 protein (Figure 37). These data show that the majority of NS1 mutants are distributed in A549 cells in a manner similar to rHK-wt NS1 localization, while several mutants were found to decrease the amount of NS1 localized to the cytoplasm. Therefore, increased cytoplasmic localization of NS1 is a host-specific phenotype associated with mouse-adapted NS1 mutants.

3.4.13.2.2 NS1 mutants do not increase NS1 accumulation in A549 whole cell lysate

The abundance of NS1 protein in infected A549 cells was determined by SDS gel electrophoresis and Western blot analysis on whole cell lysate samples (Figure 38). Compared to rHK-wt NS1 levels, the majority of NS1 mutants as well as the WSN and PR8 viruses, demonstrated similar levels of the NS1 protein. These data indicate that increased production of NS1 is a host specific phenotype for mouse-adapted NS1 mutants.

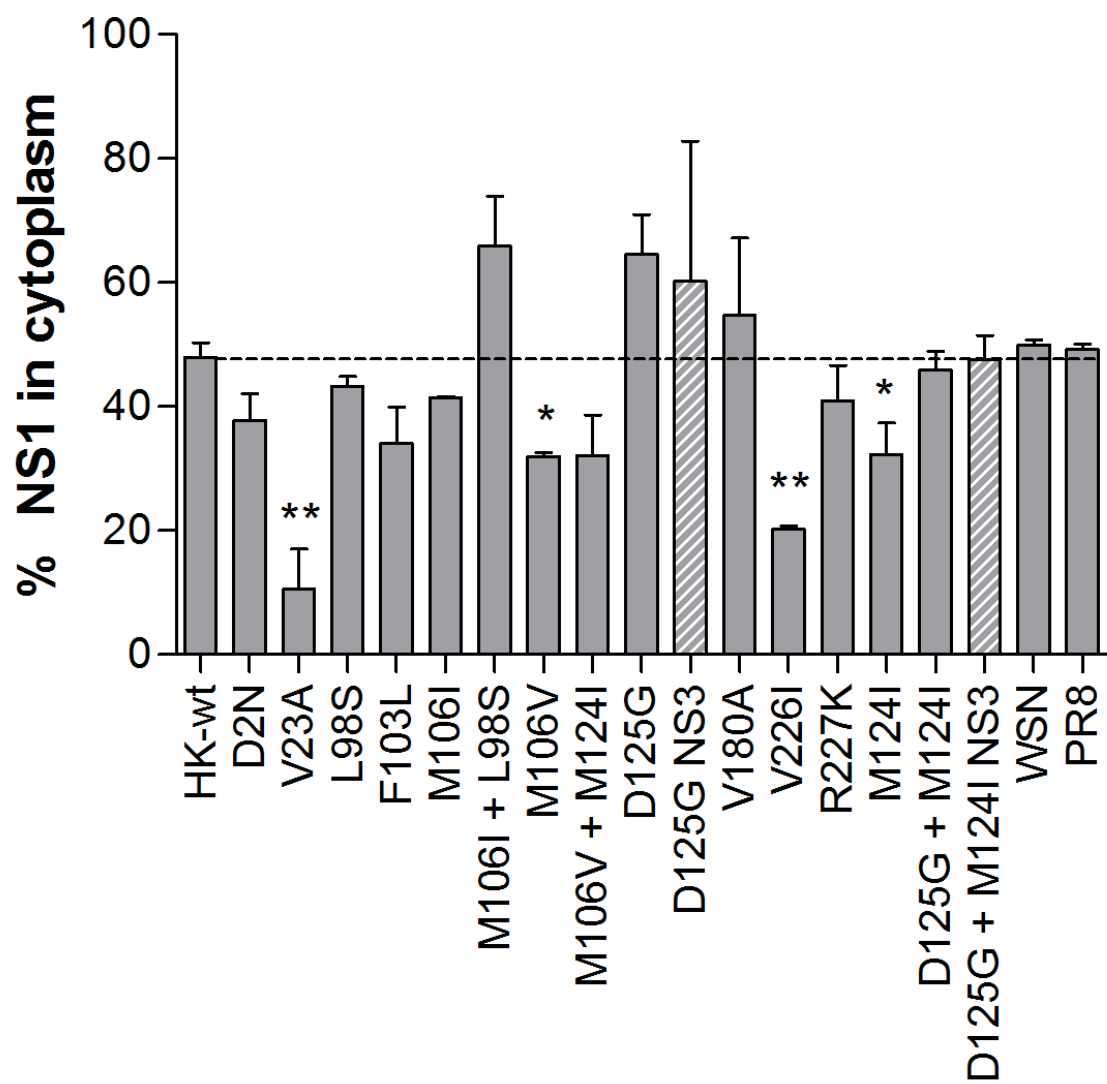


Figure 37. MA NS1 mutants alter the percentage of NS1 protein localized to the cytoplasm in infected human cells. Western blot data presented in Figure 36 was analysed by densitometry, where the amount of NS1 or NS3 protein in the nuclear and cytoplasmic fractions of a cell infected with a given virus was normalized to histone H3 and tubulin levels of the whole cell lysate, respectively. The percent of NS1 in the cytoplasm was calculated by dividing cytoplasmic NS1 by total NS1 expressed in the cell (nuclear + cytoplasmic NS1). Data represent the means $\pm$ SD (* $p < 0.05$, ** $p < 0.01$; two-tailed student's t-test).

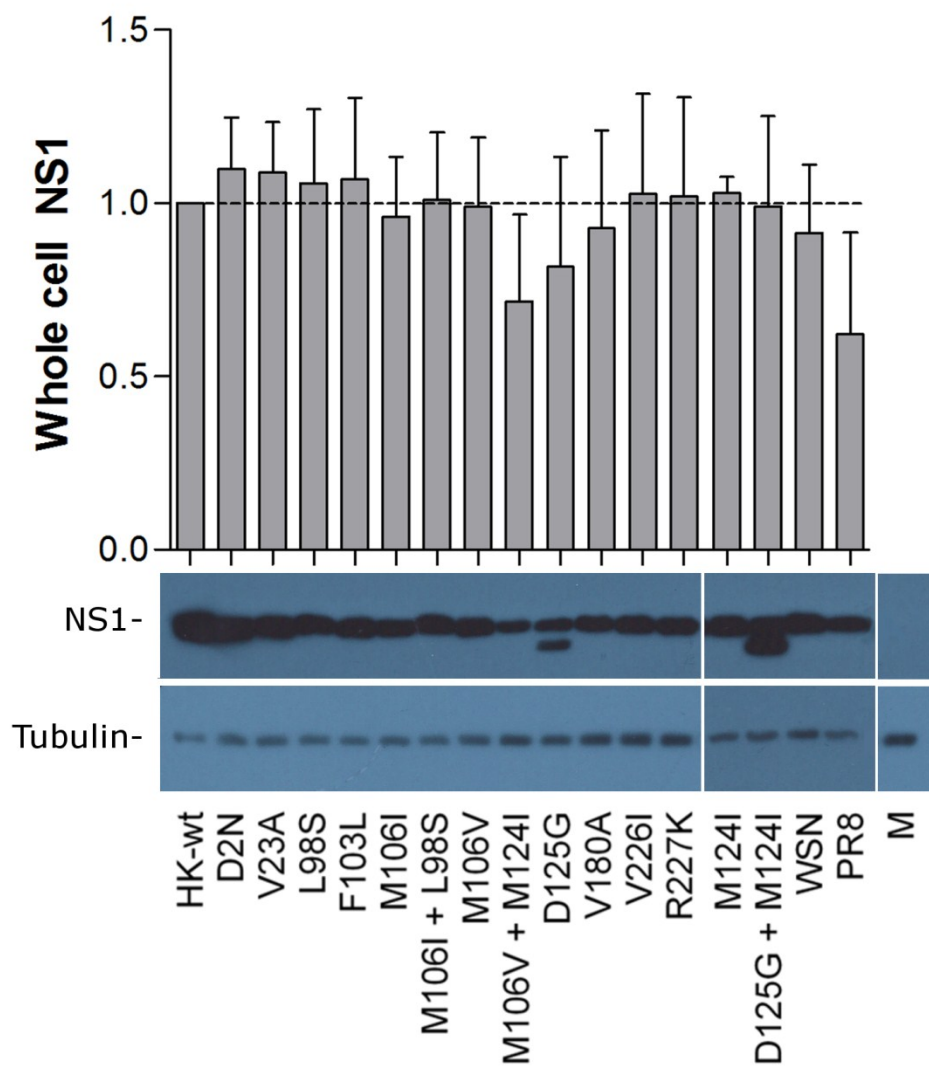


Figure 38. The majority of MA NS1 mutants do not increase the abundance of NS1 in infected human cells. Human A549 cells were infected with rHK NS mutants, the specified virus, or mock-infected with PBS. At 16 hpi a sample of the whole cell lysate was obtained, resuspended in SDS buffer and separated by SDS PAGE electrophoresis, followed by Western blot analysis to detect the NS1 protein as well as tubulin to serve as a loading control. Lower panel: a representative blot of 2-3 independent experiments. Upper panel: Densitometry analysis of whole cell NS1 normalized to tubulin value then rHK-wt NS1. Data represent the means $\pm$ SD (all $p > 0.05$; two-tailed student's t-test).

3.4.13.2.3 The majority of NS1 mutants increase nuclear NS1 accumulation in A549 cells

I next determined whether the abundance of NS1 in the nucleus of infected A549 cells was altered for NS1 mutants by Western blot analysis as per the previous protocol (n=2-3) (Figure 39). All NS1 mutants selected upon adaptation to the mouse did increase the amount of NS1 localized to the nucleus in infected A549 cells, however the average fold increase compared to rHK-wt NS1 was >3 fold less than in M1 cells (2.9 versus 9.3 fold increase). Notably, neither PR8 nor WSN IAV strains were found to increase levels of nuclear NS1. These data show that the ability of mouse-adapted NS1 mutants to increase nuclear NS1 upon in vitro infection is host range dependent.

3.4.13.2.4 The majority of NS1 mutants increase cytoplasmic NS1 accumulation in A549 cells

To analyze the effect of NS1 mutants on cytoplasmic NS1 levels in infected A549 cells, cytoplasmic fractions of infected cells were subject to Western blot analysis as per the previous protocol (n=2-3) (Figure 40). All NS1 mutants were found to significantly increase the amount of NS1 localized to the cytoplasm, where the average increase was > 12 fold compared to rHK-wt levels. Unlike nuclear NS1 expression, where the enhancement was much more pronounced in mouse cells, cytoplasmic NS1 enhancement is ~3 fold greater in human A549 cells than in M1 cells (Figure 40, Figure 32). The greatest enhancement in cytoplasmic NS1 was associated with the double mutant M106I + L98S and single mutant L98S (36 fold, p<0.00001; and 25 fold, p<0.01, respectively), which is consistent with the phenotype expressed in M1 cells. On the other hand, the double mutant D125G + M124I

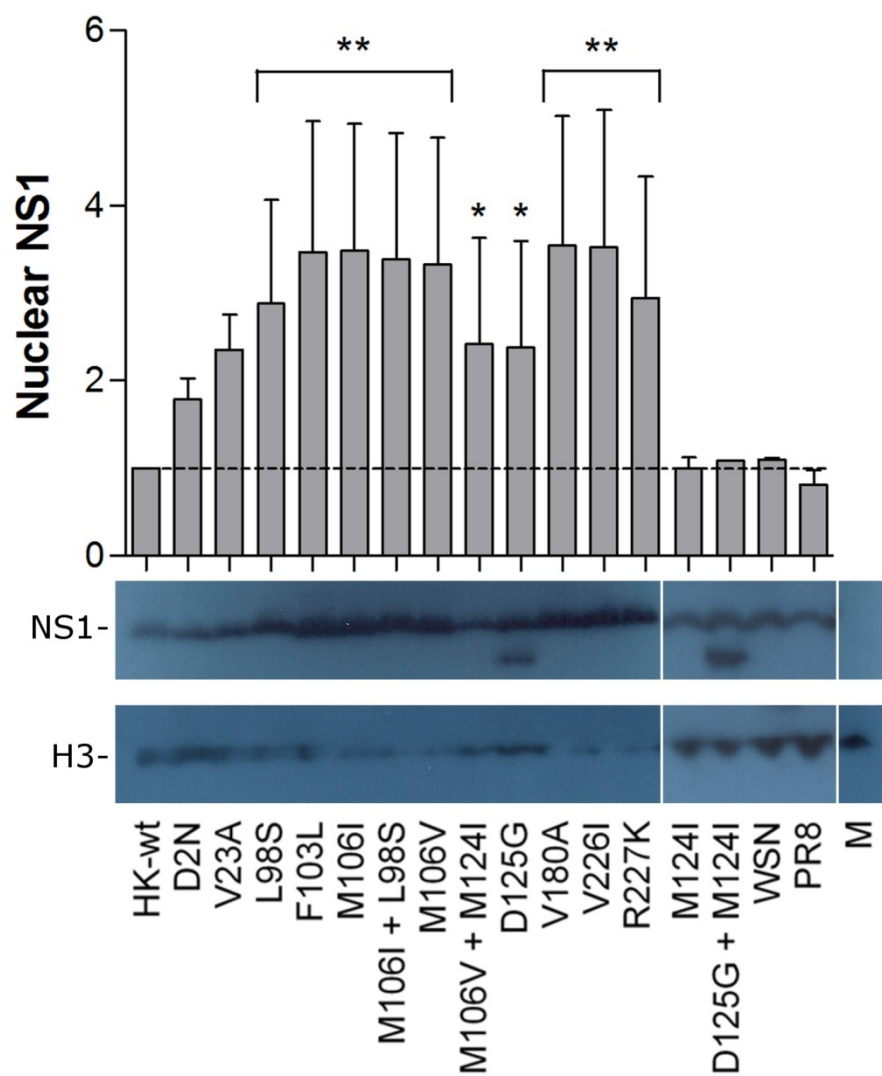


Figure 39. The majority of MA NS1 mutants increase the abundance of nuclear NS1 in infected human cells. Human A549 cells were infected with rHK NS mutants, the specified virus, or mock-infected with PBS. At 16 hpi cells were differentially centrifuged to obtain the nuclear fraction, which was then resuspended in SDS buffer and separated by SDS PAGE electrophoresis, followed by Western blot analysis to detect the NS1 protein as well as histone H3 to serve as a loading control. Lower panel: a representative blot of 2-3 independent experiments. Upper panel: Densitometry analysis of nuclear NS1 normalized to histone H3 value then rHK-wt NS1. Data represent the means $\pm$ SD (* $p < 0.05$, ** $p < 0.01$; two-tailed student's t-test).

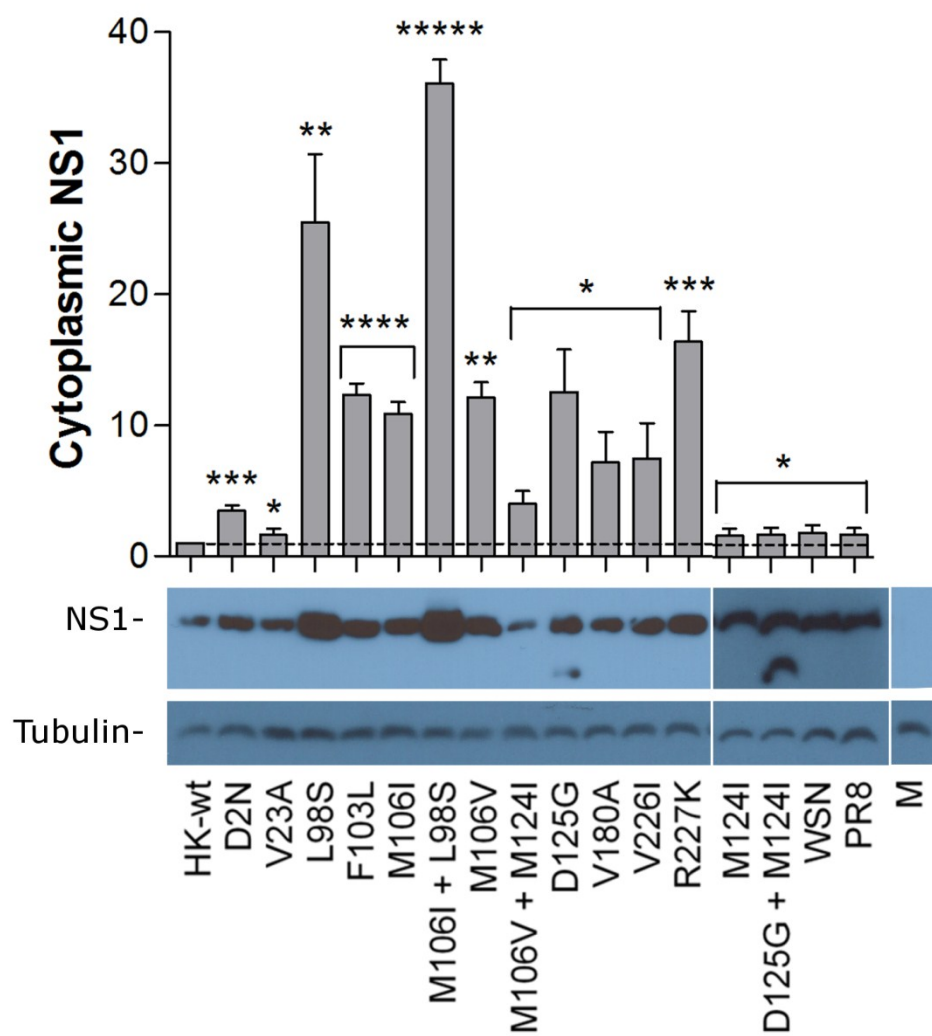


Figure 40. The majority of MA NS1 mutants increase the abundance of cytoplasmic NS1 in infected human cells. Human A549 cells were infected with rHK NS mutants, the specified virus, or mock-infected with PBS. At 16 hpi cells were differentially centrifuged to obtain the cytosolic fraction, which was then resuspended in SDS buffer and separated by SDS PAGE electrophoresis, followed by Western blot analysis to detect the NS1 protein as well as tubulin to serve as a loading control. Lower panel: a representative blot of 2-3 independent experiments. Upper panel: Densitometry analysis of nuclear NS1 normalized to tubulin value then rHK-wt NS1. Data represent the means $\pm$ SD (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, **** $p < 0.0001$, ***** $p < 0.00001$; two-tailed student's t-test).

did not exhibit increased cytoplasmic NS1 in A549 cells (Figure 40), when in M1 cells enhancement surpassed 5 fold (Figure 32). Prototypic mouse-adapted strains WSN and PR8, like D125G + M124I, also exhibited levels of cytoplasmic NS1 that were comparable to rHK-wt. These data suggest increased cytoplasmic localization of the NS1 protein is a phenotype associated with but not limited to adaptation to a novel host and thus can operate in multiple hosts.

