Characterisation of novel bat influenza A virus NS1 proteins

Hannah L. Turkington

MRC - University of Glasgow Centre for Virus Research

Supervisor: Dr Benjamin G. Hale

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DECLARATION

I, Hannah L. Turkington, declare that the following thesis (of approximately 30,400 words in length) has been written entirely by me, and is representative of the work carried out by me over the course of the previous year (unless otherwise stated). The work described here has also not been submitted in any other thesis for a higher degree.

Certain figures used in this thesis were obtained from other sources (components of Figures 2 and 3), or were generated using other previously published figures for inspiration (Figure 1). In all cases the source or inspiration for the figures used here is referenced appropriately.

Date…….. Signed……

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List of Abbreviations

CARD	caspase recruitment domain
CPSF30	cleavage and polyadenylation specificity factor 30kDa subunit
DAPI	4’,6’-diamidino-2-phenylindole
DC	dendritic cell
DMEM	Dulbecco’s Modified Eagle Medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
dNTPs	deoxynucleotide triphosphates
dsRNA	double stranded ribonucleic acid
ED	effector domain (NS1)
EDTA	ethylenediaminetetraacetic acid
eIF2α	eukaryotic initiation factor 2 alpha
eIF4F	eukaryotic initiation factor 4F
EMEM	Eagle’s Modified Medium
FF-luc	Firefly luciferase
FBS	fetal bovine serum
GFP	green fluorescent protein
GST	glutathione S-transferase
HA	haemagglutinin
hPAF1C	human PAF1 complex
HRP	horseradish peroxidase
HSV-TK	herpes simplex virus thymidine kinase
IAV	influenza A virus
IF	immunofluorescence
IFN-β	interferon-β
IP	immunoprecipitation

IRES	internal ribosome entry site
IRF-3	interferon regulatory factor 3
ISG	interferon stimulated gene
iSH2	inter-Src Homology 2 domain (p85β)
LAR	luciferase assay reagent
LB	Luria Broth
M1	matrix protein
M2	ion channel protein
MCS	multiple cloning site
MOPS	3-(N-morpholino)propanesulfonic acid
mRNA	messenger ribonucleic acid
NA	neuraminidase
NDV	Newcastle Disease virus
NEP/NS2	nuclear export protein/non-structural protein 2
NES	nuclear export signal
Neu5Ac	N-Acetylneuraminic acid
NLS	nuclear localisation signal
NoLS	nucleolar localisation signal
NP	nucleoprotein
NS1	non-structural protein 1
OAS	oligoadenylate synthetase
PA	polymerase acidic
PABPII	polyadenine binding protein II
PAn	polymerase acidic N-terminal
PB1	polymerase basic protein 1
PBS	phosphate buffered saline
PBS-T	phosphate buffered saline-Tween 20 (0.1%)
PCR	polymerase chain reaction

PI3K	phosphoinositide 3-kinase
PIV5	parainfluenza virus 5
PKR	protein kinase R
PR8	A/Puerto Rico/8/34 virus
PVDF	polyvinylidene fluoride
RBD	RNA-binding domain (NS1)
Ren-luc	Renilla luciferase
RIG-I	retinoic acid-inducible gene 1
RT-PCR	reverse transcription polymerase chain reaction
RT-qPCR	real time-quantitative polymerase chain reaction
SARS	severe acute respiratory syndrome
SDS-PAGE	sodium dodecyl sulphate - polyacrylamide gel electrophoresis
SeV	Sendai virus
SOC	super optimal broth
STAT	Signal Transducer and Activator of Transcription
svRNA	virus-derived small RNA
Tx98	A/swine/Texas/4199-2/1998
rUd	recombinant Udorn virus
rWSN	recombinant WSN virus
TNF-α	tumour necrosis factor alpha
UDB	urea disruption buffer
UTR	untranslated region
UV	ultraviolet
vRNP	viral ribonucleoproteins complex
WB	western blot
zsG	zs Green fluorescent protein

SUMMARY

Novel influenza A virus (IAV) genomes were recently discovered in two South American bat species and were designated the unique subtypes of H17N10 and H18N11. Concerns are presented for cross-species transmission as the introduction of novel IAV subtypes into naïve populations is often linked to severe disease outbreaks. Previous studies have synthesised chimeric bat IAVs, with the six internal H17N10 gene segments and the HA and NA gene segments from laboratory IAVs, and have shown that reassortment between IAVs from bats and other species is unlikely (Juozapaitis *et al.*, 2014, Zhou *et al.*, 2014). It was also revealed that the H17N10 NS1 protein can antagonise the interferon-β (IFN-β) response in human cells to a level comparable to that of a human IAV NS1 (PR8 H1N1). However, in the context of a chimeric bat IAV, carboxy-terminal truncations affecting this ability had less of an effect on virus replication *in vivo* compared to the human virus. This suggests that there may be functional differences between these NS1 proteins.

This study sought to further characterise the H17N10 NS1 to include the H18N11 NS1 protein, and to compare their molecular functions with a human H1N1, avian H5N1 and avian H7N9 NS1 proteins. Functions of the bat IAV NS1 proteins were predicted with a preliminary sequence alignment analysis, which also elucidated their high sequence divergence. Functional characterisation studies determined that, like all NS1 proteins, the two bat IAV NS1 proteins are able to antagonise the IFN-β response in a range of cell types from different species. In that regard, interactions with the host proteins RIG-I and Riplet were confirmed for the H17N10 NS1. However, unlike many human-adapted IAV NS1 proteins, the two bat NS1 proteins were unable to block general gene expression in human and bat cells. In fact, the bat IAV NS1 proteins appear to enhance gene expression, particularly in bat cells, however the mechanism behind this was not determined.

A major laboratory interest was to discover neither of the bat IAV NS1 proteins can interact with the human p85β subunit of phosphoinositide 3-kinase (PI3K), representing the first naturally occurring NS1 proteins not to bind. Other NS1 proteins are postulated to activate PI3K to benefit IAV replication. Attempts to restore p85β binding with six single residue substitutions in the H17N10 NS1 were unsuccessful, suggesting multiple residue changes have occurred to abrogate binding. Future work will focus on determining if the bat IAV NS1 proteins bind p85β subunits in the species from which these genomes were identified, or indeed if they have evolved to bind other bat cellular factors. Furthermore, these NS1 proteins could be utilised for studying the functions of other NS1 proteins binding and activating the PI3K pathway.

Chapter 1: INTRODUCTION

1.1 Influenza A viruses (IAV)

1.1.1 Orthomyxoviridae

Influenza A viruses (IAVs) belong to the family *Orthomyxoviridae* which includes five other genera; influenza B viruses, influenza C viruses, Isaviruses, Thogotoviruses and Quaranjaviruses (Plarre et al., 2012, Leahy et al., 1997, Presti et al., 2009, Krossoy et al., 1999). These viruses all have negative-sense, single stranded and segmented RNA genomes (Bouvier and Palese, 2008).

Influenza A, B and C viruses are evolutionary distinct genera, with differences in viral morphology, number of gene segments and viral proteins encoded (Palese and Shaw, 2013). A further distinction lies in the natural host-range of these viruses; influenza B and C viruses have narrow host ranges encompassing solely humans and seals for influenza B viruses (Osterhaus et al., 2000) and humans and pigs for influenza C viruses (Kimura et al., 1997). IAVs however have the remarkable propensity for infecting a wide range of species that include humans, birds, pigs, horses, dogs, various sea mammals and cats (Palese and Shaw, 2013).

The wide range of IAVs necessitates the need for further classification according to the nature of their surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA), of which 18 subtypes of HA (H1 to H18) and 11 subtypes of NA (N1 to N11) have been described to date (Mehle, 2014).

1.1.2 Influenza virus and disease

Influenza A, B and C viruses are all capable of causing seasonal epidemics of influenza disease (Bouvier and Palese, 2008). These epidemics peak annually between December and March and can affect up to 5 million people worldwide, causing up to 500,000 deaths (WHO, 2014). Influenza is characterised by the sudden onset of a high fever, cough, headache, and muscle and joint pains. The majority of otherwise healthy people recover within a week (WHO, 2014), however, in high risk groups such as the elderly, infants or pregnant women, influenza can lead to severe disease or even death (WHO, 2014). What is of greater concern, however, is the occasional occurrence of

pandemic influenza disease which presents a much higher risk to the general population.

1.1.3 Pandemic influenza

Within the *Orthomyxoviridae*, IAVs are solely responsible for pandemic disease, partly due to their ability to infect a wide host range. Three IAV pandemics have occurred in the 20th century, including the Spanish influenza (H1N1) of 1918, the Asian influenza (H2N2) of 1957 and the Hong Kong influenza (H3N2) of 1968 (Kilbourne, 2006). The deadliest of which was the 1918 pandemic which was responsible for up to 50 million deaths worldwide (Taubenberger and Morens, 2006). The first influenza pandemic of the 21st century was caused by the H1N1 influenza virus in 2009, which exhibited a case-fatality rate of only 0.4%, compared to that of the 1918 influenza which was 2.4% (Al Hajjar and McIntosh, 2010, Fraser et al., 2009).

The ability of different IAVs to infect the same host leads to the potential production of novel viral strains though genetic reassortment due to the segmented nature of the viral genome. Co-infection of the same host cell with two or more different IAVs can result in the reassortment of viral gene segments. This can theoretically result in the production of 254 different progeny viral genotypes from two different IAVs (Palese & Shaw, 2013). Reassortment can lead to the antigenic shift of the HA or NA glycoproteins, more specifically, a major antigenic change resulting in an immunologically distinct HA or NA that permits high infection rates in the presence of an immunologically naïve population (Palese & Shaw, 2013).

The last three IAV pandemics of 1957, 1968 and 2009 have been caused by reassortment events. The H2N2 subtype from the 1957 Asian pandemic contained avian HA, PB1 and NA gene segments reassorted with the circulating human H1N1 IAV segments, whereas the 1968 Hong Kong pandemic was caused by a virus containing avian HA and PB1 segments reassorted with the remaining human IAV gene segments from the circulating 1957 H2N2 strain (Scholtissek et al., 1978, Kawaoka et al., 1989). These reassortment events are documented in **Figure 1.** The 2009 H1N1 pandemic strain was generated from multiple reassortment events and included the NA and M segments from an avian-derived Eurasian swine lineage, HA, NP and NS segments from a classical swine lineage, PB2 and PA segments from an

avian-derived swine triple reassortment lineage, and finally the PB1 segment from a human-derived triple reassortment swine lineage (Garten et al., 2009).

1.1.4 IAV viral proteins

Previous characterisation of the IAV genome in the 20th century presented the accepted view that the 8 IAV genome segments encoded for 10 canonically expressed proteins, however recent analyses have revealed the possible expression of 7 further IAV proteins, most of which are likely to be strain-specific (Dubois et al., 2014), Palese & Shaw, 2013). The expression of these potential 17 proteins from their particular gene segments is shown in **Figure 2.** Encoded on the 8 genomic segments are the 10 canonically expressed proteins, the roles of which will be discussed in the context of the viral replication cycle; PB1, PB2 and PA which form the polymerase complex, the nucleoprotein (NP), surface glycoproteins haemagglutinin (HA) and neuraminidase (NA), the outer membrane ion channel (M2), the matrix protein (M1), the nuclear export protein (NEP/NS2), and the multifunctional virulence factor (NS1) (Palese & Shaw, 2013). The less-well described proteins include PB1-N40, PB1-F2, PA-X, PA-N182, PA-N155, M42 and NS3, the various functions of which will be discussed later.

1.1.5 IAV replication cycle – viral entry

The IAV surface glycoprotein HA is responsible for binding sialic acid receptors on the host cell surface, resulting in endocytosis and membrane fusion of the viral particle (Skehel and Wiley, 2000). This is shown in **Figure 2a/b**. IAVs attach to the terminal sialic acids present on glycoproteins and glycolipids, which can be linked to the penultimate galactose by an α-2,3-linkage or an α-2,6-linkage, the two predominant forms found in nature (Rogers and Paulson, 1983). From this, receptor-binding specificity of different HA proteins has emerged, with human IAVs usually recognising the α-2,6-linkage whereas avian IAVs usually recognise the α-2,3-linkage (Connor et al., 1994).

Binding of the viral HA and host cell sialic acid receptor results in endocytosis of the viral particle. Inside the endosome a low pH activates the fusion of viral and endosomal membranes due to conformational changes in the HA glycoprotein (Skehel and Wiley, 2000).