4. Discussion

The NS1 protein is a known IAV virulence factor, and associated with a primary function of IFN antagonism. NS1 is very multifunctional; the protein binds numerous viral and host proteins, as well as RNA, and likewise engages in many functions during virus infection (54). Few reports have characterized the phenotypes associated with NS1 proteins from virulent variants or from viruses adapted to a novel host. Experimental evolution of influenza A virus in the mouse was previously demonstrated to force selection of virulent variants with mutations in multiple genes (9-11, 65, 119, 141). Moreover, the use of this non-biased approach led to identification of adaptive mutations in the polymerase and hemagglutinin proteins that increased virus replication and virulence (10, 11, 71, 140, 141). In my research, I characterized the NS1 mutations selected upon HK virus experimental evolution under ideal conditions of selection in the mouse and identified key NS1 mutations responsible for adaptation and virulence in a novel host.

Moreover, given that numerous *in vivo* and *in vitro* phenotypes were evaluated, it was then possible to identify phenotypic trends associated with NS1 adaptation. To this end, I created a heat map of NS1 mutant phenotypes in respect to rHK-wt (Figure 41), by assigning a color ranking to values presented as fold change relative to rHK-wt values (Table 2) to aid in the interpretations and analyses of trends and patterns that will be discussed in the following sections.

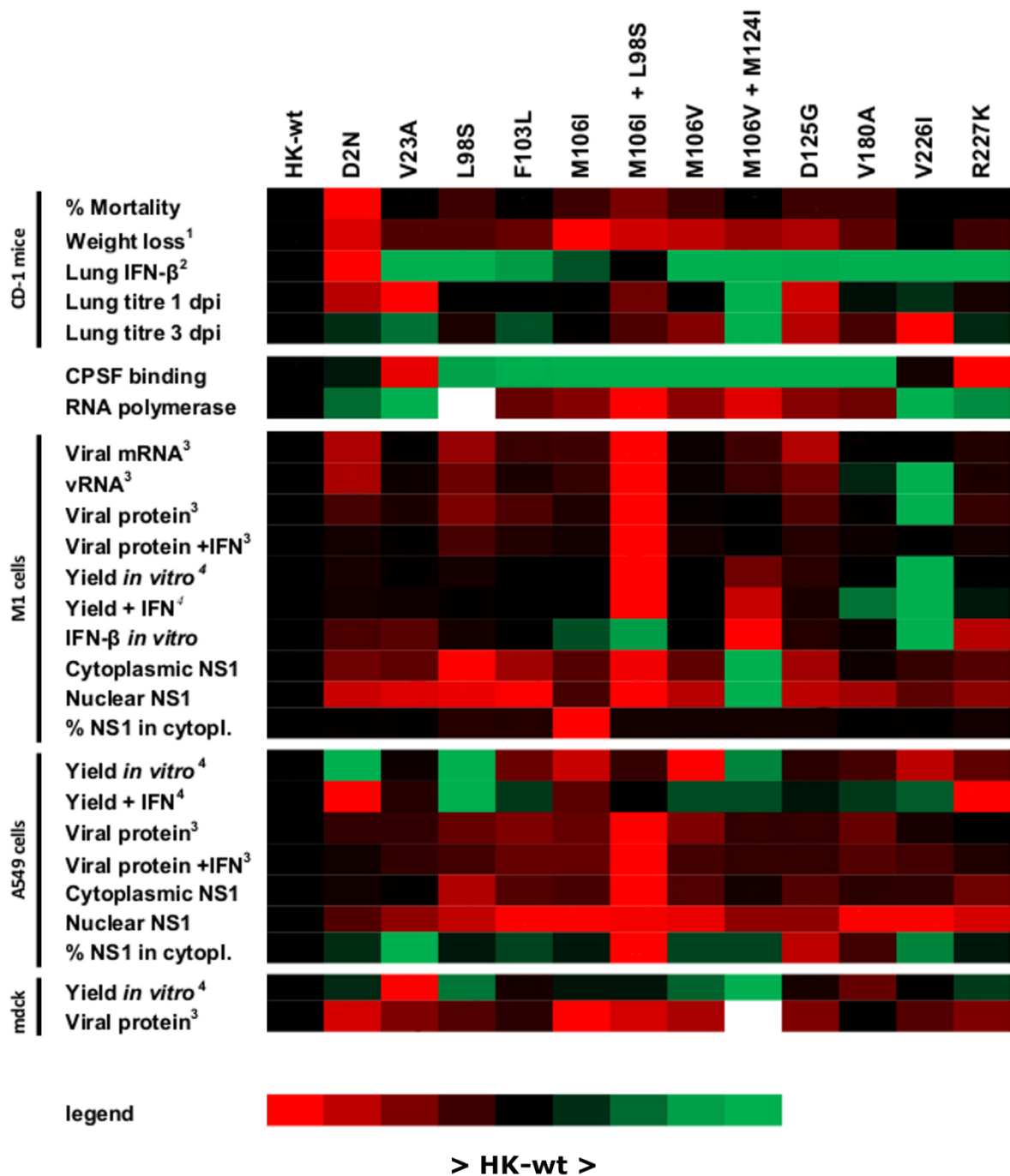


Figure 41. Adaptive properties of rHK viruses expressing MA NS1 mutations. Heat map illustrating adaptive phenotypes relative to rHK-wt value, where green scale depicts phenotypes lesser than rHK-wt, and red scale depicts phenotypes greater than rHK-wt. Table 2 presents the values used to create the heat map, where data was calculated as a fold change relative to rHK-wt value, with the exception of % mortality. Microsoft excel automated color scheme was used to create the heat-map.

		HK-wt	D2N	V23A	L98S	F103L	M106I	M106I + L98S	M106V	M106V + M124I	D125G	V180A	V226I	R227K
CD-1 mice	% Mortality	0	83	0	20	0	20	40	20	0	20	20	0	0
	Weight loss ²	1	3.4	1.9	1.9	2.1	3.8	3.3	3.1	2.7	2.9	2.0	1.0	1.7
	Lung IFN-β ³	1	1.4	0	0	0.12	0.54	1.0	0	0	0.03	0	0	0
	Lung titre 1 dpi	1	12.0	16.4	1.0	1.0	1.3	7.6	1.1	0.02	13.4	0.92	0.74	2.35
	Lung titre 3 dpi	1	0.75	0.38	1.5	0.57	1.1	2.3	3.2	0.03	4.1	2.2	5.3	0.79
Mouse M1	Viral mRNA ⁴	1	13	1.5	11.4	5.0	5.5	18.6	1.8	5.6	13.4	1.2	1.0	3.4
	vRNA ⁴	1	17	2.4	11	3.7	5.9	25	2.2	6.2	11	0.9	0.5	4.0
	Viral protein ⁴	1	1.9	1.4	2.6	2.0	1.4	4.3	1.1	1.0	2.0	1.1	0.6	1.7
	Viral protein +IFN ⁴	1	1.6	1.2	3.1	2.0	1.6	8.4	1.6	1.1	2.1	1.4	1.1	1.6
	Yield in vitro ⁵	1	12.5	3.9	10.7	2.8	1.6	121	1.3	53.9	20.5	1.0	0.74	1.2
	Yield + IFN ⁵	1	8.1	6.9	3.4	1.6	1.4	100	1.2	78.4	12.0	0.50	0.23	0.90
	IFN-β in vitro	1	6.0	7.0	2.5	1.3	0.6	0.2	1.1	18.1	3.4	1.8	0.1	13.3
	Cytoplasmic NS1	1	4.1	3.6	8.2	5.5	3.4	7.8	3.6	0.1	5.6	1.5	2.5	3.4
	Nuclear NS1	1	11.0	11.9	12.8	13.5	4.5	13.6	10.0	0.7	10.3	9.2	5.6	8.0
	% NS1 in cytopl.	1	2.1	1.8	6.0	5.9	36.1	3.9	3.7	2.8	2.5	1.0	1.7	3.6
Human A549	Yield + IFN ⁴	1	0.6	1.1	0.6	1.8	2.5	1.4	2.9	0.7	1.3	1.5	2.4	1.7
	Viral protein ⁴	1	2.4	1.2	0.05	0.7	1.5	1.0	0.6	0.6	0.9	0.7	0.5	2.4
	Viral protein +IFN ⁴	1	1.2	1.2	1.4	1.5	1.4	2.0	1.5	1.2	1.2	1.4	1.1	1.0
	Cytoplasmic NS1	1	1.1	1.3	1.4	1.6	1.6	2.5	1.4	1.3	1.3	1.5	1.4	1.2
	Nuclear NS1	1	3.5	1.7	25.5	12.3	10.9	36.1	12.1	4.0	12.6	7.2	7.5	16.4
	% NS1 in cytopl.	1	1.8	2.4	2.9	3.5	3.5	3.4	3.3	2.4	2.4	3.5	3.5	3.1
	Yield in vitro ⁵	1	0.8	0.2	0.9	0.7	0.9	1.4	0.7	0.7	1.3	1.1	0.4	0.9
MDCK	Viral protein ⁴	1	0.8	2.0	0.4	1.1	0.9	0.9	0.5	0.1	1.1	1.4	1.0	0.7
	CPSP30 F2F3 binding	1	1.5	1.3	1.2	1.1	1.6	1.5	1.4	nd	1.3	1.0	1.2	1.3
	RNA polymerase	1	0.87	2.1	0.08	0.02	0	0	0	0	0	0	1.1	2.2
		1	0.7	0.5	nd	2.7	3.2	5.2	3.3	4.7	3.2	2.8	0.5	0.6

Table 2. Adaptive properties of rHK viruses expressing mouse-adapted NS1 mutations¹

Phenotypes associated with MA NS1 mutations were evaluated relative to rHK-wt value.¹ All values are fold change relative to rHK-wt value; where 0 represents below the limit of detection by the respective assay, with the exception of % mortality, which is expressed as values independent of rHK-wt.² Assessed at respective dpi with maximum weight loss for each respective virus.³ Fold change value of lung IFN- β level at day 1 pi.⁴ Fold change of overall mRNA or vRNA expression (NP + M1 + NS1) or overall viral protein expression (M1 + NS1), respectively.⁵ Relative maximum titre obtained during time course.

4.1 NS1 mutations selected upon mouse adaptation

4.1.1 Pattern of selection

Upon serial passage in the mouse lung, the human influenza A virus (IAV) isolate A/Hong Kong/1/1968 (H3N2) (HK-wt) acquired advantageous mutations that were associated with increased replication and virulence in the mouse. Prior to adaptation, HK-wt was incapable of inducing disease in the mouse ($\text{MLD}_{50} > 10^{7.7}$ PFU) (11). However, the resulting mouse-adapted strains were capable of killing 50% of CD-1 mice with a dose as small as 11 PFU (MA63, Figure 3a). This dramatic (> 7 log) increase in virulence was associated with 11 nonsynonymous mutations per viral genome, selected during 21 serial passages in the mouse lung (141).

In a genomic study of adaptive evolution of 36 strains selected following 20 and 21 passages in the mouse lung, 18 MA isolates acquired 11 mutations in the NS1 gene occurring as single or double nucleotide point mutations (141) (Table 1). Evolution of the NS1 protein involved multiple instances of positive selection including parallel evolution that was demonstrated by directional evolution at position 106, where two independently passaged populations selected different amino acid mutations at the same site (M106V and M106I), and convergent evolution was demonstrated at position 125, where two additional independently passaged populations selected the same NS1 mutation (D125G). The pattern of mutation in respect to the NS1 primary sequence suggests regions of the NS1 protein are more functionally relevant to adaptive mutation upon passage in a novel host.

4.1.2 MA NS mutations that induce mutations in the NEP gene

Several NS1 mutations selected upon mouse adaptation are within the overlapping reading frame that encodes NEP, the spliced gene product of the mRNA expressed from gene segment 8 that functions in nuclear export of vRNPs (123). Of the 18 MA viruses that possessed NS1 gene mutations, 4 induced amino acid substitutions in the NEP protein (Table 1). The MA variants 62 and 63 selected the NS1 V180A mutation, which concurrently induced the NEP mutation S23P. Notably the MA63 virus was the most virulent of NS mutant MA viruses (Figure 2, Figure 3). The NS gene segment of MA93 (NS1 D2N) that induced the highest level of mortality in the mouse when expressed as a rHK NS mutant (Figure 5), contained two NEP mutations, D2N and E108K. Since both the NS1 and NEP proteins have the first 10 amino-terminal aa in common, it is possible that NS1 and NEP mutations within this region involve a common mechanism of action, however this remains to be investigated. Lastly, MA112 (R227K) induced NEP mutation G70S. This current study has not separated the effects of these NEP mutations from their associated NS1 mutations, given the restrictions of independently mutating overlapping codons when expressed as a recombinant NS mutant virus. Use of a modified gene segment 8 construct that separates the NS1 and NEP genes could address this concern, however this system is limited in that it does not maintain the temporal expression patterns including the ratio of NS1:NEP gene expression observed in typical IAV infection (137).

4.1.3 Convergence with NS1 mutations selected in other highly virulent strains

Several NS1 mutations selected upon HK virus mouse adaptation have been independently selected in highly pathogenic IAV isolates. The HPAI H5N1 strain A/Hong

Kong/156/1997 (HK H5N1) caused a fatal infection in humans and contains NS1 mutations 23A, 103L, and 106I (50). The novel pH1N1 that emerged in 2009 also possessed the NS1 mutation V23A (41). Carboxy terminal NS1 mutations 226I and 227K are also found in the 1918 Spanish influenza isolate A/Brevig Mission/1/1918 (H1N1) (1918) (146). The 1918 NS1 gene has been shown to contribute to virulence in infected Macaques (4) and delay IFN induction in ferrets (110). Expression of 226I and 227K as single mutations on the HK NS1 gene was not associated with increased virulence in the mouse, as measured by mortality and body weight loss (Figure 5). However, both mutations decreased IFN- β production in vivo, and for the 226I mutation, this phenotype was associated with >5 fold increased virus replication in the mouse lung (Figure 8). Our findings in the mouse model demonstrate that the 226 and 227 aa residues of the 1918 virus NS1 gene may be operating to contribute to the increased virulence demonstrated in vivo. These incidences of convergence with highly pathogenic strains further support the role of MA NS1 mutations in host adaptation, and given the appropriate genetic context, the ability of MA NS1 mutations to increase virulence in multiple host species.

4.1.4 Convergence with NS1 mutations selected upon experimental evolution to a novel host

Previous studies on the experimental evolution of IAV to a novel host include selection of several NS1 mutations identified in my research. In parallel with our studies, the NS1 mutation D125G (GAT-GGT) was selected upon mouse adaptation of the human isolate A/Aichi/2/68 (H3N2) (119). The adaptation of duck H9N2 virus to quail was found to select for a NS1 mutation at position 106 (M106T) (64), and provides another example of

directional evolution at this codon. Given that mutation at site 106 was the most common NS1 mutation site associated with mouse adaptation of HK viruses (Table 1), this study further supports that site 106 is an important modulator of NS1 gene function. A complimentary study performed by Samar Dankar (previous Brown Lab PhD candidate) demonstrated mutations F103L and M106I are capable of increasing virulence of IAV strains unrelated to the HK virus: A/Puerto Rico/8/34 (H1N1), a virus with known virulence properties in the mouse that possesses NS1 amino acids 103S and 106I, and both the recombinant WSN virus expressing the NS1 gene from the chicken isolate, A/Chicken/Beijing/1/1995 (H9N2) and the recombinant A/PR/8/34 virus possessing the A/HK/156/1997 (H5N1) NS1 gene, which both possess 103L and 106I mutations (18). These studies constitute multiple demonstrations that NS1 mutations selected upon HK mouse adaptation are capable of enhancing virulence in unrelated IAV isolates.

4.1.5 Virulence of NS mutant MA viruses

MA HK viruses possessing NS1 mutations demonstrated increased virulence in CD-1 mice, where the MA63 virus (NS1 V180A; NEP S23P) induced the highest level of virulence, with a > 6.6 log reduction in MLD_{50} from rHK-wt, while all other assayed MA viruses with the exception of MA111 (D125G) reduced MLD_{50} by > 1.2 logs (Figure 3a). Upon expression in the HK wild-type genetic background, MA NS1 mutants D2N and M106I + L98S induced the highest levels of virulence following infection with 5×10^6 PFU (83% and 40% mortality, respectively), while M106I, M106V, D125G and V180A also induced mortality in the mouse (20% mortality) (Figure 5). In a recent study, the NS1 protein of A/PR/8/34 (H1N1) was found to interact with all other IAV proteins (154). In addition, every NS1 mutant MA virus

acquired mutations in additional virus genes. Therefore epistasis is likely influencing the ability of a MA NS1 mutant to enhance virulence in the mouse and awaits further analysis.

4.1.6 IFN resistance of NS mutant MA viruses

A panel of MA NS1 mutant viruses were previously assayed for their sensitivity to IFN- β in respect to virus replication in mouse M1 cells (Figure 4; by Shuai Wang). All MA NS1 mutants were found to be significantly more resistant to the effects of IFN- β than rHK-wt (<4 fold versus 14-fold reduction in viral titre upon IFN pre-treatment) ($p < 0.05$). Upon reverse engineering rHK NS1 mutant viruses, I was then able to assess the extent to which this MA virus IFN-resistant phenotype was due to mutations in the NS1 gene. In vitro infection of both untreated and IFN- β pre-treated mouse M1 cells led to enhanced virus replication for the majority of rHK NS mutants indicating that NS mutants enhanced virus growth in both in the presence and absence of IFN pre-treatment compared to rHK-wt (Figure 12). Consistent with the previous experiment (Figure 4), rHK-wt IFN sensitivity was estimated at 16 fold reduction (growth in untreated cells/growth upon IFN- β pre-treatment; 72 hpi) (Figure 42). However, only NS1 mutants V23A and M106V + M124I were more IFN resistant than rHK-wt (9 and 11- fold reduction, respectively) by this assay. The remainder of the NS1 mutants demonstrated similar or increased sensitivity to IFN- β pre-treatment in respect to virus replication in the absence of IFN- β , with NS1 mutants L98S and V226I the most sensitive (>50 fold reduction in viral titre) (Figure 42). This data suggests that additional virus mutations selected upon mouse adaptation contribute to the IFN resistant phenotype of NS1 mutant MA viruses, and that the ability of MA NS1 mutations to induce IFN resistance may be due to interactions with additional mutant

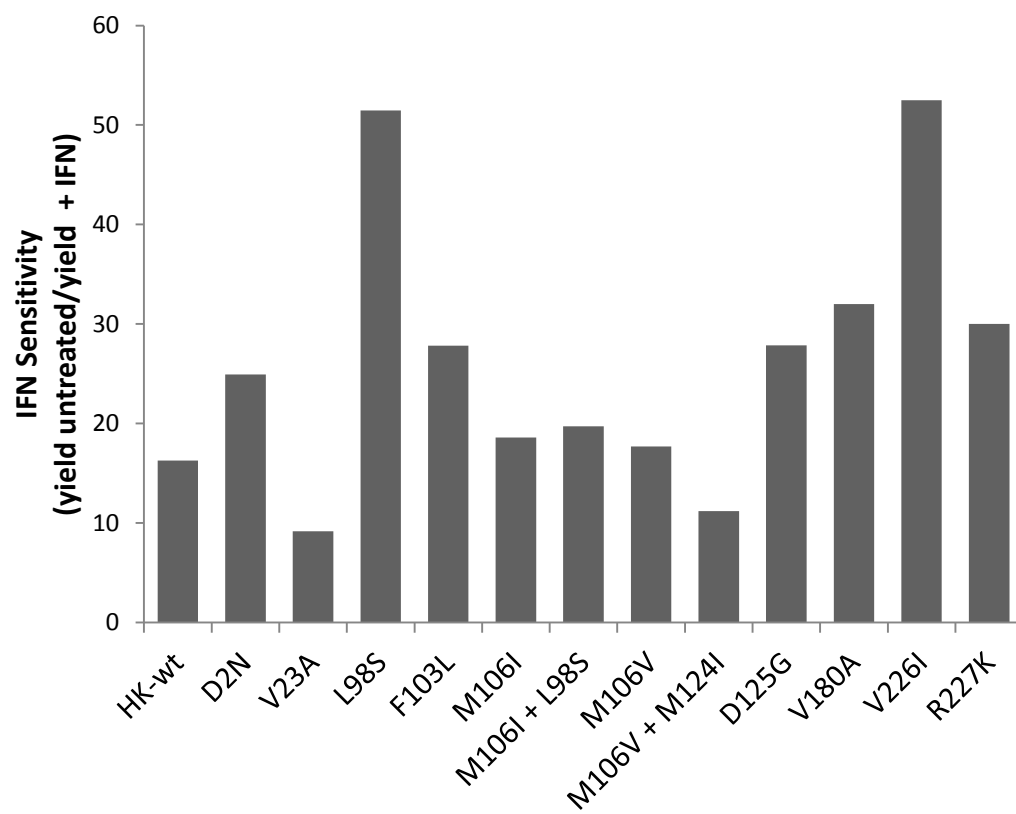


Figure 42. IFN sensitivity of NS1 mutant rHK viruses in infected mouse cells. Mouse M1 cells were either left untreated or pre-treated for 24 hours with IFN- β (200 U/mL MEM) then infected at MOI = 2 with rHK NS1 mutants or rHK-wt virus and virus titre was assayed 12-72 hpi by plaque assay on MDCK monolayers (Figure 12). IFN sensitivity was quantified by dividing 72 hpi yield in untreated cells by 72 hpi yield in IFN-primed cells.

viral proteins (due to epistatic effects of mutations not present in the rHK backbone). Additional recombinant viruses expressing various combinations of HK-wt and MA virus gene segments would need to be engineered and assayed in order to determine the genetic requirements for IFN resistance in NS1 mutant MA viruses. Most NS1 mutants exhibited a decreased sensitivity to IFN treatment in respect to virus yield compared to rHK-wt, I did observe a higher level of virus replication in the presence of IFN (as well as absence of IFN), that in itself constitutes an ability to counteract the IFN response.

4.2 NS mutants alter subcellular localization of the NS1 protein in a host-specific manner

The final objective of my research aimed to determine whether host adaptation altered cellular localization of the NS1 protein, and if so, if this property was linked to other adaptive phenotypes. The NS1 protein performs a number of functions during influenza virus infection by binding cellular factors in both the nucleus and the cytoplasm of infected cells (54). Following its synthesis by host translation machinery, nascent NS1 is found in the cytoplasm of infected cells, then rapidly localizes to the nucleus (89). Most NS1 proteins, including the HKNS1, possess two NLS, NLS1 at aa 34-38 and NLS2 at aa 203-237 (47), which by interaction with importin- α (109), function in driving nuclear localization of NS1. The NS1 protein also contains one NES (aa 137-147), which may contribute to cytoplasmic localization of the NS1 protein reported at later times following infection (156). Regulation of nuclear export appears to be complex, as the adjacent region to the NES (aa 148-161) inhibits NES activity in transfected cells but is unmasked in infected cells in a mechanism dependent upon the protein's secondary structure (91). Cellular localization of NS1 protein

appears to be a function of multiple counteracting activities such as NLS (1+2) versus NES versus NES + regulator activities that appear to involve other viral proteins or functions.

Although only the two C-terminal distal MA NS1 mutations, V226I and V227K, are directly located in a known site involved in NS1 localization (NLS2), previous reports have shown that NS1 mutagenesis at sites beyond NLS and NES domains affect NS1 localization (20, 56, 87, 91), including aa residues at sites 103 and 106, which have been associated with the ability of NS1 to bind CPSF30 (76). Moreover, previous reports had shown subcellular localization of the NS1 protein was a host-dependent phenotype (90, 109, 183), and that inhibition of NS1 nuclear localization by site-directed mutagenesis is associated with increased IFN production and virus attenuation (25, 114). Although there has been no previous report on the role of host adaptation on NS1 localization in infected cells, my research demonstrates that host adaptation altered the ability of the NS1 protein to localize, and this altered subcellular distribution was associated with other adaptive phenotypes such as enhanced IFN antagonism and viral gene expression.

4.2.1 Effect of NS1 mutants on cellular NS1 distribution in infected mouse M1 cells

The majority of NS1 mutants dramatically altered subcellular localization of the NS1 protein upon in vitro infection of mouse M1 cells. Upon rHK-wt virus infection, the majority the NS1 protein was localized in the nucleus, supported by both Western blot analysis of cell fractions collected at 16 hpi, and IF confocal microscopy of cells fixed from 4 to 24 hpi (Figure 23a, Figure 28). However, NS1 mutant M106I + L98S consistently increased the percentage of cells positive for NS1 in the cytoplasm compared to rHK-wt when assayed by IF

confocal microscopy (Figure 23, Figure 25), and this property gained dominance in a temporal fashion (Figure 27b).

Western blot analysis of whole cell, nuclear, and cytoplasmic fractions collected 16 hpi supported the IF confocal microscopy results. A greater amount of NS1 localized in the cytoplasm (>2 fold increase compared to rHK-wt) for both NS1 mutants demonstrating the greatest increased prevalence of cytoplasmic NS1 positive cells, M106I + L98S and D125G (Figure 23-25), as well as the majority of MA NS1 mutants and two prototype mouse-adapted viruses PR8 and WSN (Figure 29, Table 2). However, the double mutant D125G + M124I had a similar percentage of NS1 localized to the cytoplasm compared to rHK-wt, which is discordant with the observed NS1 staining in IF-labeled infected cells.

Quantification of the Western blot protein bands to determine the proportion of cells with NS1 localized to the cytoplasm only accounted for the distribution of the NS1 protein, as the NS3 band was excluded from the NS1 densitometry analysis. It is possible that the NS3 protein contributes to the increased cytoplasmic staining in D125G + M124I infected M1 cells IF-labeled with a NS1 polyclonal antibody that recognizes both NS1 and NS3 proteins.

The overall distribution pattern of NS1 in infected M1 cells was remarkably consistent for the majority of MA NS1 mutants. Most mutants increased NS1 abundance in the whole cell lysate, nucleus, and cytoplasm of infected M1 cells (Figure 41). Moreover, the fold enhancement compared to rHK-wt levels was greatest in nuclear NS1 (average 10 fold enhancement) than in cytoplasmic NS1 (average 4.5 fold enhancement) for every NS1 mutant.

4.2.2 Effect of NS1 mutants on cellular NS1 distribution in infected human A549 cells

The effect of NS1 mutants on subcellular NS1 localization was quite different in human A549 cells. First of all, the rHK-wt NS1 protein was visualized localizing to the cytoplasm in infected A549 cells, which never was observed in mouse M1 cells (Figures 23, 26 and 33). Thus IF microscopy demonstrated human rHK virus expressed NS1 protein in both the nucleus and cytoplasm of human cells but only the nucleus of mouse cells as described in the previous section which may constitute a part of the host-dependent deficits for the rHK-wt virus seen in mouse cells. Moreover, in A549 cells, no MA NS1 mutants were found to increase the percentage of cells positive for cytoplasmic NS1 compared to rHK-wt staining (Figures 33-34), further establishing the host-dependent phenotypes associated with these mutations. Therefore, the majority of MA NS1 mutants did not visually alter subcellular localization of NS1 in infected human A549 cells.

Western blots of whole A549 cell lysate, nuclear, and cytoplasmic fractions collected 16 hpi were analyzed to further quantitate NS1 distribution. The overall distribution pattern of NS1 in A549 cells infected with NS1 mutants was quite divergent from M1 cell infection (Table 2, Figure 41). NS1 mutants did not significantly increase the percentage of NS1 localized to the cytoplasm (Figure 37), and overall NS1 abundance (whole cell lysate) was similar to rHK-wt levels for the majority of NS1 mutants (Figure 38). Moreover, although most NS1 mutants enhanced NS1 accumulation in both the nucleus and the cytoplasm in A549 cells, the majority of NS1 mutants demonstrated a greater fold enhancement in cytoplasmic NS1 (>12 fold average) than in nuclear NS1 (< 3 fold average), which was the inverse of the trend observed in infected M1 cells (Table 2). These data indicate NS1

mutations selected upon mouse adaptation alter subcellular localization of the NS1 protein in infected human A549 cells, although NS1 distribution in the cell appears to be affected by a host-dependent mechanism, as the phenotype expressed in mouse M1 cells differs greatly from that in human A549 cells.

4.2.3 Limitations of experimental approaches

By analyzing the subcellular localization of the NS1 protein using both IF microscopy and Western blot analysis of infected cell fractions, I have shown that NS1 mutations selected upon mouse adaptation alter NS1 localization in a host cell-dependent mechanism. However, the data obtained from the two independent experimental approaches did not always agree. NS1 was detected in the cytoplasm of infected mouse cells by Western blot analysis of the cytoplasmic cellular fraction for all NS1 mutants and rHK wt viruses, with the exception of NS1 mutant M106V + M124I (Figure 32). However, confocal microscopy of IF labeled cells showed a lack of cytoplasmic NS1 for rHK-wt and most NS1 mutations, (except M106I + L98S, D125G, and D125G + M124I) (Figure 25, Figure 27b). One possible explanation for the inability to visualize Cy3-labeled NS1 protein in the cytoplasm for the majority of NS1 mutants as well as rHK-wt virus infection is that the cells are fixed with formalin, which fixes tissues by crosslinking cellular proteins, rendering the NS1 protein anchored in both its location and in its interaction with other fixed proteins. The NS1 protein binds numerous cellular factors, some of which localize to the nucleus (PABPN1, CPSF30, NXF1/TAP), and others that localize to the cytoplasm (PABP1, eIF4GI, PKR, RIG-I, hStaufen, p85 β). I hypothesize that cytoplasmic NS1 exists primarily in complexes with other proteins, possibly to a greater extent than nuclear NS1, and that at low amounts, the

protein is unable to be detected by IF microscopy, but if the abundance of cytoplasmic NS1 surpasses a certain threshold, it is not titrated out by interacting partners and can be visualized by immunofluorescence.

I also observed a difference in the extent of the relative increases in mutant NS1 protein in relation to HK-WT NS1 protein in the Western blots of infected cell fractions. For both M1 and A549, the increased amounts of NS1 seen in the cytoplasmic and nuclear fractions relative to HK-wt were greater than the increases seen for lysates of whole cells (Table 2), which strongly suggests that the MA NS1 proteins were more stable to the fractionation procedure than HK-wt NS1. This suggests differences in ability of HK-wt NS1 to be recovered from subcellular fractions compared to mutant NS1 proteins, and may be dependent in part on the smaller amounts of HK-wt NS1 that would be disproportionately affected by a degradative loss.

One possible explanation is the NS1 protein stability is altered due to NS1 mutations in a cell fraction-specific manner, where NS1 mutations increase protein stability in cytoplasmic and nuclear fractions. All fractions were processed in parallel so there is little chance experimental error is a factor. Further experiments are required to determine whether protein stability is altered by NS1 mutations in a manner dependent upon the cellular location of the protein. A possible experimental approach would be to pulse chase infected cells with ³⁵S labeled Cys and Met residues then collect cell fractions as per the previous protocol and subject the fractions to SDS PAGE electrophoresis and autoradiography. Alternately, I could repeat the cell fraction experiment using an

alternative protocol in the presence of protease inhibitors. As the data stands, it is unclear whether NS1 mutations alter NS1 protein stability upon cell fractionation.

4.2.4 Role of host adaptation in NS1 expression and cellular distribution

By ^{35}S pulse labeling both mouse and human infected cells, I showed the majority of NS1 mutants increased the rate of viral protein synthesis, where the greatest enhancement was demonstrated in mouse cells (Table 2). Consistent with this observation, virus replication was also enhanced to a greater extent in mouse cells than in human cells, relative to rHK-wt infection, which implies the rHK-wt virus was markedly attenuated in mouse but not human cells (Table 2). I have demonstrated that NS1 abundance of MA NS1 mutants is increased more in nuclear than cytoplasmic fractions in infected mouse cells, while in human cells, NS1 is increased more in the cytoplasmic fraction. I hypothesize that the nuclear translocation of the rHK-wt NS1 protein is host-restricted, and thus repressed in mouse M1 cells. Upon mouse adaptation, NS1 mutants are alleviated from this repression by an ability to interact with an unknown host-specific translocation factor, and thus in M1 cells, enhanced NS1 abundance in the nucleus exceeds enhanced NS1 abundance in the cytoplasm (Table 2). In the same vein, nuclear translocation of the rHK-wt NS1 protein occurs without inhibition in human cells, as the virus had already evolved to high virulence in the human host. Therefore, given NS1 as well as other assayed viral proteins is synthesized at a heightened rate for the majority of NS1 mutants in infected A549 cells, adaptive NS1 mutations are associated with increased NS1 protein in both the nucleus and the cytoplasm. Mutant NS1 protein levels are enhanced to a greater extent in the cytoplasm compared to the nucleus of infected A549 cells, which suggests NS1 that

mutations selected upon mouse adaptation increase levels of cytoplasmic NS1 due to enhanced NS1 protein synthesis and /or NES activity for the NS1 protein (Table 2).

MA NS1 mutations could also be altering NS1 protein shuttling to favour cytoplasmic localization. The NS1 protein has 2 NLS, therefore NS1 is by default a nuclear protein and an action must occur to get it back to the cytoplasm. As NES sequences are usually groups of L (or I) residues with amino acids between them (43, 191), MA mutations such as 103L and 106I could be converting this region into an additional NES. Upon 103L and 106I mutation, the HK-wt NS1 sequence from 103 to 109, FMLMPKQ, would then become **LMLIPKQ**. Alternately, this sequence could increase activity of the known NES at aa 138-147 (FDR**LETILL**). Additional experiments on the temporal localization of MA NS1 proteins could determine whether MA NS1 mutations result in an additional NES or enhance the activity of the NES at site 138-147, and whether such an adaptive phenotype is host-dependent.