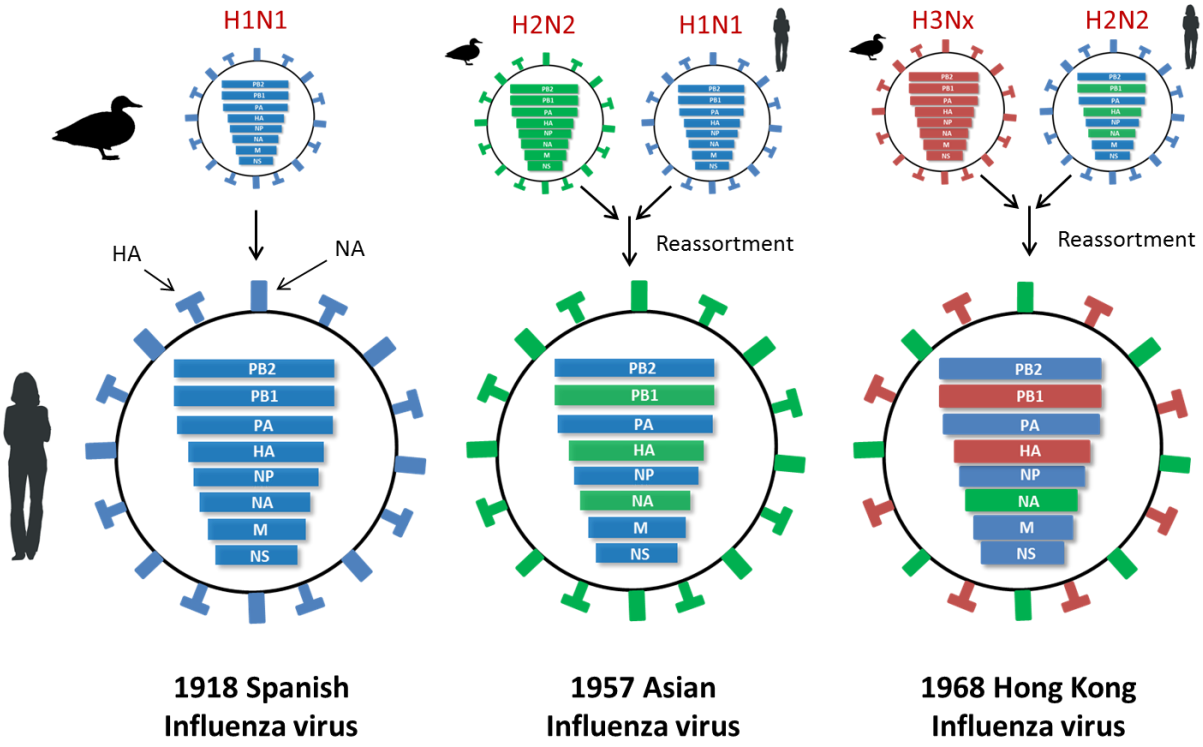


Figure 1. Schematic for the generation of pandemic influenza A viruses. The three IAV pandemics of the 20th century were caused by the presence of a novel IAV subtype entering the human population. The Spanish influenza of 1918 was caused by the direct transmission of an avian H1N1 virus into the human population. The 1957 Asian influenza pandemic was the result of a reassortment event between an avian H2N2 and a human H1N1 virus. The resultant pandemic H2N2 virus contained the PB1, HA and NA gene segments from the avian H2N2 virus and the remaining gene segments from the human H1N1 virus. The 1968 Hong Kong influenza pandemic was the result of another reassortment event between a novel avian H3Nx virus and the circulating 1957 H2N2 virus. The resulting H3N2 pandemic virus contained PB1 and HA gene segments from the avian H3Nx virus and the remaining segments from the 1957 H2N2 virus (figure was adapted from (De Clercq, 2006).

Viral proteins translated from the IAV genome

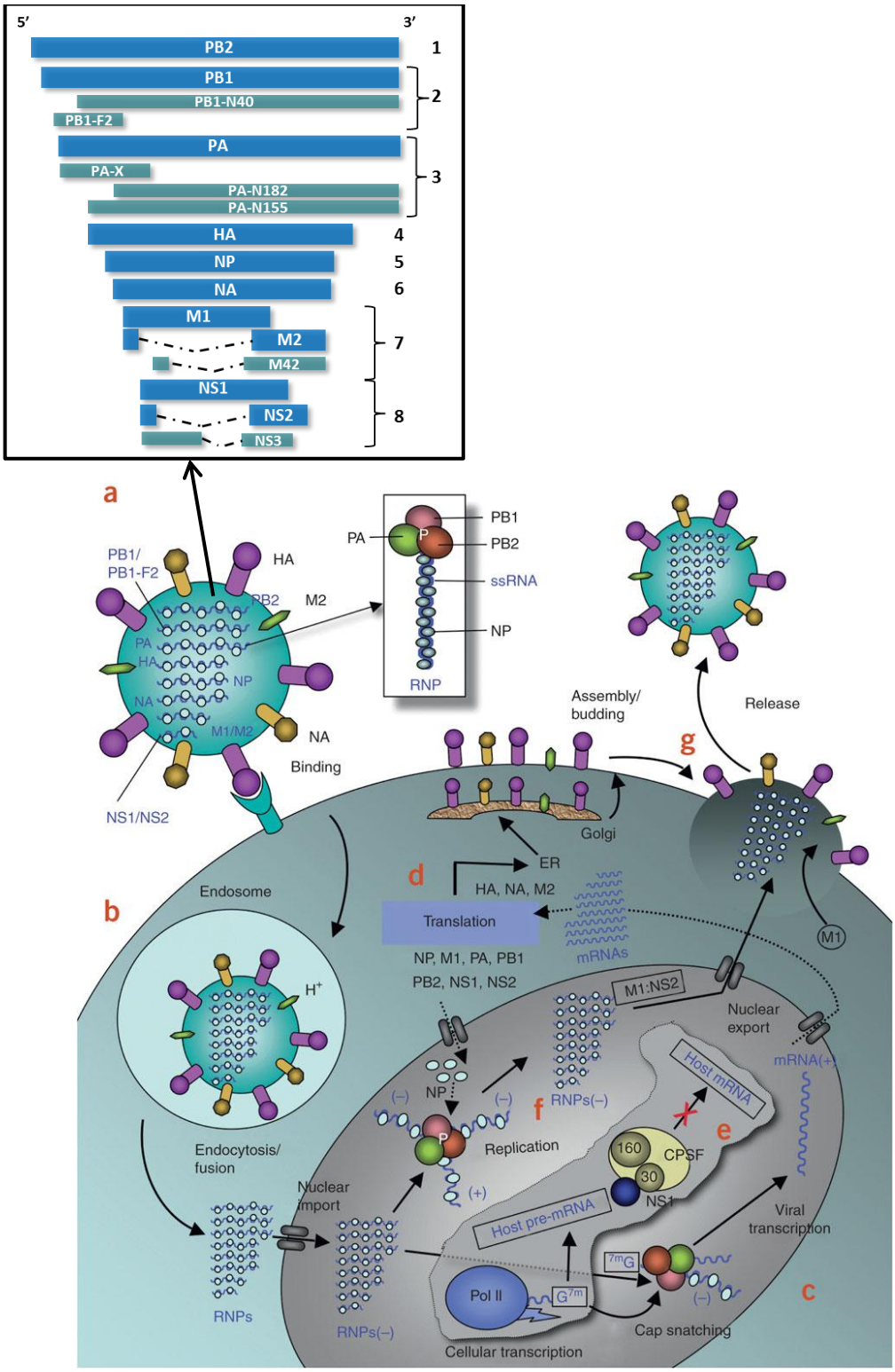


Figure 2. IAV replication cycle a) IAV particle contains 8 genomic segments with the expressed proteins from each indicated. **b)** Viral HA mediates sialic acid binding to the cell surface followed by endocytosis of the virus. Low pH triggers HA-mediated membrane fusion and M2-mediated release of viral RNPs into the cytoplasm. NP-encoded nuclear localisation signals mediate nuclear import. **c)** Viral polymerase synthesises mRNA with 5' caps cleaved by PB2 from host pre-mRNAs. **d)** Viral mRNAs are exported to the cytoplasm for translation. **e)** NS1 protein specifically blocks host gene expression. **f)** **V**iral polymerase also mediates replication of viral RNPs for transport to the cytoplasm and **g)** vRNPs are packaged into a new virion at the cell surface which is released. Diagram adapted from (Das et al., 2010).

An acidic pH in the endosome also facilitates the flow of protons selectively through the M2 ion channel, allowing acidification of the virus whilst in the endosome and resulting in viral uncoating (shown in **Figure 2b**) (Pinto and Lamb, 2006). Viral ribonucleoproteins (vRNPs) consisting of the RNA genomic segments, each associated with the trimeric viral polymerase (comprised of PB1, PB2 and PA) and the nucleoprotein, NP, are then released into the cytoplasm (Portela and Digard, 2002, Pinto and Lamb, 2006).

1.1.6 IAV replication cycle – nuclear import of vRNPs

A characteristic event in the IAV replication cycle is that viral transcription and replication occur in the nucleus of the host cell. The vRNPs must therefore be imported into the nucleus, a process that is mediated by nuclear localisation signals (NLSs) present in all of the viral components of the vRNP complex (PB1, PB2, PA and NP) (reviewed in (Boulo et al., 2007)). The two NLSs present on NP are necessary and crucial for the nuclear import of vRNPs (Wu et al., 2007, Cros et al., 2005).

1.1.7 IAV replication cycle – viral transcription and replication

In the nucleus, synthesis of viral mRNA is initiated by the PB2 subunit of the RNA polymerase complex, which binds the 5' cap of host cell pre-mRNAs (shown in **Figure 2c**) (Honda et al., 1999). This allows the endonuclease activity-containing PA subunit to cleave the pre-mRNAs 10 to 13 nucleotides downstream from the cap (Plotch et al., 1981, Dias et al., 2009). Viral transcription is then initiated from the 3' end of the cleaved template, with chain elongation catalysed by the major catalytic subunit of the RNA polymerase complex, PB1 (Kobayashi et al., 1996).

Replication of vRNA does not require 5' cap priming and involves the synthesis of full-length cRNA copies of vRNA (shown in **Figure 2f**). The exact mechanism regulating the switch between viral transcription and replication are not yet clear. However, as newly synthesised cRNA must be encapsidated, it has been suggested that the availability of NP plays a role in controlling this switch (Portela and Digard, 2002). The nuclear export protein (NEP) has also been reported to be a key player in regulating the switch between transcription and replication, specifically by structurally altering the transcription or replication machinery (Robb et al., 2009). Furthermore, it has been postulated that expression of IAV-derived small RNAs (svRNAs) during IAV

infection correlates with the accumulation of vRNA and the switch from transcription to genome replication, through interactions with the viral polymerase (Perez et al., 2010).

1.1.8 IAV replication cycle – packaging and budding

After their synthesis in the nucleus, viral RNPs are exported to the cytoplasm in a process facilitated by NEP (O'Neill et al., 1998) (shown in **Figure 2d**). Upon reaching the cell membrane, vRNPs are then packaged into new viruses (shown in **Figure 2g**). In particular, the M1 protein is essential for virus assembly and budding, and has been shown to interact with both viral RNPs and the cytoplasmic tails of the two glycoproteins HA and NA (Ali et al., 2000, Rossman and Lamb, 2011). NEP has also been found to play a role in efficient virion formation and budding, through its interaction with the plasma membrane-associated F_1F_0-ATPase (Gorai et al., 2012). Additionally, M2 is involved in viral budding and is believed, upon its recruitment, to stabilise the budding site and to consequently mediate membrane curvature of the budding virus, finally causing membrane scission and particle release (Rossman and Lamb, 2011).

The second surface glycoprotein NA acts to promote virus release from infected cells by preventing aggregation of viral particles by enzymatically cleaving the linkage between terminal sialic acids and the penultimate galactose residue on host cells (Colman, 1994, Wagner et al., 2002).

1.1.9 Recently identified IAV proteins and their functions

Two further proteins produced from segment 2 of the IAV genome, PB1-F2 and PB1-N40, are translated in different open reading frames from each other (Palese & Shaw, 2013). PB1-F2 is a strain-specific mitochondrial protein that targets the inner mitochondrial membrane to induce apoptosis specifically in human immune cells (Chen et al., 2001, Gibbs et al., 2003). PB1-F2 has also been reported to bind the IFN-β signalling protein MAVS to inhibit the production of IFN-β (Varga et al., 2012). PB1-N40 is an N-terminally truncated version of PB1 whose function during viral infection remains unknown, though it has been hypothesised to be involved in the regulation of transcription and replication (Wise et al., 2009, Tauber et al., 2012).

Segment 3 encodes additional proteins; PA-X, produced by ribosomal frameshift, and two N-terminally truncated versions, PA-N155 and PA-N182. PA-X is produced from an overlapping reading frame (X-ORF), retaining the N-terminal endonuclease domain of PA but with a functionally distinct C-terminus produced from the X-ORF by +1 ribosomal frameshifting (Jagger et al., 2012). PA-X was found to repress cellular gene expression and modulate the host response to IAV infection to reduce pathogenicity (Jagger et al., 2012). PA-N155 and PA-N182 were identified as smaller proteins translated from the PA mRNA from start codons at amino acid positions 155 and 182 respectively (Muramoto et al., 2013). Functional attributions for these novel proteins have yet to be assigned, however there is some indication that PA-N155 may play a role in viral replication, though this is yet to be determined (Muramoto et al., 2013).

Segment 7 was previously thought to encode only two proteins; M1 and M2 (Allen et al., 1980). In 2012, it was discovered that segment 7 of the IAV genome can encode a third M2-related protein termed M42 (Wise et al., 2012). M42 was found to contain a distinct ectodomain, but is non-essential for virus replication and expression seems likely to occur in only a small number of IAV strains (Wise et al., 2012). This was predicted from sequence analysis that revealed the presence of weak splice donor sites for M42 mRNA production in the major human-infecting IAV subtypes (Wise et al., 2012).

Lastly, until recently it was thought that segment 8 of the IAV genome also encoded only two proteins; NS1 and NEP, however the latest analysis has suggested a third non-structural protein can be produced, termed NS3 (Lamb and Choppin, 1979, Selman et al., 2012). NS1 and NEP are differentially synthesised due to splicing of the viral mRNA (Lamb and Choppin, 1979). In 2012, the existence of the protein NS3 was discovered to be a strain-specific occurrence due to the acquisition of a single nucleotide mutation, A374G of segment 8 (Selman et al., 2012). This mutation, and subsequent production of a novel protein spliced from NS1, was found in the context of a human IAV evolving in a mouse host and led to an increase in viral growth and antagonism of the IFN-β response (Selman et al., 2012). NS3 is an isoform of NS1, with an internal deletion of the codons 126 to 168 in NS1 (Selman et al., 2012). Furthermore, the A374G mutation was identified in 33 IAV strains that have naturally or experimentally adapted from avian hosts to mammalian ones, including humans,

suggesting NS3 production may be associated with viral host switching (Selman et al., 2012).