4.2.5 Role of altered NS1 subcellular localization in additional adaptive phenotypes

Host-specific subcellular distribution of the NS1 protein may play a role in adaptive phenotypes associated with NS1 mutations. One of the most robust phenotypes associated with MA NS1 mutations is the ability to increase viral gene expression, which was quantified at both the protein and mRNA level in infected mouse cells, and at the protein level in infected human and canine cells (Table 2). Moreover, using recombinant WSN viruses expressing a subset of HK MA NS genes, I demonstrated HK MA NS mutants enhance protein synthesis in mouse, human and canine infected cells irrespective of the virus's genetic backbone (Figure S4). Given the robust enhancement of viral mRNA production in infected mouse cells (Table 2), it is likely increased mRNA production contributes to

enhanced levels of viral protein synthesis. Although mRNA production was not quantified in human cells, I predict the enhancement would be far less than that observed in mouse cells. MA NS1 mutants demonstrated an enhancement of nuclear localization of the NS1 protein in mouse cells, while in human cells the HK-wt protein was already present at high levels in the nucleus. Therefore I hypothesize that the robust enhancement of viral protein synthesis associated with NS1 mutants is greatest in infected mouse cells because NS1 mutations increase nuclear localization of the NS1 protein, which leads to increased enhancement of viral polymerase activity and ultimately viral mRNA production. Increased viral mRNA production leads, in turn, to greater levels of viral protein synthesis. Moreover, given the NS1 protein is also more abundant in the cytoplasm for the majority of NS1 mutants compared to HK-wt NS1, cytoplasmic NS1 can more effectively promote initiation of viral protein synthesis by binding to viral mRNA and recruiting it to cellular translational factors. However I cannot discount the possibility of NS1 mutants favourably affecting binding affinity for translational factors such as eIF4GI and PABPI. Nonetheless, altered host-dependent subcellular localization of NS1 mutants is likely to contribute to host-dependent enhancement of viral gene expression.

Interferon antagonism was another NS1 property enhanced by the majority of NS1 mutants, and likewise this adaptive phenotype may be partially attributed to altered subcellular localization of the NS1 protein. Although I have shown the majority of NS1 mutants reverse CPSF30-F2F3 binding affinity, NS1 also blocks IFN mRNA production by binding polyA binding protein nuclear 1 (PABPN1) (15), as well as by interacting with host mRNA nucleocytoplasmic export machinery, NXF1/TAP (151), which leads to the inhibition

of nuclear export of processed mRNA. Increased nuclear NS1 may allow greater inhibition of host mRNA processing and export, while increased cytoplasmic NS1 may enhance NS1 cytoplasmic functions including recruiting cellular translational machinery (3, 12), which would also lead to a greater inhibition of host gene, including IFN gene, expression. In the cytoplasm of infected cells, NS1 inhibits IFN production at the level of RIG-I, by directly binding RIG-I to block IFN mRNA transcription activation (51, 111, 128), by binding the RIG-I activator TRIM25 (38), and by binding dsRNA. Thus the increased cytoplasmic NS1 observed for MA NS1 mutants would increase all of these functions, culminating in a greater inhibition of IFN production.

In addition, increased cytoplasmic NS1 may also contribute to an enhanced ability of NS1 to circumvent the IFN response. Cytoplasmic NS1 binds dsRNA, which sequesters it and prevents the activation of PKR and 2'5'-OAS (86, 114, 115, 174)(93, 115, 174). NS1 also inhibits dsRNA-independent PACT-mediated PKR activation (86). Moreover, cytoplasmic NS1 is responsible for blocking STAT-1 signalling induced by type I IFN, which results in suppression of IFN-stimulated gene transcription (76). Both cytoplasmic and nuclear NS1 localization contribute to properties of IFN antagonism. By increasing the prevalence of NS1 in both these cellular compartments, MA NS1 mutants limit the amount of IFN production in vivo (Figure 8) and suppress the effects of IFN on viral replication (Figure 12) and viral protein synthesis (Figure 15) in mouse cells. Robust IFN-resistant viral protein synthesis was also demonstrated for a subset of assayed HK MA NS mutants when expressed on the WSN genetic backbone (Figure S5).

4.3 NS mutants enhance virulence of the HK virus in the mouse

All NS1 mutants selected upon mouse adaptation enhanced HK virus virulence in the mouse and/or increased virus replication in the mouse lung (Table 2). However, NS1 mutants D2N, M106I + L98S, and D125G exhibited the most pronounced virulence in the mouse (Figure 42). Unlike the D2N mutant, mutants M106I and D125G were able to increase virus replication in the mouse lung while either maintaining or reducing IFN- β production in the mouse lung compared to rHK-wt (Figure 42) thus suggesting that enhanced IFN antagonism is an important regulator of viral replication. Further analyses on individual phenotypes associated with virulence are found in the following passages.

4.3.1 Survival and body weight loss, body temperature

The most striking phenotype induced by 7 of 12 rHK NS1 mutants was ability to induce mortality in the mouse due to a single or double mutation in the NS1 gene (Figure 5). Each NS1 mutant associated with mortality in the mouse was also found to exacerbate morbidity as measured by body weight loss, where body weight loss increased by > 1.9 fold compared to rHK-wt infection (Table 2, Figure 5). Mouse IAV infection has been previously associated with a significant fall in body temperature (5). From day one to day 14 pi (or final day of survival), mice infected with NS1 mutants that induced mortality demonstrated a statistically increased loss in body temperature compared to mice infected with rHK-wt ($p=0.007$, two-tailed t-test). On the other hand, NS1 mutants that did not induce mortality in the mouse resulted in a temperature change that was statistically similar to rHK-wt infected mice. Therefore, both body weight loss and a fall in body temperature were predictive factors for mortality following infection with rHK NS1 mutants.

4.3.2 Increased lung pathology, virus spread and virus replication in the lung

NS1 mutants D2N and M106I + L98S as well as single mutants M106I and L98S and rHK-wt were assessed for a more extensive analysis of the adaptive phenotype in vivo in the mouse. Compared to mice infected with rHK-wt infected, mice infected with all assayed NS1 mutants demonstrated exacerbated pathology and virus spread in the lung at 2 and/or 6 days pi, where NS1 mutant M106I + L98S exhibited the most pronounced phenotype due to a synergistic effect of each mutation (Figure 6).

Interestingly, NS1 mutant D2N, which induced the highest extent of mortality, was undetectable by IF microscopy of mouse lungs collected 6 days pi (Figure 6, Figure 41). These observations correlated with virus growth of NS1 mutant D2N in the lungs of mice infected with a low intranasal dose, where at day 1 D2N surpassed rHK-wt titre by 57 fold, but dropped to rHK-wt levels at day 3, and by day 7 virus titre dropped below the limit of detection (Figure 7). NS1 mutant D2N was one of the few NS1 mutants that was unable to suppress IFN- β production in the mouse lung (Figure 8). Moreover, heightened lung pathology was detected in lungs infected with NS1 mutant D2N following 6 days pi (Figure 6). These observations suggest NS1 mutant D2N establishes high virulence in the mouse by reaching high viral load early following infection that in turn triggers a strong antiviral response that subsequently functions to inhibit virus replication at later time points (6-7 days pi) that is sufficient to clear the virus to levels below detection. On the other hand, NS1 mutant M106I + L98S did not reach the same level of mortality in the mouse as D2N (40% mortality versus 83% mortality; Figure 5), however lung pathology and virus spread in the lung was more pronounced, and M106I + L98S replicated to viral titres that were similar

or greater than D2N in the mouse lung while maintaining the rHK-wt level of IFN- β production in the lung early in infection. This suggests that mutations at site 106 and 98 may enhance the ability of NS1 to suppress IFN production that serves to maintain the property of enhanced replication for longer times post-infection.

4.3.3 NS mutants affect IFN- β induction and virus titre in the mouse lung

The ability to reduce IFN- β production in vivo while maintaining or significantly increasing viral titre in the mouse lung was one of the most universal phenotypes associated with the panel of NS1 mutations selected upon mouse adaptation. Several possible mechanisms include altered NS1 localization and/or altered binding affinity to host factors including the nuclear protein PABPI, which could increase host gene expression inhibition, as well as to cytoplasmic proteins RIG-I and 2'5'-OAS, and viral RNA. The majority of NS1 mutants increased both nuclear and cytoplasmic levels of NS1 infected mouse cells, therefore the increased abundance of the protein alone may heighten interaction levels with these cellular factors. Currently it is not known whether this phenotype is host specific; infection of primary human cells and assessment of human type I IFN production would be a possible experimental approach to begin to address this question.

4.4 CPSF30-F2F3 binding affinity, suppression of IFN production, and RNA polymerase activity: phenotypic trends

The functional role of CPSF30:NS1 interaction during virus infection has been the subject of controversy for IAV researchers for over a decade. Several previous reports from the same laboratory suggested a role for CPSF30:NS1 interaction in interferon antagonism (78, 124), while other independent studies have associated the ability of NS1 to bind

CPSF30 with general inhibition of host gene expression, which was not a requirement for suppression of IFN production (55, 76, 169). Since the majority of NS1 mutants inhibited IFN production in vivo in the mouse lung (Figure 8), we wanted to see whether there was a correlation of IFN inhibition and CPSF30-F2F3 binding affinity. Notably, the majority of NS1 mutations did affect CPSF30-F2F3 binding affinity, with 8 of 12 mutants significantly decreasing affinity and 2 of 12 mutants significantly enhancing affinity compared to rHK-wt NS1 (Figure 10). Decreased binding affinity to CPSF30-F2F3 was not associated with decreased abundance of the NS1 protein in the nucleus, as the majority of NS1 proteins increased nuclear NS1 accumulation in both human and mouse cells (Table 2). We also did not see a correlation between increased CPSF30-F2F3 binding and increased ability to suppress IFN- β production; 6 of the NS1 mutants reduced binding affinity below the limit of detection, which correlated with the phenotype of significantly decreased IFN- β production in the mouse lung 1 dpi, with the exception of mutant M106I + L98S (Figure 8, Figure 10, Figure 41). This observed lack of association between CPSF30-F2F3 binding and IFN suppression is in direct conflict with reports from previous studies, where inhibition of CPSF30 binding by site-directed mutagenesis at amino acid residues 103 and 106 or region 184-188 led to increased IFN- β production (78, 124). However, other studies of IAV NS1 proteins that are unable to bind CPSF30 show that their respective viruses (mouse-adapted A/PR/8/34 (H1N1) (169) and pandemic 2009 H1N1 isolates (55)) maintain the ability to suppress IFN- β production but lose the ability to block general host gene expression; where restoration of CPSF30 binding by site-directed mutagenesis resulted in slightly increased or reduced virulence, respectively.

However, I did observe a correlation between CPSF30-F2F3 binding affinity and RNA polymerase activity, where all NS1 mutants that lost the ability to bind CPSF30-F2F3 were found to increase RNA polymerase activity, which was not the case for NS1 mutants that retained or surpassed HK-wt NS1 binding affinity to CPSF30-F2F3 (Figure 41). A previous study showed an interaction between NS1 and polymerase complex in respect to the NS1:CPSF30 complex in H5N1-infected cells (78). My data suggests that NS1 mutants unable to bind CPSF30 may be interacting with a component of the viral polymerase complex independent of CPSF30 binding. Moreover, it is possible that CPSF30 binding may sequester NS1 to limit the extent to which NS1 can associate with the viral polymerase complex, where alleviation of CPSF30 binding may in fact enhance NS1 interaction with the RNA polymerase complex and result in increased viral polymerase activity. Or conversely since HK-wt NS1 protein inhibited polymerase activity but the CPSF binding negative mutations did not, it is possible that the NS1 binding to the viral polymerase is inhibitory to the ability of NS1 to bind CPSF30. Biochemical interaction and function studies are required to resolve these questions.

4.5 Enhancement of viral transcription and virus replication in mouse cells

Another dramatic in vitro phenotype produced by the NS1 mutants was observed upon infection of mouse M1 cells (Table 2), where virus replication was enhanced by as great as 121 fold and viral transcript production by 25 fold. Notably, both maximum phenotypes were due to the NS1 mutation M106I + L98S (Table 2). The majority of NS1 mutants, however, also enhanced virus replication and virus gene expression in M1 cells (Figure 41, Table 2).

There were several interesting trends associated with NS1 mutant phenotypes in vitro in mouse cells. First of all, all NS1 mutants except M106V + M124I shared phenotypes of increased NS1 abundance in the nucleus and increased viral mRNA and viral RNA production (Table 2). Moreover, enhancement of viral transcription, as measured by viral RNA and mRNA production as well as RNA polymerase activity, correlated with increased virus replication, with the exception of NS1 mutant V226I (Figure 41). Increased RNA polymerase activity was associated with increased viral mRNA and viral RNA production in infected M1 cells assayed by qPCR, with the exception of NS1 mutant V180A (Figure 41). The increased transcription seen in infected M1 cells for the D2N, V23A, and R227K was not associated with increased RNA polymerase activity and suggests an indirect mechanism of action on RNA synthesis. The V226I mutant did not demonstrate increased RNA synthesis by either assay indicating that the increased replication in vivo was independent of enhanced RNA synthesis.

4.6 NS mutations are adaptive in vitro irrespective of IFN pre-treatment

Mouse M1 cells were primed with IFN- β prior to in vitro infection to determine whether adaptive phenotypes (enhanced virus replication and virus protein synthesis) were IFN sensitive or alternately were dependent upon IFN signaling, the latter implying a mechanism involving IFN antagonism. All NS1 mutants that enhanced virus replication > 1.2 fold in untreated M1 cells also enhanced virus replication > 1.2 fold in cells primed with IFN- β . Consistent with this observation, virus protein synthesis enhancement was also independent of IFN- β pre-treatment. These trends suggest that these in vitro adaptive phenotypes occur by a mechanism insensitive to and independent from IFN signalling.

However, unlike other mutants, NS1 mutants V23A and M106V + M124I enhanced viral replication to a greater extent in IFN-primed M1 cells (6.9 and 78.4 fold) than in untreated M1 cells (3.9 and 53.9 fold) relative to rHK-wt (Table 2). It is possible that V23A and M106V + M124I enhance virus replication in M1 cells by increasing IFN antagonism. A similar exception in respect to viral protein synthesis was NS1 mutant M106V. NS1 mutant M106V was shown to enhance virus protein synthesis only in M1 cells primed with IFN- β , also indicating this adaptive phenotype is dependent upon antagonism of the IFN-mediated antiviral response (Table 2).

To determine whether the effect of IFN pre-treatment on virus replication and protein synthesis was host specific, similar experiments were performed on human A549 cells, where I pre-treated cells with human IFN- α 2b (pharmaceutical grade human type I IFN approved for treatment of cancer and virus infections) or left the cells untreated prior to infection. However, unlike in mouse M1 cells, NS1 mutants that enhanced virus replication in untreated cells did not consistently demonstrate enhanced replication in IFN primed cells, and vice versa. It must be noted that compared to maximum titre produced in M1 cells, the data spread was much narrower, with maximum titres of NS1 mutants between 0.05 and 2.9 fold relative to rHK-wt (Table 2).

As for the effect of IFN on viral protein synthesis in A549 cells, NS1 mutants enhanced the rates of viral protein synthesis to moderately high levels (≤ 2.5 fold enhancement relative to rHK-wt) in both untreated and IFN-primed cells, and the difference in enhancement between cell treatment conditions never exceeded 0.5 fold (Table 2). All NS1 mutants that enhanced virus protein synthesis in untreated cells also enhanced virus

protein synthesis in cells pre-treated with IFN, with the exception of NS1 mutants V226I and R227K, which were only capable of enhancing virus protein synthesis in cells pre-treated with IFN. However, the fold enhancement was only 1.4 and 1.2 relative to rHK-wt level, respectively. With such modest increases in virus protein synthesis, there is currently little evidence to support IFN dependent mechanisms. Overall, these results support a trend, where enhancement of virus protein synthesis associated with MA NS1 mutants occurs in a host-independent mechanism insensitive to and independent from IFN signalling (all mutations but M106V, V226I, and V227K) However, enhancement of virus replication for all NS1 mutants, with the exception of V23A and M106V + M124I, was found to occur in a host-*dependent* mechanism insensitive to and independent from IFN signalling.

One possible explanation is that pre-treatment of M1 cells with murine IFN- β sustained a more robust antiviral state than pre-treatment of A549 cells with human IFN- α 2b, but this would not be expected because both types of interferon bind the same receptors although this binding may not be identical in affinity nor response. However, the A549 cell line is a lung carcinoma cell line, and numerous studies have reported tumor cells have a defective IFN response (73, 74, 178, 190) and thus the differences between M1 and A549 cells in expression levels and functionality of IFN effector proteins may have been responsible for the host-dependent effects. In response to virus infection, IFN- β production precedes and is required for IFN- α production (31). Moreover, IFN- β was reported to play a protective role in the host response to IAV infection (77). Therefore I cannot conclude that treatment with human IFN- β instead of IFN- α , nor use of a non-transformed human cell line, would have led to the same results.

4.7 Host cell specificity of gain-of-functions associate with NS mutations in vitro

4.7.1 Host specificity of virus replication enhancement

It is not surprising that NS1 mutants selected upon mouse adaptation of a human virus enhance virus replication to the greatest extent in mouse cells, where 10 of the 12 NS1 mutants enhanced virus replication by an average of 23 fold compared to rHK-wt maximum titre (Table 2, Figure 12). In a parallel virus growth experiment performed in human A549 cells, 9 of the 12 NS1 mutants enhanced virus replication (as measured by maximum virus titre) with an average of 1.8 fold increase compared to rHK-wt (Table 2, Figure 17). When the same study was performed in MDCK cells, a canine cell line highly permissive to IAV infection, only 4 NS1 mutants enhanced maximum virus yield, with an average of 1.4 fold increase compared to rHK-wt titre (Figure 20, Table 2). In contradiction to this observed trend, several NS1 mutants induced a greater replicative advantage in A549 cells than M1 cells. NS1 mutants M106I, M106V, and R227K all increased virus replication in mouse M1 cells, however the fold increase relative to rHK-wt titre was greater in A549 cells (Table 2). Interestingly, NS1 mutants V180A and V226I enhanced virus replication in A549 cells but not in M1 cells (Table 2). These observations suggest that NS1 mutants M106I, M106V, and R227K, although able to increase virus replication in mouse cells and the mouse lung as well as virulence upon in vivo infection of the mouse, may require additional mutations in other gene segments to more fully demonstrate their adaptive phenotype. For NS1 mutants V180A and V226I, both express a virulent phenotype in the mouse, where V180A induced 20% mortality and both V180A and V226I increased virus titre in the lung 3 dpi (Table 2). However, virus replication as well as viral gene

expression does not appear to be enhanced upon M1 cell infection for these mutations. In vitro infection of mouse M1 cells likely does not recapitulate the intrinsic nature and complexity of in vivo infection, and this loss of complexity may not allow the adaptive properties of NS1 mutants to be consistently evident, at least for some mutations.