The recent identification of 7 previously unknown IAV proteins (since 2001) which have divergent and important influences during the virus replication cycle highlights the importance of characterising novel viral proteins in order to understand what role they may play during influenza pathogenesis.

1.2 Non-structural protein 1 (NS1)

As the functional characterisation of novel NS1 proteins was the focus of this work, the canonical structure and functions of this protein will now be discussed in greater detail. The multifunctional virulence factor NS1 is encoded on segment 8 of the IAV genome and shares its first 10 amino acids with NEP, which is produced from an alternate open reading frame due to a splice site (Lamb and Lai, 1980). NS1 mRNA is co-linear to the viral RNA transcript, translation of which produces a protein that has a strain-specific length of as little as 202/215 residues, but which is usually between 230 and 237 residues in length (Lamb and Lai, 1980).

1.2.1 Structure of NS1

The NS1 protein is comprised of several distinct domains which are depicted in **Figure 3A and B**. The N-terminal domain consists of residues 1 to 73 and is termed the RNA-binding domain (RBD) (Liu et al., 1997). The C-terminal domain consists of residues 88 to 202 and is termed the effector domain (ED) (Bornholdt and Prasad, 2008). The RBD and ED are capable of forming independent homodimers, both of which have been observed by X-ray crystallography (Liu et al., 1997, Hale et al., 2008a, Bornholdt and Prasad, 2006). The two main functional domains of NS1 are connected by a flexible inter-domain linker, and additionally there is a disordered C-terminal tail region found after residue 202 (Hale et al., 2008c). Lengths of the inter-domain linker and the C-terminal tail regions vary depending on IAV strains; linkers can be between 10 and 15 residues in length whereas C-terminal tails can vary between 11 and 33 residues (Hale, 2014).

1.2.1.1 RNA-binding domain

The RBD of NS1 assembles into a symmetrical homodimer containing three α-helices per monomer (Liu et al., 1997). The formation of the RBD dimer is essential for the binding of double-stranded RNA (dsRNA) which occurs via interactions between the phosphate backbone of dsRNA and highly conserved, basic and hydrophilic RBD residues (Yin et al., 2007). Residues that have been identified as crucial for the binding of dsRNA include Arg35, Arg38, Lys41, Ser42 and Thr49, and are highlighted in **Figure 2B** (Cheng et al., 2009). Specifically, Arg38 has been identified as being the only residue that is absolutely essential for the binding of dsRNA (Wang et al., 1999). Lys41 was shown to also contribute strongly to this binding, as an alanine mutant at this position results in a 10-fold decrease in dsRNA binding ability (Wang et al., 1999). Additionally, the serine at position 42 has been shown to undergo specific phosphorylation, with substitution to alanine resulting in a 10-fold decrease in dsRNA-binding ability (Hsiang et al., 2012).

1.2.1.2 Effector domain

The NS1 ED homodimer consists of monomers each comprising 7 β-strands and 3 α-helices (Bornholdt and Prasad, 2006). The actual interface for ED dimerisation is, however, disputed. Two models have been proposed, the strand-strand dimer and the helix-helix dimer, based on observations in X-ray crystallography structures (Bornholdt and Prasad, 2006, Hale et al., 2008a). The strand-strand form was the first to be observed in a crystal structure of an NS1 from the A/Puerto Rico/8/34 (PR8) strain, with the interface occurring between the N-terminal β-strands of each monomer (Bornholdt and Prasad, 2006). Later, a different dimerisation interface was observed in the crystal structure of an NS1 from the A/Duck/Albany/6/76 strain, and this was termed a helix-helix dimer due to the interface occurring between the long α-helices of each monomer (Hale et al., 2008a).

Further studies have confirmed that the helix-helix dimer is the relevant dimer form in solution, suggesting that this interface is the biologically relevant one. This was based on observations that the helix-helix form buries a greater surface area than the strand-strand form, and additionally that mutation of tryptophan 187 (W187), a residue which is critical for helix-helix dimerisation, abolishes dimer formation in solution (Hale et al., 2008a, Aramini et al., 2011, Kerry et al., 2014). Additionally, the residues involved

in the helix-helix dimer interface are highly conserved across all IAV strains, with W187 seen to be present in all IAVs that have been sequenced (Hale et al., 2008a, Aramini et al., 2011).

1.2.1.3 Full-length NS1

Whilst there have been crystal structures of full-length NS1 proteins, the precise conformations required for the biological functioning in the context of the host cell still remain unclear (Bornholdt and Prasad, 2008, Carrillo et al., 2014). The first full-length crystal structure of an IAV NS1 (H5N1) suggested that the ED and RBDs interact with neighbouring NS1 molecules in an alternating manner (with their respective domains) to produce a long tubular structure in which to bind dsRNA (Bornholdt and Prasad, 2008). A second crystal structure of another NS1 (H6N6) revealed that whilst the RBD remains a stable homodimer, the ED is capable of shuffling between 'open' (i.e. monomeric), 'semi-open' and 'closed' (dimeric) conformations due to the flexibility of the inter-domain linker (Carrillo et al., 2014). This conformational elasticity of the NS1 ED had previously been proposed, where the monomeric (or 'helix-open') form can preferentially interact with certain host proteins such as the cleavage and polyadenylation specificity factor (CPSF30), whose binding surface on the NS1 ED overlaps the dimerisation interface thus making this interaction and ED dimerisation mutually exclusive (Kerry et al., 2011, Das et al., 2008). The 'helix-closed' or dimeric ED is instead preferential for the binding of dsRNA in the context of full-length NS1, where abrogation of ED dimerisation has been shown to result in decreased dsRNA binding by the entire NS1 molecule (Kerry et al., 2011, Ayllon et al., 2012b). Thus it may be that alternating monomeric and dimeric NS1 ED conformations are related to the stage of IAV infection and the function of NS1 required at that particular time. The functions of the IAV NS1 protein during the viral replication cycle will now be discussed in detail.

1.2.2 Localisation of NS1

During the early stages of IAV infection, NS1 primarily localises to the nucleus of the host cell, however at later stages of infection NS1 is more prominently located in the cytoplasm of the cell (Greenspan et al., 1988, Melen et al., 2007). There have been two NLSs identified in the NS1 protein, an N-terminal NLS1 and a C-terminal NLS2 (Greenspan et al., 1988, Melen et al., 2007). The critical amino acids in the NLS1 were

found to be the same required for binding dsRNA; R35, R38 and K41, and it is thought that this signal mediates nuclear import via the classical importin α/β pathway (Melen et al., 2007). A functional NLS2 sequence requires the presence of basic arginine or lysine residues at positions 219, 220, 231 and 232, with this signal also functioning as a nucleolar localisation signal (NoLS) (Melen et al., 2007). Thus, these particular signals are strain-specific due to the varying C-terminal tail lengths of different NS1 proteins.