NS1 mutant V23A was the only mutant capable of enhancing virus replication by ≥ 2 fold in MDCK cells (Figure 20, Table 2). In reference to the fact that the remainder of NS1 mutants did not induce even a 2 fold enhancement in virus replication in MDCK cells, the HK-wt virus itself grew to $> 10^8$ PFU/mL, which is an extremely high viral titre to be reached by any IAV isolate in cell culture. Therefore any replicative advantages conferred by NS1 mutations would be difficult to quantify given the permissiveness and lack of restraints of this experimental system. If the MOI was decreased 10 or 100 fold, perhaps NS1 mutants would be capable of inducing measureable enhancement of virus replication in MDCK cells.

4.7.2 Host specificity of viral protein production

Similar to virus replication, NS1 mutants enhanced virus protein production to the greatest extent in mouse M1 cells, where 8 of 12 NS1 mutants enhanced virus protein synthesis ≥ 1.4 fold compared to rHK-wt levels, and NS1 mutant M106I + L98S demonstrated the greatest enhancement (4.3 fold increase) (Figure 15, Table 2). In A549 cells, 6 of 12 NS1 mutants increased virus protein synthesis ≥ 1.4 fold compared to rHK-wt levels, and again NS1 mutant M106I + L98S demonstrated the greatest enhancement (2 fold increase) (Figure 19, Table 2). In MDCK cells, virus protein synthesis was enhanced for fewer NS1 mutants, and to a lesser extent, and the most pronounced phenotype was from NS1 mutant M106I (1.6 fold enhancement) (Figure 21, Table 2). NS1 mutants V23A, D125G, and

R227K were found only to enhance virus protein synthesis in mouse M1 cells, suggesting the mechanism of enhancement was host-restricted for these mutations. One possible explanation could be the enhancement of nuclear NS1 abundance, which was greater in mouse cells than in human cells (Table 2). This increased nuclear localization may contribute to enhanced viral mRNA production (Figure 13a), leading to increased viral protein synthesis (Figure 15). As HK-wt NS1 protein does not exhibit restricted nuclear localization in A549 cells, MA NS1 mutants may lack the property of enhanced viral transcription in this cell line, however this has yet to be experimentally assessed.

Among all three tested cell lines, only M106I and M106I + L98S consistently enhanced viral protein synthesis, and in each case, M106I (MDCK) or M106I + L98S (M1, A549) demonstrated maximum enhancement compared to rHK-wt (Table 2). For NS1 mutant M106I, relative enhancement of virus protein synthesis was consistent among cell lines (1.4 fold in M1 and A549, 1.6 fold in MDCK, relative to rHK-wt). However, the phenotype for the double mutant M106I + L98S varied dramatically among the three cell lines (4.3 fold in M1, 2.0 fold in A549, and 1.4 fold in MDCK, relative to rHK-wt) (Table 2). The contribution of the L98S mutation appears to be driving the host-dependent phenotype of enhanced virus protein synthesis associated with NS1 mutant M106I + L98S. When expressed as a single mutation, L98S enhances virus protein synthesis in M1 and A549 cells but only slightly in MDCK cells (2.6 fold, 1.4 fold, and 1.2 fold increased values, relative to rHK-wt). This data suggests that NS1 mutant M106I enhances virus protein synthesis in a host-independent mechanism, and that expression of a second mutation, L98S, further

enhances this phenotype in a host-dependent manner (with the greatest effect seen in mouse cells).

NS1 binds several cellular factors involved in mRNA translation, including eIF4GI, PABPI, and the human isoform of staufen (3, 12, 32). Both amino acid residues 98 and 106 are within the binding region of NS1 for eIF4GI. Notably, the region to which NS1 binds eIF4GI is not conserved between human and mouse eIF4GI homologues (Genbank Gene ID NM_182917 (human) and 208643 (mouse)). Therefore, the NS1 mutant L98S may be enhancing viral protein synthesis through an increased interaction with mouse but not human eIF4GI. Currently a plasmid expressing the mouse isoform of eIF4GI is not commercially available, however if such construct was synthetically produced one could test this hypothesis by determining binding affinity by co-immunoprecipitation of the HK-wt and MA NS1 proteins.

4.7.3 Enhanced viral protein production as a predictor of enhanced virus replication

Upon in vitro infection of mouse M1 cells, the majority of NS1 mutants significantly increased viral replication (Figure 12) and enhanced viral protein synthesis ≥ 1.4 fold compared to rHK-wt levels (Figure 15, Table 2). All NS1 mutants with the exception of M106V + M124I, V180A, and V226I were positive for both phenotypes, of which all but M106V + M124I displayed a lack of enhancement in either phenotype. This suggests a correlation, or predictive relationship between enhancement of virus protein synthesis and enhanced virus replication. However, this was not the case for NS1 mutant M106V + M124I, which enhanced virus replication but not virus protein synthesis. This indicates that increased replication was not dependent on enhanced protein synthesis and furthermore

since the M106V + M124I NS1 mutant did not grow well in mice that this combination of mutations has a requirement for interaction with specific host factors or conditions to fully demonstrate its phenotypic effects. The interaction of this pair of mutations warrants further analysis of each individual mutation to determine their biological effects.

In A549 cells, enhanced virus protein synthesis (≥ 1.4 fold compared to rHK-wt levels) was associated with significantly enhanced virus replication for all NS1 mutants but L98S, V226I and R227K. The L98S mutation enhanced viral protein synthesis but not virus replication, and V226I and R227K demonstrated the inverse phenotype (Table 2). Again this data suggests enhanced virus protein synthesis is a strong, but not universal, predictor of enhanced virus replication, and that this predictive relationship is evident among more than one host species. Although both phenotypes were assessed in MDCK cells, enhancement of virus protein synthesis was not observed to correlate with enhancement of virus replication. This could be due to the highly permissive nature of MDCK cells for IAV infection, where the rHK-wt virus was already capable of producing high ($>10^8$ PFU/mL) virus titre and substantial virus protein, but also indicated a host specific aspect to enhanced protein expression for these mouse-adapted mutations.

4.8 Final Conclusions

In summary, all mutant NS1 genes selected upon serial passage of the HK virus in the mouse were adaptive and increased replication in mouse cells and lung that was in most cases associated with increased virulence in the mouse. Moreover, adaptive mutations in the NS1 protein affected subcellular localization of the protein in a host-dependent manner, likely by alleviating restricted nuclear and/or cytoplasmic transport of

the HK NS1 protein in mouse cells. The increased abundance of NS1 in the cytoplasm and nucleus in infected cells may explain at least in part several of the other phenotypes where mutant NS1 proteins were more effective in antagonizing IFN, enhancing virus replication and increasing viral gene expression. Heightened virus replication was associated with increased gene expression and IFN antagonism in vitro in mouse cells. IFN antagonism was found to be independent of the ability of the NS1 protein to bind the cellular factor CPSF30 F2F3 domain. However, CPSF30-F2F3 binding affinity was inversely associated with enhanced viral RNA polymerase activity. Several NS1 mutants also increased virus replication and virus gene expression in human and canine derived cell lines, indicating host-independent adaptive phenotypes. This research has emphasized the role of NS1 as a virulence factor in IAV infection, and identified several novel phenotypes associated with host adaptation and virulence.

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6. Contributions of Collaborators

This work was supported by research performed by other members of Dr Earl Brown's Lab.

Jian-Jun Jia, a research technician in Dr Earl Brown's lab, assisted in the Q-PCR of viral RNA and viral mRNA (Figure 13), where I performed the virus infection and Jian-Jun Jia collected and quantified the RNA samples.

Dr Jihui Ping, a previous post-doctoral fellow in Dr Earl Brown's lab, performed the mini-genome assay (Figure 14) and drafted the figure depicting these results.

Dr Shuai Wang, previous visiting PhD student in Dr Earl Brown's lab, performed the following experiments: IFN- β production in mouse M1 cells following in vitro infection with parental WSN, HK, rWSN HK NS mutants, and HK MA viruses (Figure S2), IFN sensitivity of rWSN HK NS mutants in mouse M1 cells (Figure S3) (joint effort between N Forbes and S Wang), and IFN sensitivity of mouse-adapted viruses with NS1 mutations upon infection of mouse M1 cells (Figure 4). All figures associated with these experiments were drafted by Shuai Wang.

Mohammed Selman, a previous honours student in Dr Earl Brown's lab, performed all analyses relating to the characterization of the NS3 protein, and drafted Figure 22B.

The **pathology department** at the University of Ottawa assisted in performing frozen sections on perfused lungs, and in the H & E stain.

The **Animal Care Veterinary Facility** at the University of Ottawa assisted in daily wellness checks on mice infected with IAV.

7. Appendix

The following collection of data demonstrates the phenotype of a subset of MA NS1 mutants when expressed on the A/WSN/33 (H1N1) (WSN) virus genetic backbone as rWSN HK NS mutant viruses. This research preceded the evaluation of MA NS1 mutants when expressed on the HK parental backbone as rHK NS mutants. The WSN reverse genetics system was readily available at the time of this research, and in addition the WSN virus, unlike the HK virus, was virulent in the mouse. Therefore I hypothesized the expression of HK NS1 mutants on the WSN backbone would allow the contribution of MA NS1 mutations to the virulence of MA viruses to be readily observed. However, the expression of the NS1 gene of HK virus origin on the WSN virus backbone (rWSN NS HK WT) attenuated the virus in vivo (Previous data from Brown lab, not shown) and resulted in increased IFN- β production following in vitro infection compared to infection with the parental WSN and HK viruses (Figure S2). However, the rWSN HK NS mutant viruses demonstrated that NS1 mutations selected upon mouse adaptation increased the ability of NS1 to antagonize the IFN response in vitro and the ability to increase virus protein synthesis in mouse, human, and canine cells. In addition, virus protein synthesis was found to be more resistant to the effects of IFN compared to rWSN HK NS levels. Overall, the data presented in this section shows NS1 mutations selected upon HK virus evolution in the mouse are adaptive, and enhance HK NS1 functions when expressed in both HK and WSN virus genetic backbones.

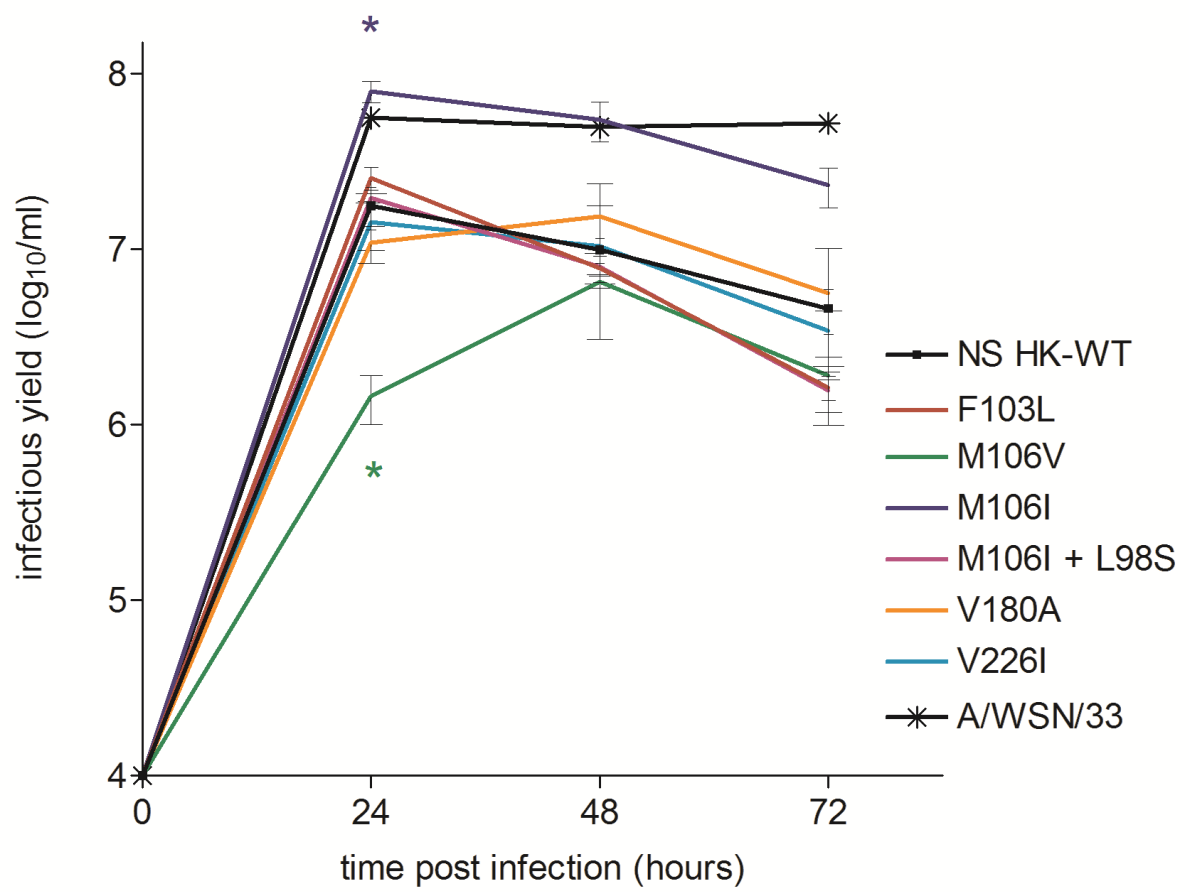
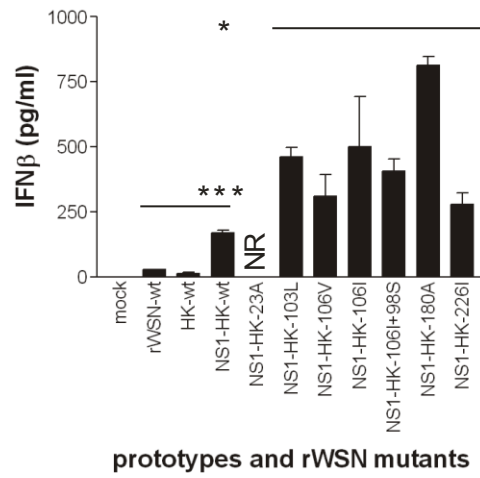


Figure S1. Virus replication of MA NS1 mutants expressed on the rWSN virus backbone.

Confluent monolayers of MDCK cells were infected with rWSN viruses possessing HK NS wt or HK NS mutant genes or the parental WSN virus (n=3) at an MOI of 0.02. At specified time points cell supernatant was sampled and virus titre was quantified by plaque assay on MDCK cell monolayers. Values represent means $\pm$ SD (*p<0.05, two-tailed student's t-test).

(a)



(b)

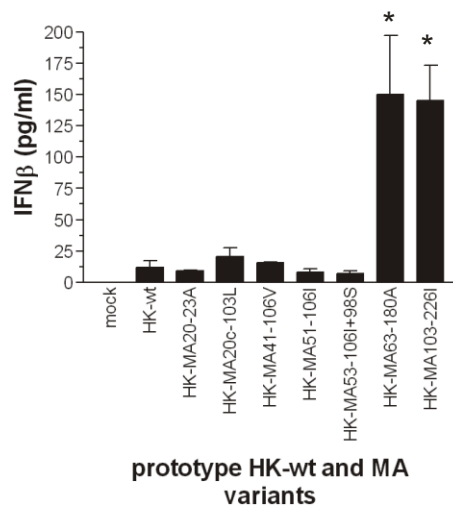


Figure S2. IFN- β production in mouse M1 cells following in vitro infection with parental WSN, HK, rWSN HK NS mutants, and HK MA viruses. Confluent monolayers of mouse M1 cells were infected at MOI=2 with indicated parental (HK and WSN), rWSN viruses possessing HK NS wt or HK NS mutant genes (a), or HK-MA viruses (b) (n=3). For HK-MA viruses, the virus name is followed by the NS1 mutation. At 24 hpi, cell supernatants were tested for IFN- β levels by an ELISA specific to murine IFN- β . NR: not reported. Data represent means $\pm$ SD (* p<0.05, ***p<0.001, two-tailed student's t-test). This assay was performed in collaboration with Shuai Wang, a previous lab member.

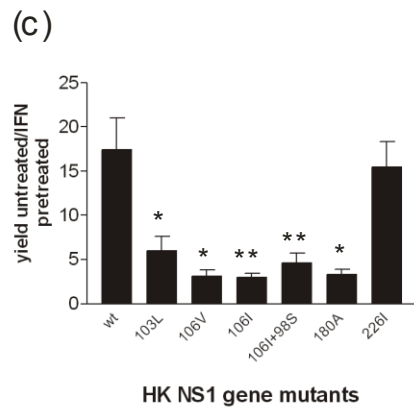
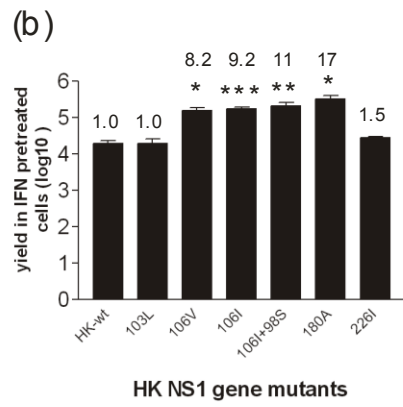
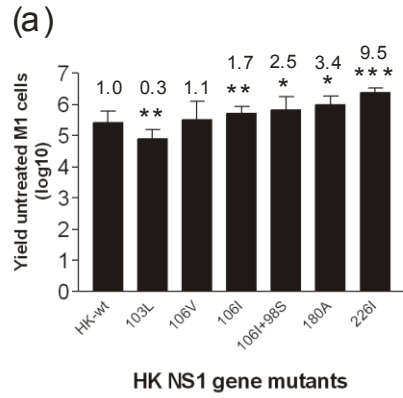


Figure S3. IFN sensitivity of rWSN HK NS mutants: virus replication in mouse M1 cells.

Confluent monolayers of mouse M1 cells were either left untreated (a) or pre-treated with 200 U/mL mouse IFN- β for 24 hour (b). Following treatment, cells were washed twice with 1X PBS then infected at an MOI of 2 with rWSN viruses possessing HK NS wt or HK NS mutant genes (n=3). At 24 hpi, cell supernatant was collected and subject to plaque assay on MDCK cell monolayers. A-B: Data represents means $\pm$ SD of virus titre for specified cell condition. Fold change relative to rWSN HK NS WT titre was indicated above each data column. C: IFN sensitivity was calculated as the fold change of virus titre due to IFN pre-treatment; virus titre (untreated cells) divided by virus titre (IFN treated cells) (*p<0.05, **p<0.01, ***p<0.001, two-tailed student's t-test). This assay was performed by myself and Shuai Wang, a previous lab member.

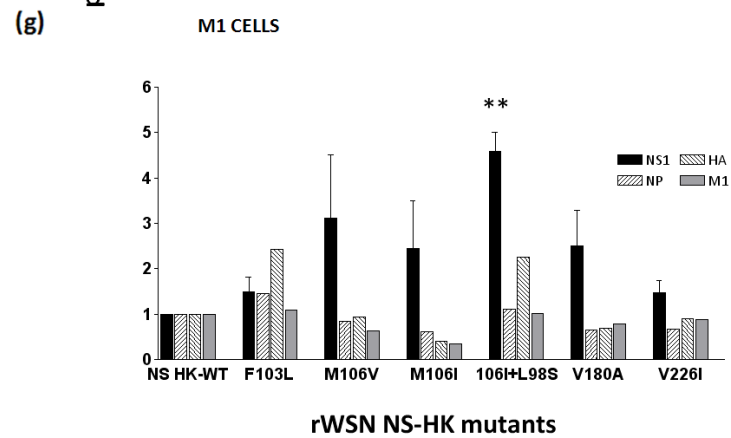
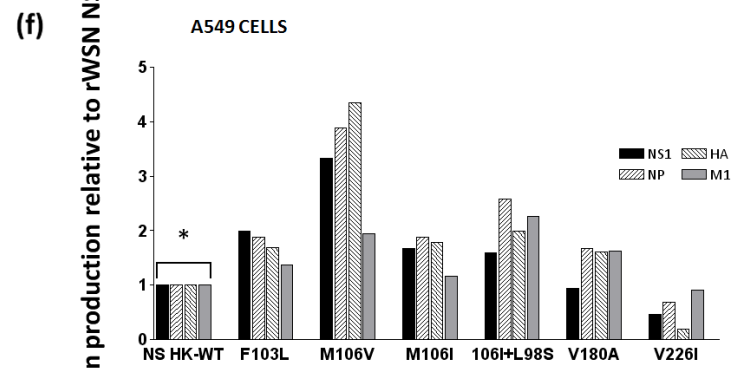
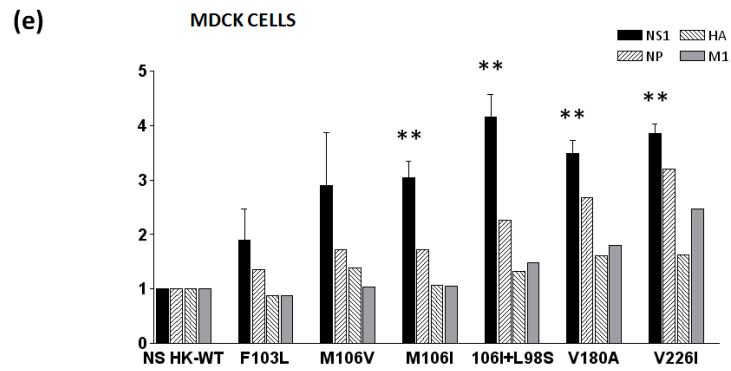
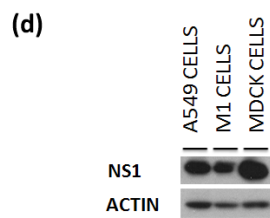
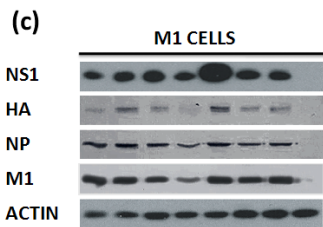
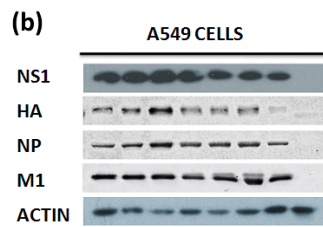
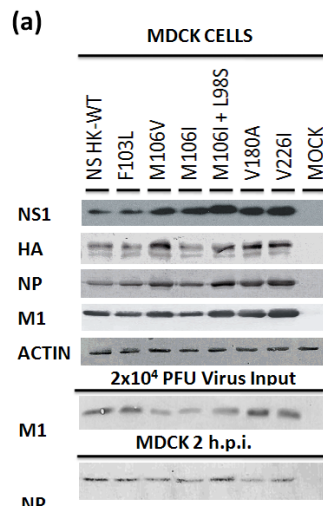


Figure S4. Effect of MA NS1 mutations on WSN virus protein synthesis in infected canine, human and mouse cells. (A) Confluent monolayers of MDCK cells were infected at an MOI = 2 with rWSN viruses possessing HK NS wt or HK NS mutant genes for 8 h before cell lysis collection for western blotting using rabbit anti-NS1 or anti-WS virus for HA, NP, and M1 proteins and anti-actin for loading control. Input virus and cellular proteins detectable at 2 hpi are shown to indicate standardization of infection. (B) Confluent monolayers of human A549 cells were infected and probed as described for (A). (C) Confluent monolayers of mouse M1 cells were infected and probed as described for (A) (D-F) Graphs representing relative levels of accumulated protein detected by densitometry of blots (A-C) normalized relative to HK-wt protein level and actin. The levels of NS1 proteins represent the average and standard error of duplicate experiments of NS1 protein synthesis in MDCK and M1 cells (* $p < 0.05$, ** $p < 0.01$, two-tailed student's t-test).

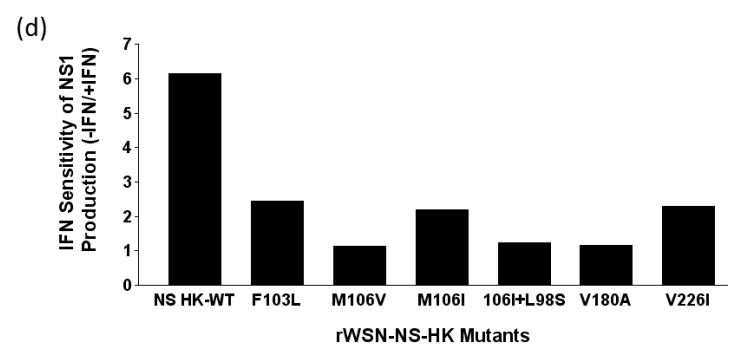
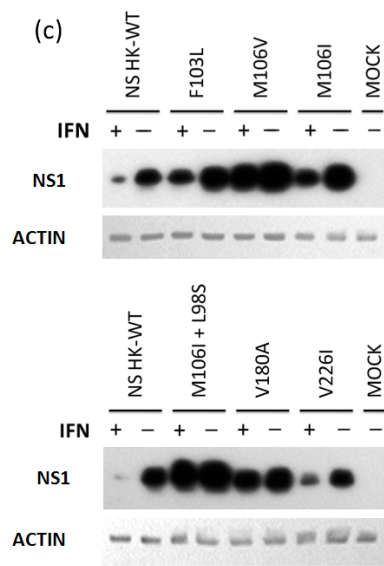
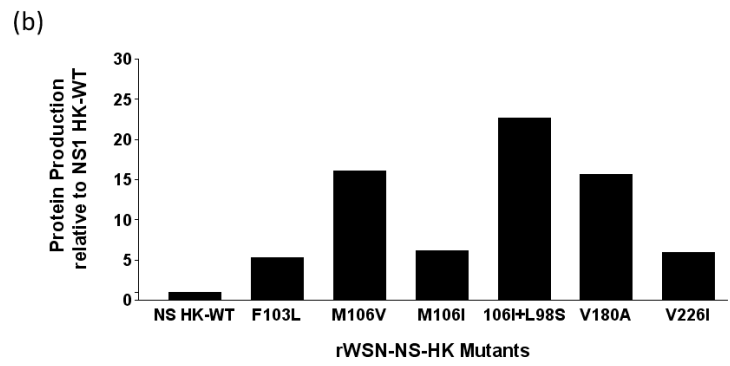
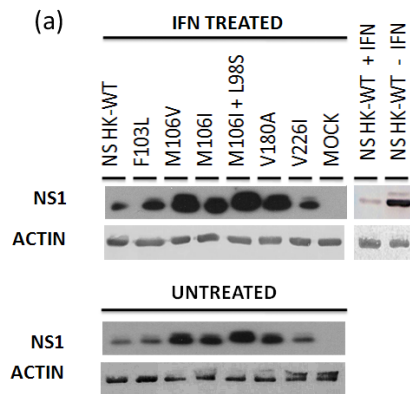


Figure S5. HK MA NS1 mutations expressed on the WSN virus confer increased protein synthesis following IFN β pre-treatment in mouse cells in vitro. (A) M1 cells were untreated or pre-treated with 200 U/ml of IFN β for 24 hour before infection with rWSN viruses possessing HK NS wt or HK NS mutant genes (MOI=2, n=3) and assay of NS1 protein synthesis as described in previous figure. (B) Densitometry analysis of NS1 protein production relative to rWSN HK NS and normalized to actin (C) NS1 protein production in treated and untreated infected cells infected with a given virus are shown side by side in the same gel for comparison. (D) IFN sensitivity of NS1 protein production, as measured by the band density of NS1 in untreated cells divided by the band density of NS1 in treated cells for the specified NS1 mutant (band density calculated from blots in panel C).

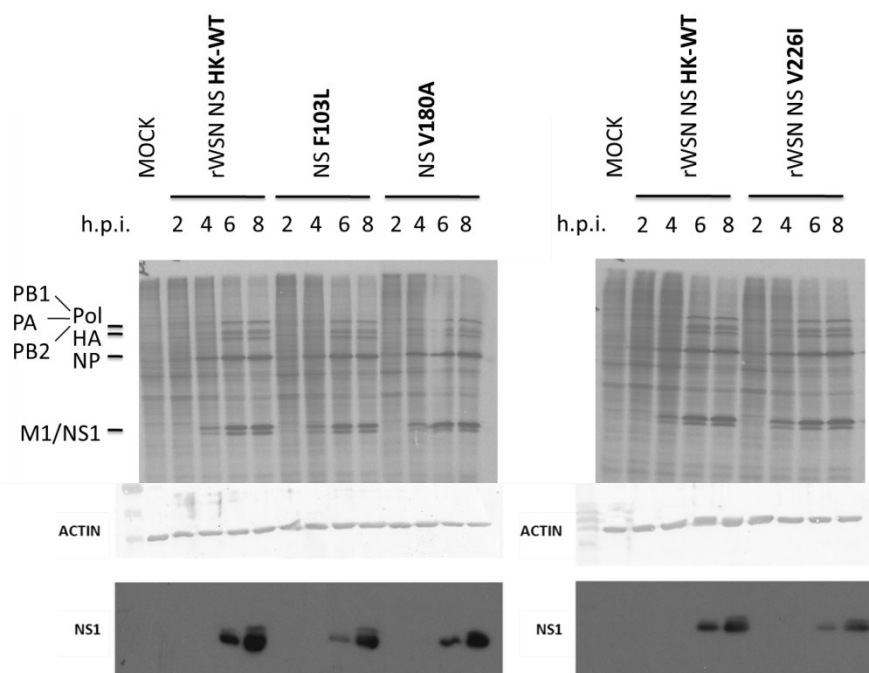
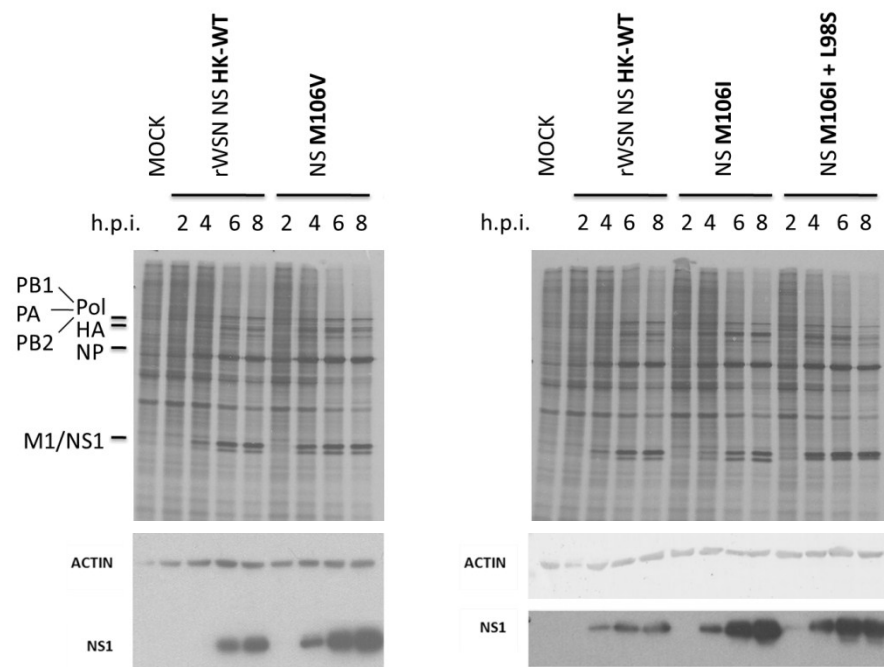


Figure S6. rWSN HK NS mutants increase rate of virus protein synthesis in MDCK cells.

Confluent monolayers of MDCK cells were infected at an MOI = 2 with rWSN viruses possessing HK NS wt or HK NS mutant genes. At 2, 4, 6, and 8 hpi cells were washed with 1 x PBS, then pulsed for 1 hour with 80 uCi/mL ³⁵S radiolabeled Cys and Met amino acids diluted in serum-free MEM deficient in Cys and Met. Following pulse, cell lysate was collected and subject to autoradiography as well as Western blot analysis (as per the previous Western blot protocol, detecting NS1 and actin proteins). Viral protein bands are indicated to the left of autoradiography images.