NS1 translocation into the cytoplasm is mediated by the presence of a hydrophobic nuclear export signal (NES) which is located between residues 134 and 147 of the ED (Li et al., 1998). This signal is specifically inhibited by 14 residues that are adjacent and downstream of the NES, therefore nuclear export of NS1 requires the alleviation of this specific inhibition, however the molecular mechanisms regulating this remain unknown (Li et al., 1998).

1.2.3 Function of NS1 during IAV infection

Among the many roles attributed to the NS1 protein during IAV infection are the abilities to disrupt host cell antiviral defences, particularly the interferon (IFN-α/β) response (Hale et al., 2008c), the ability to bind and activate the phosphoinositide 3-kinase (PI3K) signalling pathway (Hale et al., 2006), the favoured translation of viral mRNAs over host cell mRNAs (Aragon et al., 2000), inhibition of host cell adaptive immune responses (Fernandez-Sesma et al., 2006), and control of host cell apoptosis (Schultz-Cherry et al., 2001, Zhirnov et al., 2002). Many of these functions arise from the ability of NS1 to interact with a plethora of host cellular factors, of which a selection is shown in **Figure 3A.**

1.2.3.1 Antagonism of the innate immune response

During viral infection, the first-line antiviral defence mechanism for cells is the type I interferon (IFN) or IFN-α/β response, which is stimulated by the presence of viral factors for example viral RNA. IFN secretion induces the up-regulation of many IFN-stimulated genes (ISGs) which are responsible for the establishment of a cellular antiviral state (reviewed in (Randall and Goodbourn, 2008). It is generally accepted that the major role of the NS1 protein during IAV infection is the antagonism of this IFN response. Early observations using a mutant IAV deficient in NS1 showed that this virus could not replicate in wild type mice, but was able to do so in mice with

defective IFN signalling (STAT1-/-) pathways (Garcia-Sastre et al., 1998). It has been postulated that presence of the NS1 protein allows IAV to replicate proficiently whilst evading the immune system, for up to two days post-infection (Moltedo et al., 2009).

There are a number of functional mechanisms that the NS1 protein employs in order to antagonise host cell IFN responses. NS1 is able to interfere with the pre-transcriptional production of IFN itself, firstly through the RBD's ability to sequester dsRNA and therefore preventing its detection by the pattern-recognition receptor RIG-I which has been shown to preferentially recognise short genomic sequences of IAV (Kowalinski et al., 2011, Baum et al., 2010). Another study found that an IAV encoding an NS1 unable to bind dsRNA induced higher levels of IFN-β in cells in comparison to a virus able to sequester dsRNA (Donelan et al., 2003). It may also be the case that NS1 is able to interact directly with RIG-I to prevent induction of IFN (Guo et al., 2007). NS1 has been reported bind directly to two cellular ubiquitin E3 ligases, TRIM25 and Riplet, in order to prevent the ubiquitination of the RIG-I CARD domains necessary for downstream signalling and induction of IFN (Gack et al., 2009, Rajsbaum et al., 2012). These functions of NS1 may be responsible for NS1's ability to inhibit the activity of the transcription factors IRF-3 (Talon et al., 2000), NFκB (Wang et al., 2000) and c-Jun/ATF-2 (Ludwig et al., 2002) to prevent the pre-transcriptional production of IFN.

Antagonism of IFN production by NS1 also occurs at a global post-transcriptional level, particularly through interactions between NS1 and two cellular proteins: the 30 kDa subunit of the cleavage and polyadenylation specificity factor (CPSF30) and the poly(A)-binding protein II (PABPII). CPSF30 is responsible for cleavage and polyadenylation at the 3' end of host cellular pre-mRNAs in order to produce mature mRNAs (Li et al., 2001). The NS1 ED is able to interact with the CPSF30 F2F3 zinc finger domains preventing its normal processing activities and resulting in the selective inhibition of cellular mRNA production as viral mRNAs are processed by the viral polymerase and are thus not affected (Nemeroff et al., 1998, Das et al., 2008). Additionally, binding of the PABPII protein by NS1 further blocks the processing of host cell pre-mRNAs by preventing poly(A) tail elongation and subsequent nuclear export of mRNAs (Chen et al., 1999).

Finally, NS1 can also act to inhibit the functions of two host cell antiviral proteins; the 2'-5'-oligoadenylate synthetase (OAS) and protein kinase R (PKR). Inhibition of the

OAS/RNase L pathway is achieved by the sequestering of dsRNA by NS1, thus preventing activation OAS-mediated activation of RNase L and the subsequent degradation of viral RNA, which would inhibit viral replication (Min and Krug, 2006, Silverman, 2007). In the case of the serine/threonine kinase PKR, binding of NS1 to PKR (through residues 123-127 of the ED) blocks its activation by either dsRNA or the protein PACT and prevents phosphorylation of PKR substrates such as the eukaryotic translation initiation factor 2α (eIF2α) which would detrimentally effect viral protein production (reviewed in (Clemens and Elia, 1997), (Li et al., 2006), (Min et al., 2007). The ability of NS1 to bind dsRNA or PACT themselves may also contribute to this antagonism (Tawaratsumida et al., 2014, Cheng et al., 2009).

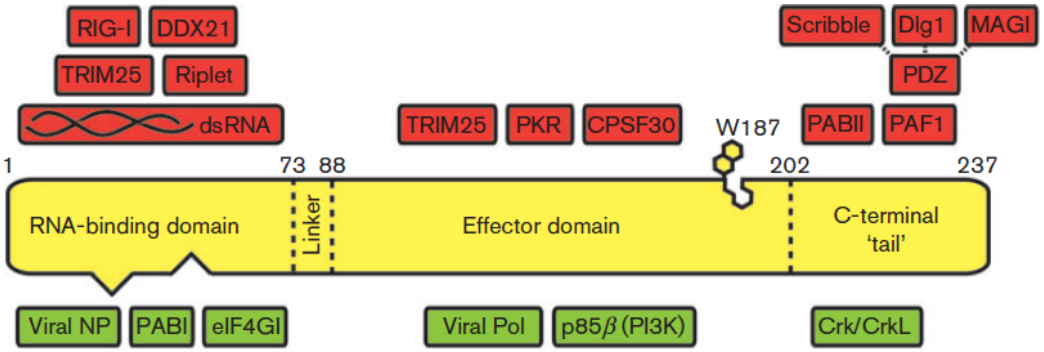
1.2.3.2 Antagonism of the adaptive immune response

The adaptive immune response in a virus-infected host is the second-line of defence after the innate immune response, and the NS1 protein is also capable of interference with this system. In particular, it was shown that NS1 is responsible for reducing the pulmonary pro-inflammatory cytokines TNF-α and IFN-γ to increase pathogenesis during IAV infection in mice (Hyland et al., 2006). It has also been reported that NS1 can attenuate the maturation of dendritic cells (DCs) by down-regulating specific genes and therefore reducing the production of necessary chemokine and cytokine factors (Fernandez-Sesma et al., 2006). This abrogation of DC maturation prevents DC-mediated priming of T-helper cells for the production of IFN-γ (Fernandez-Sesma et al., 2006).