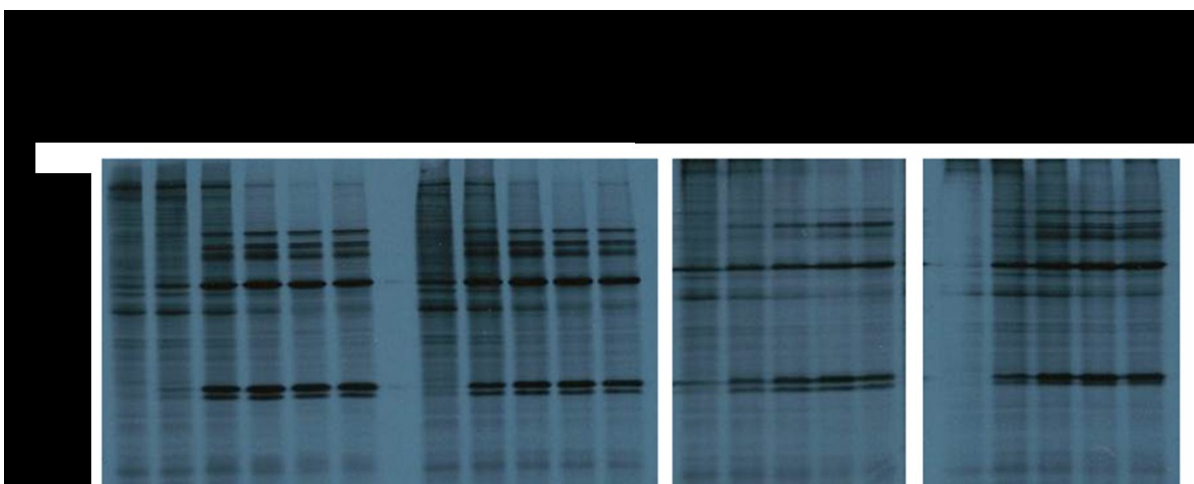
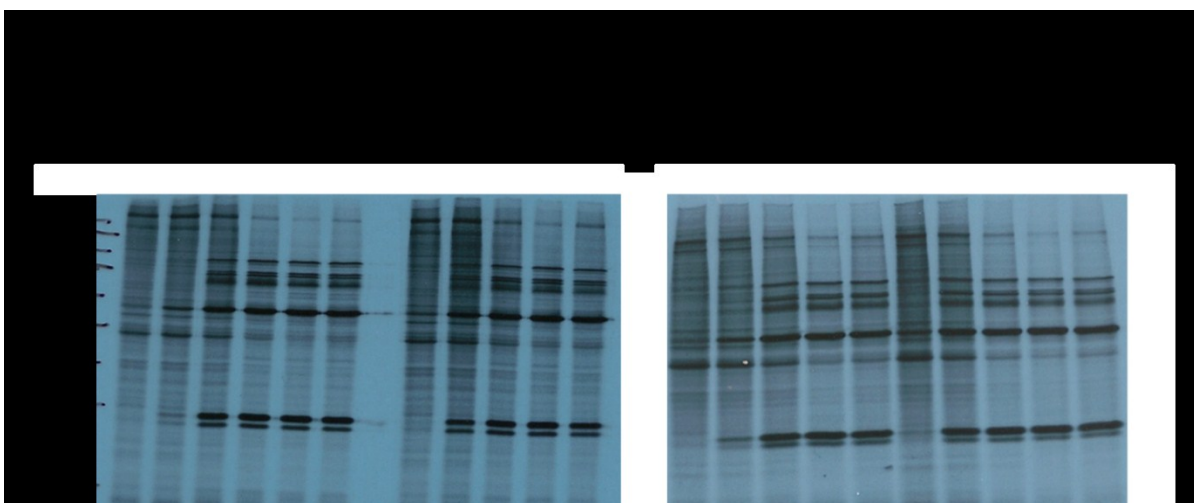
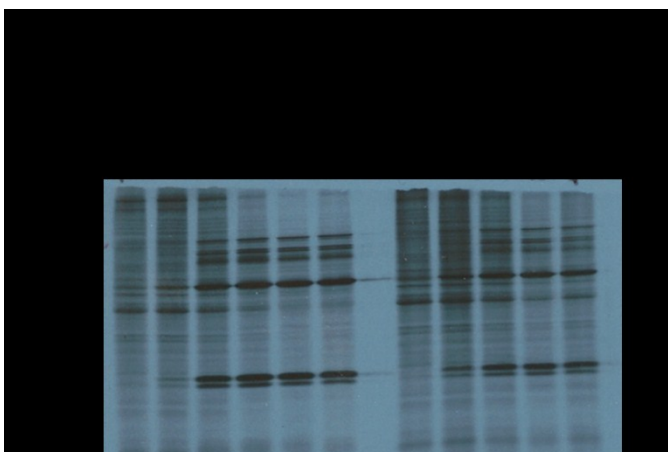
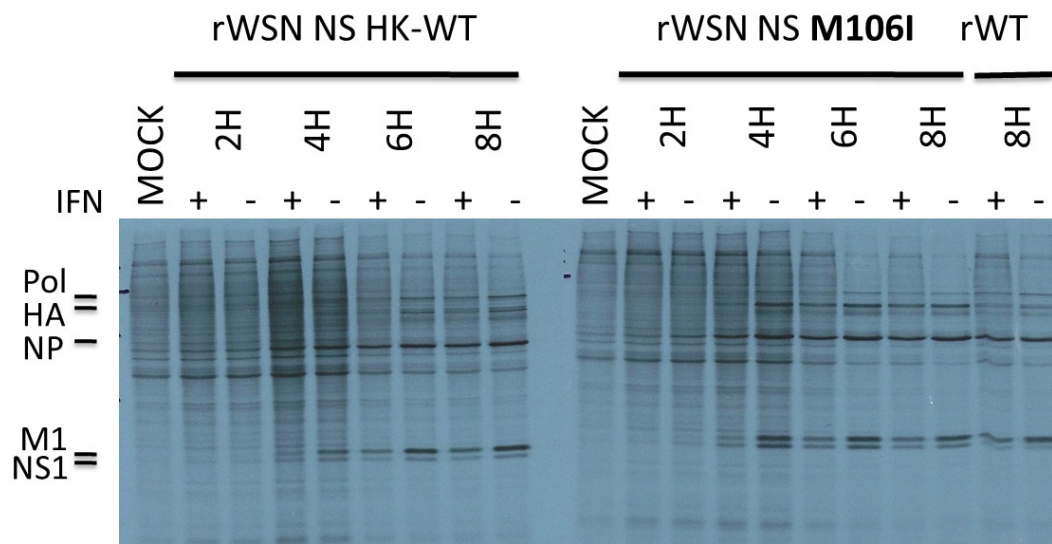
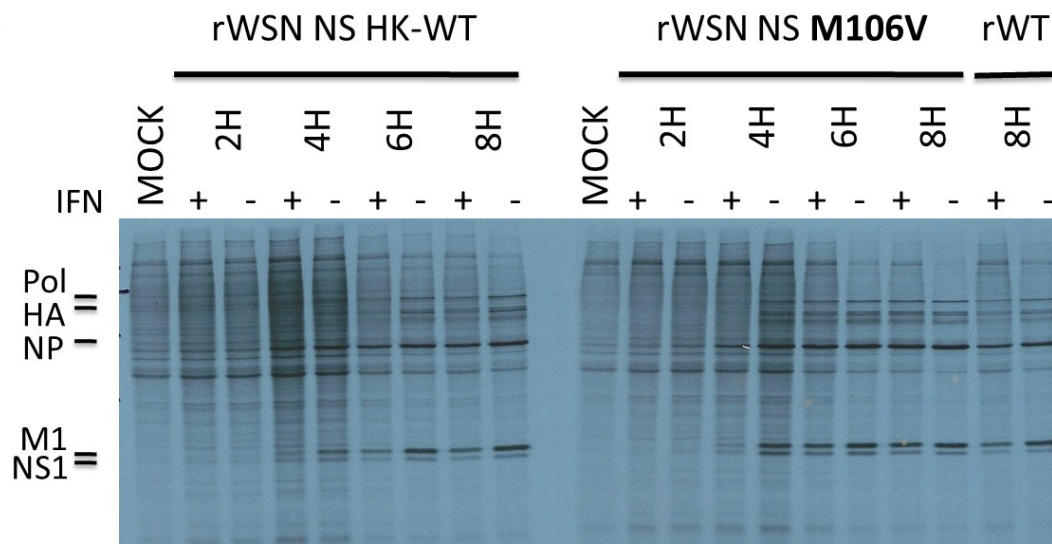
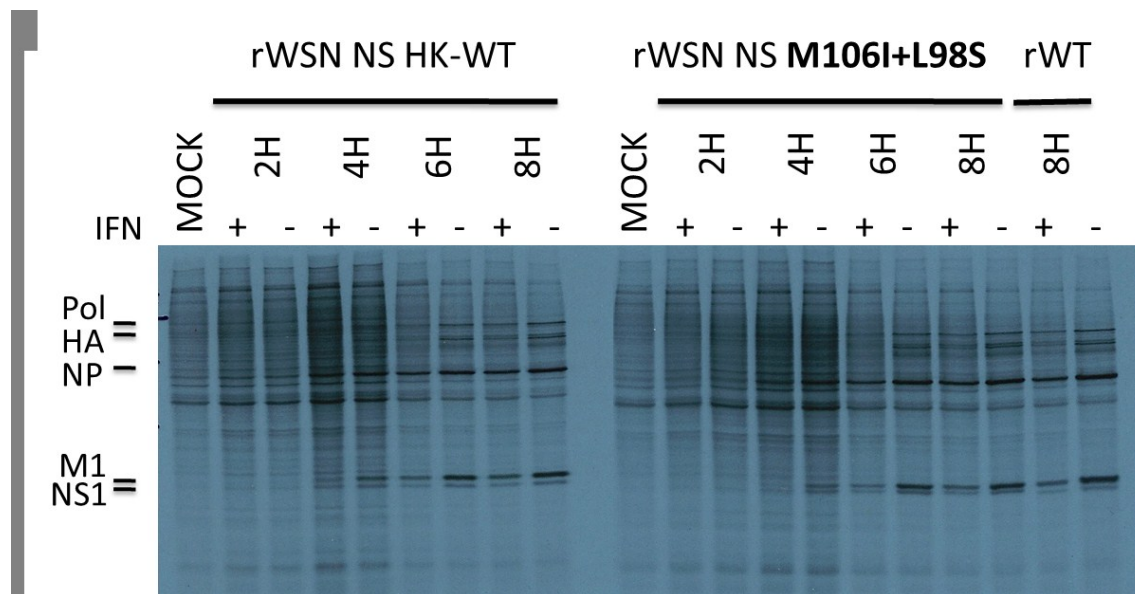


Figure S7. rWSN HK NS mutants increase rate of virus protein synthesis in A549 cells.

Confluent monolayers of A549 cells were left untreated or were treated for 6h with 200 U/mL human IFN α 2b. Following treatment, cells were infected at an MOI = 2 with rWSN viruses possessing HK NS wt or HK NS mutant genes. At 2, 4, 6, and 8 hpi*, untreated cells were washed with 1 x PBS, then pulsed for 1 hour with 80 uCi/mL ^{35} S radiolabeled Cys and Met amino acids diluted in serum-free MEM deficient in Cys and Met. Following pulse, cell lysate was collected and subject to autoradiography analysis. Viral protein bands are indicated to the left of autoradiography images.* IFN-treated cells were pulsed only at 8hpi and are indicated as 8+IFN in figure.

A.





B.

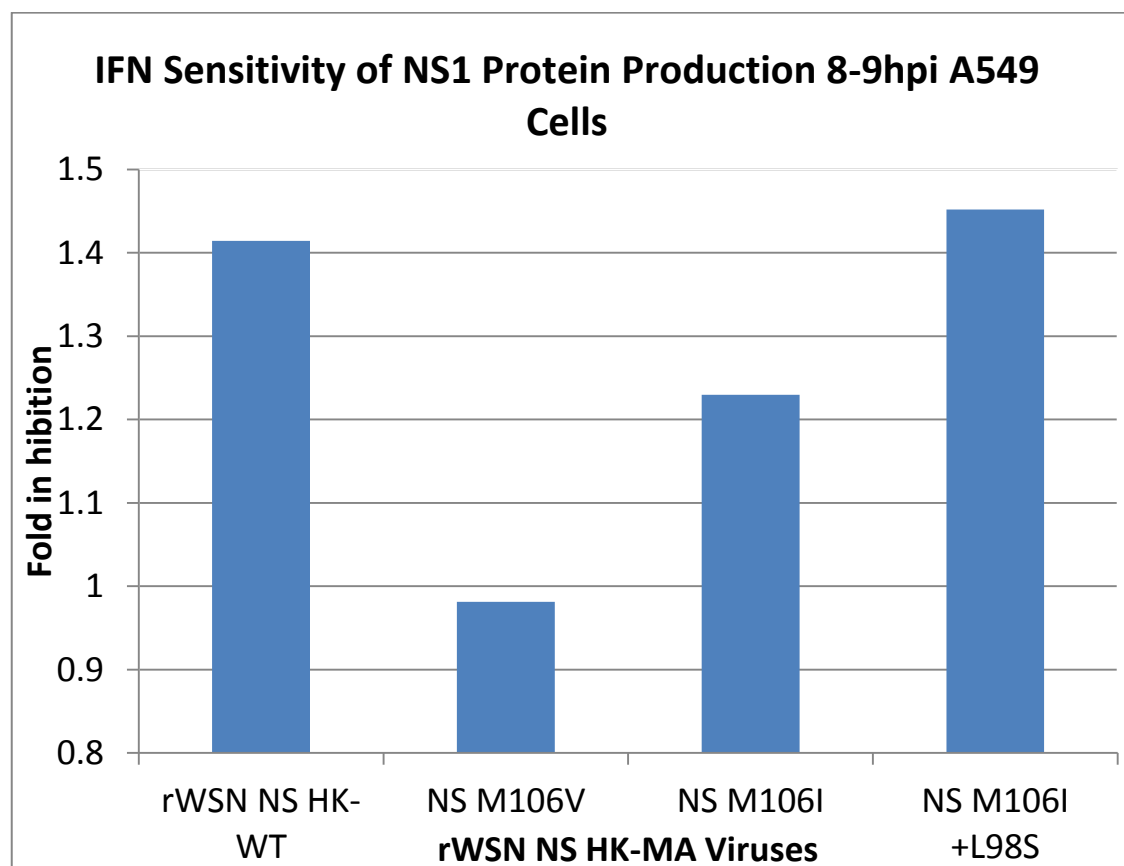


Figure S8. Effect of IFN on several rWSN HK NS mutant virus protein production in A549 cells. Confluent monolayers of A549 cells were left untreated or were treated for 24h with 1000 U/mL human IFN α 2b. Following treatment, cells were infected at an MOI = 2 with rWSN viruses possessing HK NS wt or HK NS mutant genes. At 2, 4, 6, and 8 hpi, cells were washed with 1 x PBS, then pulsed for 1 hour with 80 uCi/mL ^{35} S radiolabeled Cys and Met amino acids diluted in serum-free MEM deficient in Cys and Met. Following pulse, cell lysate was collected and subject to autoradiography analysis (A). Viral protein bands are indicated to the left of autoradiography images. (B) IFN sensitivity of virus protein production was determined by densitometry analysis of NS1 bands from cells pulsed at 8 hpi, where the density of untreated cells was divided by the density of cells pretreated with IFN.

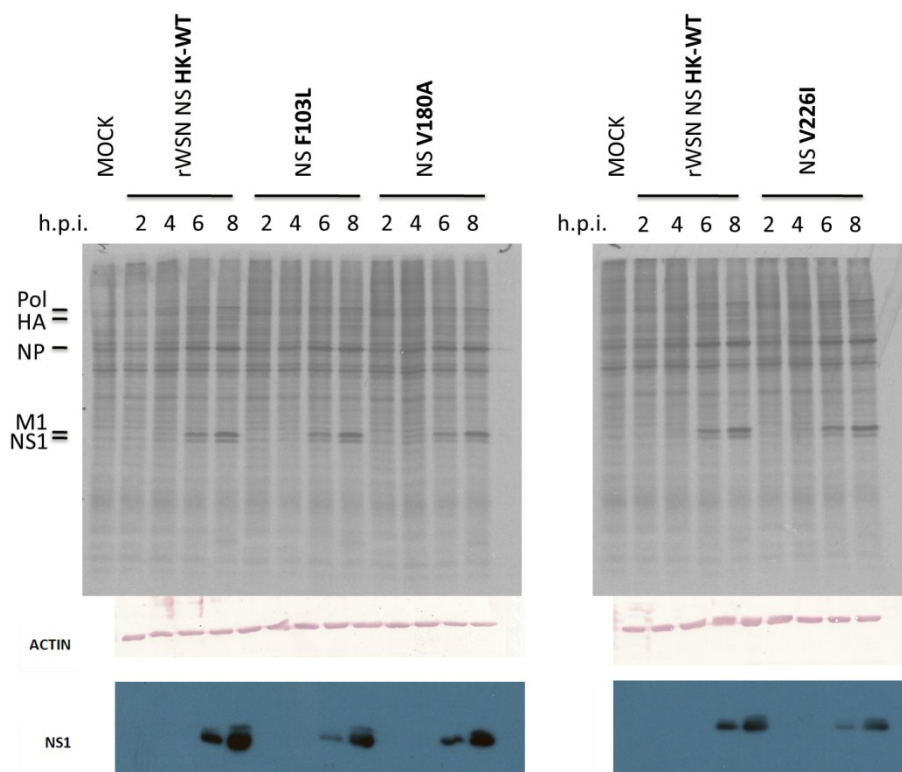
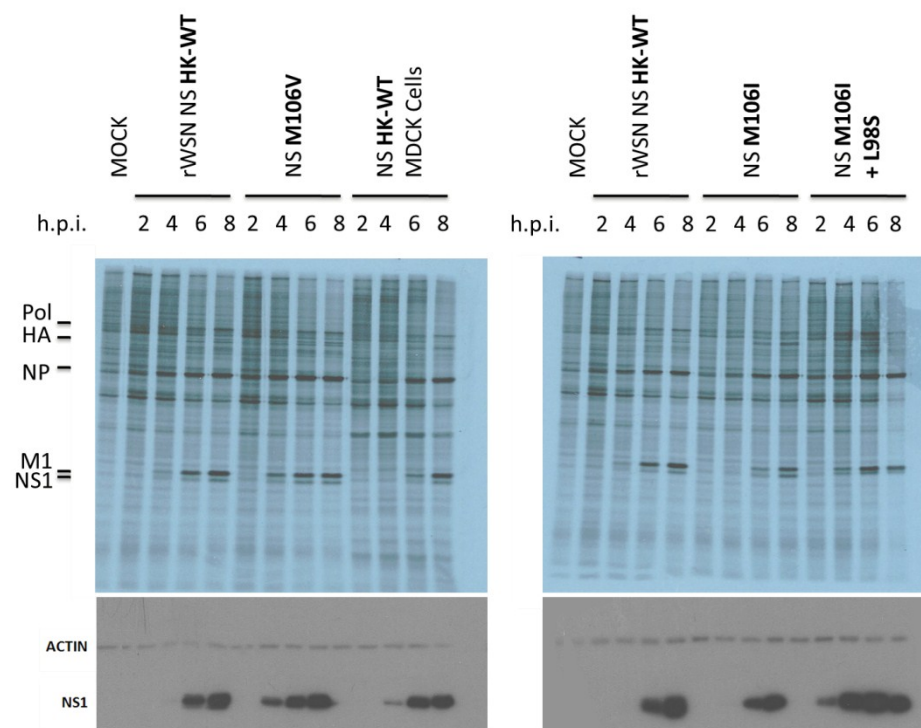
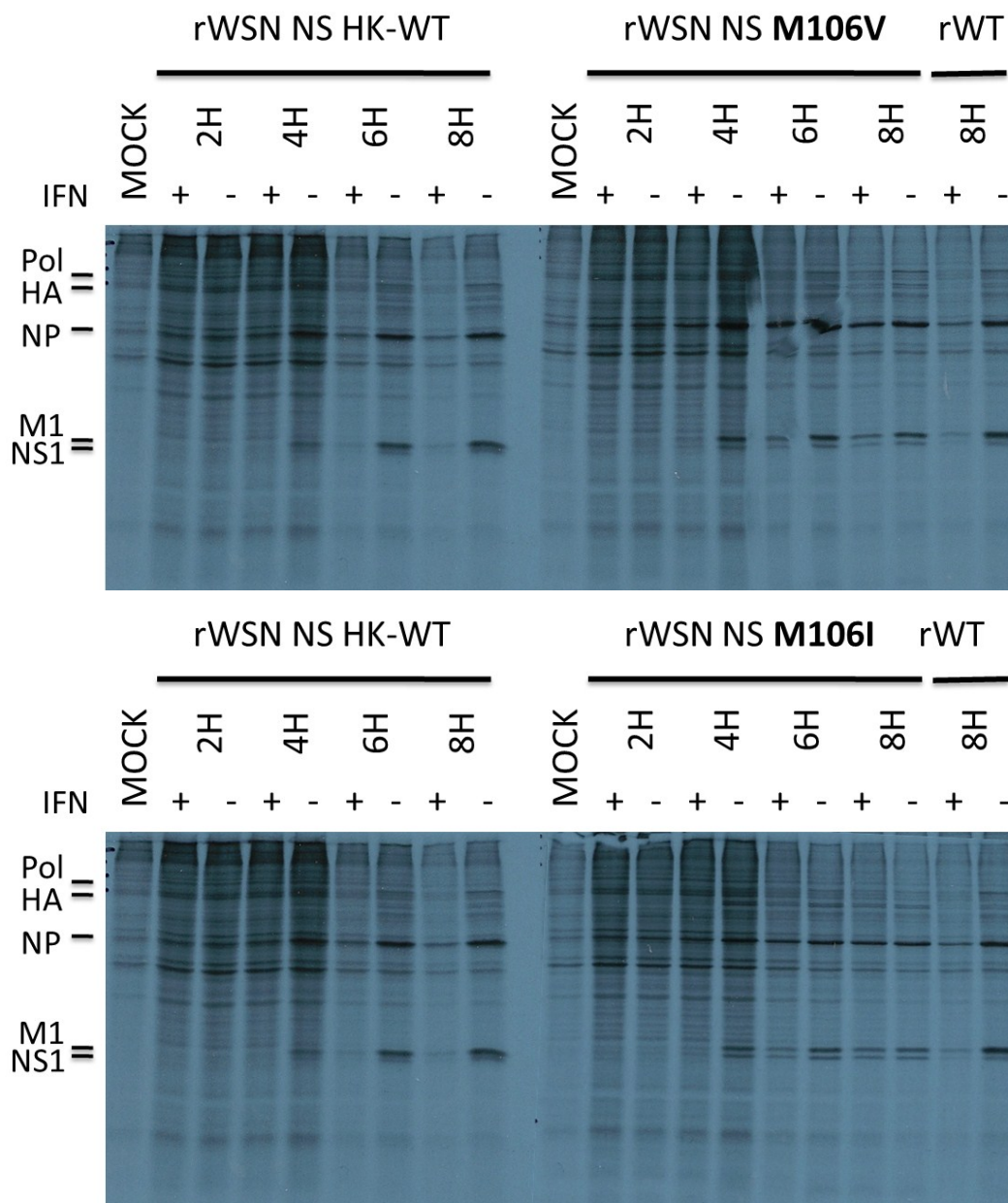
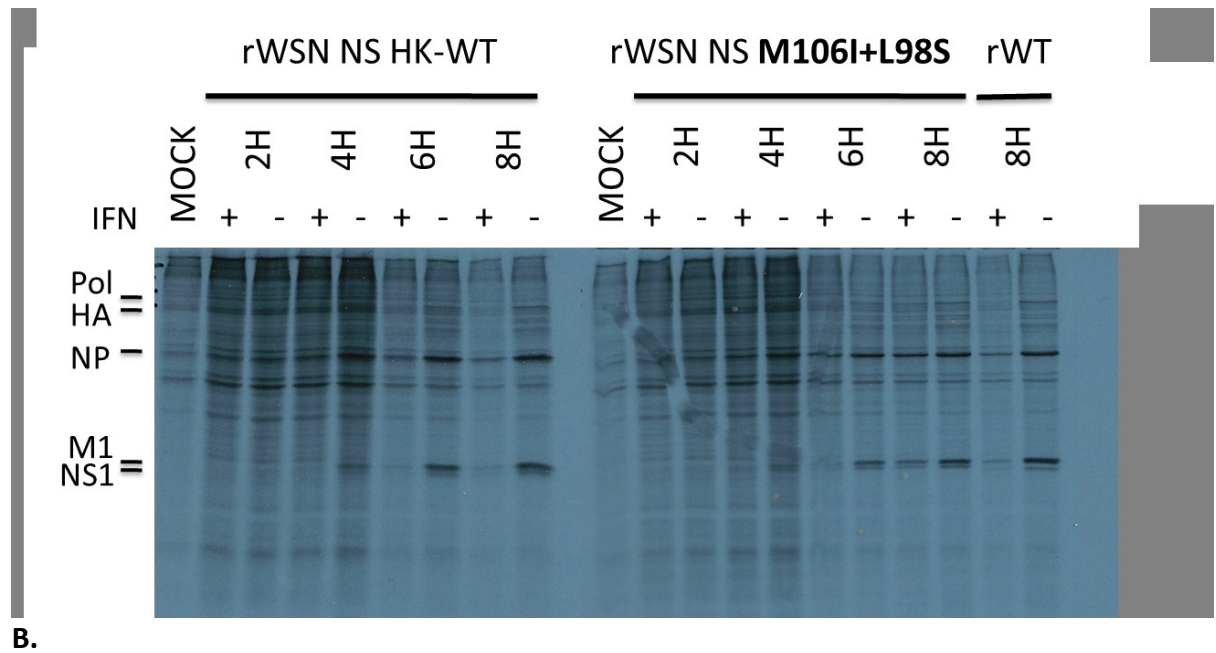


Figure S9. rWSN HK NS mutants increase rate of virus protein synthesis in mouse M1 cells.

Confluent monolayers of M1 cells were infected at an MOI = 2 with rWSN viruses possessing HK NS wt or HK NS mutant genes. At 2, 4, 6, and 8 hpi cells were washed with 1 x PBS, then pulsed for 1 hour with 80 uCi/mL ^{35}S radiolabeled Cys and Met amino acids diluted in serum-free MEM deficient in Cys and Met. Following pulse, cell lysate was collected and subject to autoradiography as well as Western blot analysis (as per the previous Western blot protocol, detecting NS1 and actin proteins). Viral protein bands are indicated to the left of autoradiography images.

A.





IFN Sensitivity of NS1 Protein Production 8-9hpi M1 Cells

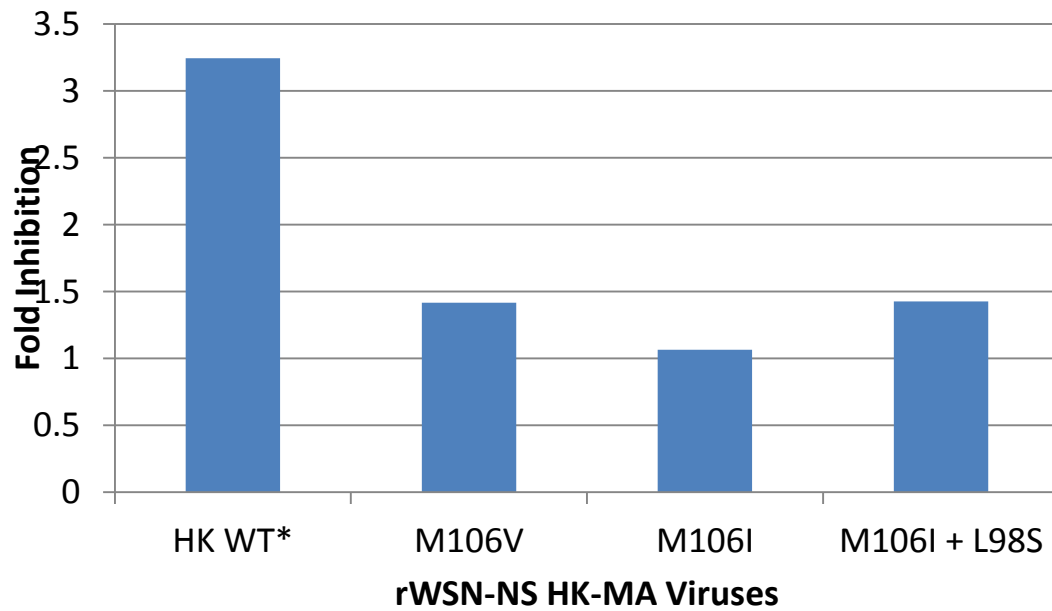


Figure S10. Effect of IFN on rate of virus protein synthesis in M1 cells for several rWSN HK NS mutants. Confluent monolayers of M1 cells were left untreated or were treated for 24h with 200 U/mL human IFN β . Following treatment, cells were infected at an MOI = 2 with rWSN viruses possessing HK NS wt or HK NS mutant genes. At 2, 4, 6, and 8 hpi, cells were washed with 1 x PBS, then pulsed for 1 hour with 80 uCi/mL ^{35}S radiolabeled Cys and Met amino acids diluted in serum-free MEM deficient in Cys and Met. Following pulse, cell lysate was collected and subject to autoradiography analysis (A). Viral protein bands are indicated to the left of autoradiography images. (B) IFN sensitivity of virus protein production was determined by densitometry analysis of NS1 bands from cells pulsed at 8 hpi, where the density of untreated cells was divided by the density of cells pretreated with IFN.

NICOLE FORBES

Curriculum Vitae

Education

PhD Candidate, Biochemistry, Microbiology and Immunology

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University of Ottawa, Ottawa, Ontario

Thesis: *Multifunctional adaptive NS1 mutations are selected upon human influenza A virus evolution in the mouse*

Anticipated defence date: November 2012

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May 2007

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Bishop's University, Lennoxville, Quebec

May 2003

Previous Employment

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1/2009-4/2009)

Supervisors: Dr Ken Dimock, Dr Earl Brown, Dr Miguel Rodriguez

Course marker for undergraduate courses: Virology, and Pathogens in the Environment;

Lab demonstrator for undergraduate introductory biochemistry course (Bilingual)

Laboratory Technician, University of Ottawa

(10/2007-12/2007)

Ottawa, Ontario; Department of Microbiology and Immunology

Supervisor: Dr Earl Brown

Propagated Influenza A virus isolates, extracted and purified genomes for sequencing

Supervisor, Starbucks Coffee Company

(9/2007-2/2008)

Ottawa, Ontario

Summer Graduate Research Student, OHRI

(5/2007-8/2007)

Ottawa, Ontario

Supervisor: Dr Robin Parks

Engineered oncolytic Adenovirus vectors to selectively target cancer cells

Honours Research Student, Dalhousie University

(9/2006-5/2007)

Halifax, Nova Scotia; Department of Microbiology

Supervisor: Dr Roy Duncan

Explored the mechanism of Reovirus FAST protein p14 cellular fusion enhancement

Bartender and Supervisor, The Grawood (8/2005-5/2007)

Halifax, Nova Scotia; Dalhousie University

Supervisor: Greg Wright

Trained incoming staff, managed and tended bar

Co-op Lab Technician, Maxxam Analytics (1/2006-8/2006)

Bedford, Nova Scotia

Supervisor: Vicki Green

Analyzed food, soil, and water samples for the presence of pathogenic bacteria

Co-op Lab Technician, Oland Brewery (5/2005-12/2005)

Halifax, Nova Scotia

Supervisor: Paul Chau

Collected brewery samples and performed chemical and microbiological analyses

Honours and Research Prizes

1. University of Ottawa Faculty of Medicine Award of Excellence

December 2011; \$500

2. Cedarlane Best Student Oral Presentation Award, Canadian Society of Microbiologists

Annual Conference

June 2011; \$500

3. 3rd place Prize, PhD Student Poster Presentation, Department of Microbiology and Immunology, University of Ottawa

May 2011; \$50

4. Graduated with Honours from both Bishop's University and Dalhousie University

Scholarships

Queen Elizabeth II Graduate Scholarship in Science and Technology

September 2011-August 2012; \$15,000

Ontario Graduate Scholarship in Science and Technology

September 2010-August 2011; \$12,000

University of Ottawa Excellence Scholarship

September 2010- August 2012; \$6500 annually

University of Ottawa Graduate Student Admissions Scholarship

January 2008-August 2010; \$6650 annually

Leadership Roles

1. Student ambassador for University of Ottawa Faculty of Medicine Graduate Program recruitment: *Fall 2011*
2. Biochemistry, Microbiology and Immunology Graduate Students Association
VP Internal: *April 2011- May 2012*
VP Communication: *January 2010-March 2011*
VP Academics: *January 2009-December 2010*
3. Biochemistry, Microbiology, and Immunology Departmental Bulletin
Editor-in-chief: *April 2010-September 2011*
4. Ottawa Regional Microbiology Symposium Co-organizer: *2009-2010*
5. Microbiology and Immunology Journal Club Coordinator: *November 2008-September 2010*

Publications

1. Ping, J., Selman, S., Tyler, S., **Forbes, N.E.**, Keleta, L., and E.G. Brown (2012). Low pathogenic avian influenza A/Turkey/Ontario/6213/1966(H5N1) is the progenitor of highly pathogenic A/Turkey/Ontario/7732/1966(H5N9). *Journal of General Virology*; doi:10.1099/vir.0.042895-0.
2. **Forbes, N.E.**, Ping, J., Dankar, S.K., Jia, J.J., Selman, M., Keleta, L., Zhou, Y., and E.G. Brown (2012). Multifunctional adaptive NS1 mutations are selected upon human influenza virus evolution in the mouse. *PLoS ONE*; 7(2): e31839.
3. Ping, J.¹, Keleta, L.¹, **Forbes, N.E.**¹, Dankar, S., Stecho, W., Tyler, S., Zhou, Y., Babiuk, L., Weingartl, H., Halpin, R.A., Boyne, A., Bera, J., Hostetler, J., Fedorova, N.B., Proudfoot, K., Katznel, D.A., Stockwell, T.B., Ghedin, E., Spiro, D.J., and E.G. Brown (2011). Genomic and protein structural maps of adaptive evolution of human influenza A virus to increased virulence in the mouse. *PLoS One*; 6(6):e21740. ¹authors contributed equally to the work
4. Dankar, S.K., Wang, S., Ping, J., **Forbes, N.E.**, Keleta, L., Li, Y., and E.G. Brown (2011). Influenza A virus NS1 gene mutations F103L and M106I increase replication and virulence. *Virology Journal*. Jan 12;8:13.
5. Ping, J., Dankar, S.K., **Forbes, N.E.**, Keleta, L., Zhou, Y., Tyler, S., and E.G. Brown (2010). PB2 and HA mutations are major determinants of host range and virulence in mouse-adapted Influenza A virus. *Journal of Virology*. 84(20):10606-10618.
6. Liu, Q., Wang, S., Ma, G., Pu, J., **Forbes, N.E.**, Brown, E.G., and J.H. Liu (2009). Improved and simplified recombineering approach for influenza virus reverse genetics. *Journal of Molecular and Genetic Medicine*. 3:225-31.

Research Presentations

1. **Forbes, N.E.**, Ping, J., Dankar, S.K., Keleta, L., and Brown, E.G. NS1 mutations in mouse-adapted influenza A viruses increase virulence, protein synthesis, and interferon antagonism. 61st Annual conference of the Canadian Society of Microbiologists, Memorial University, St John's NL, June 2011 (Refereed; oral presentation).
2. **Forbes, N.E.**, Dankar, S.K., Ping, J., Keleta, L., and Brown, E.G. Experimental evolution of Influenza A virus: Adaptive mutations in the NS1 gene enhance viral protein synthesis and interferon resistance. American Society of Virology 2010 Meeting, University of Montana, July 2010 (Refereed; poster presentation).
3. **Forbes N.E.**, Dankar, S., Ping, J., Keleta, L., and Brown, E.G. Experimental evolution of Influenza A virus: Adaptive mutations in the NS1 gene enhance viral protein synthesis and interferon resistance. Canadian Pandemic Preparedness Meeting, Montreal, November 2010 (Refereed; poster presentation).
4. **Forbes, N.E.**, Keleta, L., Wang, S., Stecho, W., Li, Y., Liu, S., and Brown, E.G. Experimental evolution of Influenza A virus: Characterization of adaptive mutations in the NS1 gene. American Society of Virology 2009 Meeting, UBC Vancouver, July 2009 (Refereed; oral presentation).

Graduate Courses Completed

MIC8125: Innate Immunology

Completed Winter 2010

Mark: A+

MIC8700: Biology and Pathogenesis of HIV Infection

Completed Winter 2008

Mark: A

MIC8238: Advanced Topics in Bacteriology: Mechanisms of Pathogenesis

Completed Fall 2008

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MIC8236: Advanced Topics in Virology

Completed Winter 2009

Mark: A+

Laboratory Skills

Virology: Plaque purification of viral isolate, titration by plaque assay, growth assay in vitro, amplification of viral particles in fertilized chicken egg, virus purification in sucrose gradient by centrifugal fractionation, large-scale viral RNA preparation for sequencing, immunocytochemistry of viral proteins in vitro, immunofluorescence detection of viral proteins from histological samples, viral growth assays in vitro and in vivo in mouse lung

Immunology: ELISA assay for interferon β on both cell supernatant and animal tissue, trained on MAGPIX (cytokine multiplex system)

In vivo techniques: Intranasal inoculations, subcutaneous injections, handling techniques, anesthesia, euthanasia, tissue extraction (all in mouse model)

In vitro **techniques:** Maintained numerous mammalian cell lines, transfected genetic material

Proteomics: SDS-PAGE gels, western blotting by both horseradish peroxidase and alkaline phosphatase method, ^{35}S pulse and pulse chase labeling, autoradiography, in vitro transcription/translation, immunoprecipitation

Molecular Genetics: Genetic cloning (PCR, ligation, restriction enzyme digests, recombination, transformation, alanine scan mutagenesis), ligation independent cloning of Influenza gene segments, DNA and RNA purification, plasmid amplification

Bacteriology: Identification of unknown bacteria via selective media and metabolic tests, test for presence/absence of coliform bacteria, quality control of microbiological media

Microscopy: Phase microscopy, light microscopy, fluorescent microscopy, transmission electron microscopy, confocal microscopy

Statistics: t-test, z-score, proficient in excel and prism software

Organizations

Canadian Society of Microbiologists

American Society for Virology

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