1.2.3.3 Modulation of host apoptotic response

The role of the NS1 protein in the modulation of host cell apoptosis remains somewhat unclear, as there have been both pro- and anti-apoptotic functions associated with this protein. NS1 has been reported to induce apoptosis in various cultured cell types, including human epithelial lung cells (Schultz-Cherry et al., 2001, Lam et al., 2008). However, it has also been shown that NS1 is capable of delaying apoptosis in a process associated with its ability to antagonise IFN production (Zhirnov et al., 2002). It may be that the NS1 protein exhibits temporal effects on the modulation of apoptosis, whereby delaying apoptosis at early stages of IAV infection allows efficient viral replication, but up-regulation of apoptosis at later time-points could assist with the release of the virus from infected cells.

A



B

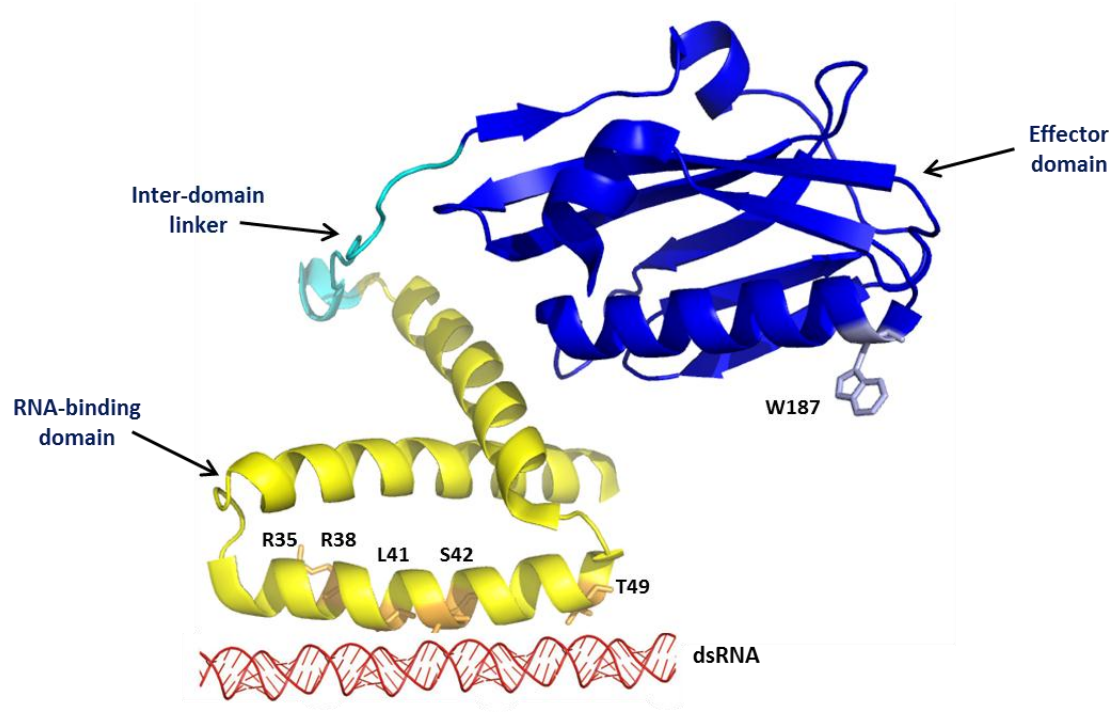


Figure 3. Structural representation of the IAV NS1 protein (A) Schematic of the NS1 functional domains and the host or viral factors it interacts with. Shown in red are the interactions that result in antagonism of the host innate immune response and in green are interactions which appear to benefit viral replication (obtained from (Hale, 2014). **(B)** Crystal structure of a monomeric NS1 protein showing the effector (blue) and RNA binding (yellow) domains linked by the inter-domain region (cyan). The interaction between the RBD and dsRNA is shown (not to scale) with the essential RBD residues for this interaction highlighted (R35, R38, L41, S42 and T49). Also highlighted is the residue W187 located in the ED which is critical for the dimerisation of the ED (with another NS1 ED). This figure was generated in PyMol using the Protein Data Bank ID 4OPH (Carrillo et al., 2014).

1.2.3.4 Preferential translation of viral mRNA

During IAV infection, NS1 is capable of promoting the preferential translation of viral mRNAs over that of host cell mRNAs (de la Luna et al., 1995). It appears that this process is facilitated by interaction between NS1 and the large subunit of the eukaryotic initiation factor 4F (eIF4F), which may result in binding of eIF4F to the 5' UTR of viral mRNAs to promote their translation over cellular mRNAs (Aragon et al., 2000).

1.2.3.5 Strain-specific functions of NS1

Studies involving NS1 proteins from various IAV strains have revealed several important strain-specific differences in the functional abilities of different NS1 proteins. Inhibition of RIG-I CARD domain ubiquitination occurs in a species-specific manner, as it was shown that NS1 proteins from human, avian, swine and mouse-adapted viruses were all able to bind human TRIM25, however none could bind mouse TRIM25, and the avian virus NS1 preferentially interacted with chicken TRIM25 (Rajsbaum et al., 2012). It was also found that the human virus NS1 could interact alternatively with mouse Riplet in order to block RIG-I ubiquitination (Rajsbaum et al., 2012).

The interaction between NS1 and host cell CPSF30 has also been documented to be strain-specific. This ability is known to be generally conserved amongst IAVs that infect humans, however the 2009 pandemic H1N1 virus was found to be unable to bind CPSF30 and thus could not block general gene expression (Hale et al., 2010). Binding was however restored through substitutions at residues R108, E125 and G189 (Hale et al., 2010). Several non-human adapted viruses are also unable to bind CPSF30, including the highly pathogenic avian H5N1 (pre-2004) and H7N9 viruses (Twu et al., 2007, Ayllon et al., 2014).

Comprehensive analysis of avian IAV sequences established that the C-terminal four residues of NS1 (residues 227-230) can form a consensus PDZ domain ligand, the amino acid sequence being either ESEV or EPEV (Obenauer et al., 2006). This motif is only found in avian IAV NS1 proteins and has been attributed roles in viral pathogenicity and in preventing apoptosis in infected cells (Liu et al., 2010, Jackson et al., 2008). In particular, insertion of the PDZ domain ligand motif residues into a human A/WSN/33 stain resulted in increased virulence in mice, an ability which may

stem from the demonstration that the NS1 PDZ domain ligand is able to bind around 30 human PDZ-containing proteins (Jackson et al., 2008, Obenauer et al., 2006).

Finally, the human H3N2 NS1 protein has been shown to harbour a unique functional ability by interacting with the human PAF1 transcription elongation complex (hPAF1C) (Marazzi et al., 2012). This NS1 is unique in containing a histone tail-like motif formed by the C-terminal amino acids 226 to 229 (ARSK) which is analogous to the histone tail sequence (ARTK) found in the N-terminus of histone H3 proteins (Marazzi et al., 2012). The H3N2 NS1 can therefore interact with hPAF1C via its PAF1 subunit to apparently target NS1 to inducible gene transcription sites to selectively reduce the transcription of antiviral host genes (Marazzi et al., 2012).

The provided examples of the differing functional capacities of various NS1 proteins from different strains of IAV highlight the importance of understanding the basis for strain-specific abilities. It is important to understand the particular mutations necessary for NS1 proteins to have a particular functional capacity, as a measure for the potential of cross species transmission, as well as viral replication and pathogenesis.

1.3 NS1 and the phosphoinositide 3-kinase (PI3K) signalling pathway

1.3.1 Class 1A PI3Ks and downstream signalling

Class IA phosphoinositide 3-kinases (PI3Ks) are heterodimeric enzymes which are comprised of a 110 kDa catalytic subunit (p110) and an 85 kDa regulatory subunit (p85), of which two isoforms of the p85 subunit are known (α and β) (Cantrell, 2001). A major downstream signalling molecule of the PI3K pathway is the serine/threonine kinase, Akt. Akt is recruited to the membrane where it is activated via specific phosphorylation at residues Thr308 and Ser473, enabling membrane dissociation and phosphorylation of cytoplasmic or nuclear downstream substrates (Alessi and Cohen, 1998, Lawlor and Alessi, 2001). Akt signalling effects include regulation of glucose metabolism, cellular apoptosis, cytoskeletal regulation and cell proliferation (Brazil and Hemmings, 2001).

1.3.2 NS1 activates the PI3K signalling pathway via p85β binding

The IAV NS1 protein is known to activate the PI3K signalling pathway during IAV infection, specifically through binding the p85β subunit of PI3K (Hale et al., 2006, Shin et al., 2007, Ehrhardt et al., 2007). NS1 interacts with the inter-Sh2 (iSH2) domain of p85β, thereby alleviating the usual inhibitory activity of this subunit upon the p110 catalytic subunit (Hale et al., 2008b). In particular, the residue tyrosine 89 was shown to be critical for binding of p85β by the NS1 ED, and is in fact conserved across all IAVs sequenced (Hale et al., 2006).

The precise biological consequences stemming from IAV activation of the PI3K pathway remain unclear. There have been polarising views in the literature regarding the regulation of apoptosis mediated by NS1-activation of Akt. Initial studies suggested that inhibition of PI3K leads to an up-regulation of apoptosis in virally infected cells (Zhirnov and Klenk, 2007, Ehrhardt et al., 2007), however a later study showed that a rUdorn-Y89F virus, incapable of binding and activating PI3K, did not up-regulate apoptosis when compared to wild-type virus (Jackson et al., 2010). What is clear is that the NS1-mediated activation of PI3K has strain-specific consequences for different IAVs, highlighted by the differences in the growth kinetics of recombinant A/Udorn/72 (rUd) and A/WSN/33 (rWSN) viruses encoding Y89F NS1 proteins, where only the rUd virus had attenuated growth kinetics due to an inability to activate the PI3K pathway (Hale et al., 2006). A further study revealed that activation of PI3K promoted the replication of the PR8 IAV, but not the WSN virus (Ayllon et al., 2012a). It is therefore of great interest to elucidate the specific downstream consequences due to IAV-activation of this pathway, and in particular why it may be beneficial for the viral life cycle.

1.4 Novel bat IAV genomes

1.4.1 Discovery of H17N10 and H18N11

In the last couple of years, RT-PCR analysis and next-generation sequencing enabled the reconstruction of two novel IAV genomes from two different species of South American bats. The first was found in samples from Guatemalan little yellow-shouldered bats (designated H17N10), and the second in samples from Peruvian flat-faced fruit bats (designated H18N11) (Tong et al., 2012, Tong et al., 2013). These

virus genomes were identified using consensus-degenerate RT-PCR primers designed to detect conserved signature sequences within the PB1 gene segment, which is highly conserved among IAVs (Tong et al., 2012, Poch et al., 1989). Rectal swabs obtained from captured bats were then screened in this RT-PCR assay, with positive samples analysed by next-generation and Sanger sequencing to identify the full-length genomes of these viruses (Tong et al., 2012, Tong et al., 2013). Serological studies to identify specific antibodies against recombinant H17 or H18 reported a high sero-prevalence consistent with a widespread occurrence of these viruses in Central and South American bat populations (Tong et al., 2013). Therefore, despite the fact that a full virus has yet to be isolated, a high sero-prevalence suggests infection of bats by these viruses.

Samples (oropharyngeal, faecal, rectal or urine) from 26 different Central European bat species were further examined using real-time RT-PCR (RT-qPCR) based on the M segment of the H17N10 virus, plus a generic IAV M segment to detect subtypes H1-H16 (Fereidouni et al., 2014). This analysis did not detect any IAV or IAV-like sequences, suggesting circulation of these specific IAVs in European bats is unlikely (Fereidouni et al., 2014). However the presence of divergent IAVs in other species of bats cannot be ruled out.

The discovery of these viruses questions the evolutionary origin of influenza viruses, as both H17N10 and H18N11 are highly divergent lineages, indicating that they may have been evolving separately in bats for a significant period of time (Tong et al., 2012, Tong et al., 2013). Indeed, the two virus genomes are highly distinct from even each other, with a greater genetic diversity between them than that present across all other IAVs sequenced (Tong et al., 2013). Again this highlights the possibility for further distinct IAVs to be discovered in additional species of bats.

A major concern stemming from the discovery of these viruses is the potential for them to cross species barriers and cause disease in humans or other animals, particularly as they represent novel subtypes. This is particularly concerning due to the potential for novel IAV subtypes to cause pandemic disease in new populations, as previously discussed. Therefore, characterisation of these bat IAVs is essential for determining any zoonotic potential.

1.4.2 Characterisation of HA and NA glycoproteins

Early structural and functional characterisation of the HA and NA glycoproteins of the two bat IAVs highlighted major differences in comparison with other IAV subtypes. Sequence analysis of the H17 HA showed it shares, on average, 45% amino acid sequence identity to the H1-H16 IAV subtypes, compared to the 49% identity shared among these 16 subtypes (Tong et al., 2012). Crystal structures of the H17 HA protein revealed that its overall structure is similar to that of other IAV HAs (Zhu et al., 2013, Sun et al., 2013). However, these structures highlighted a putative receptor binding site that is structurally divergent from that of other IAV HAs, namely due to significant mutations of key residues in this area (Zhu et al., 2013).

A further study showed that the H17 HA protein was unable to bind canonical human or avian sialic acid receptors due to its altered receptor-binding site (Sun et al., 2013). It is possible that the bat HA proteins are able to recognise alternate receptors other than sialic acids, or indeed different sialic acids, and may still work in tandem with the divergent NA protein to facilitate viral docking and release from host cells, but through as yet unidentified interactions. There is also the possibility that the bat IAVs use proteinaceous receptors for cell entry, a prospect supported by the knowledge that different viruses in the *Paramyxoviridae* family can use sialic acid or protein receptors as their means of entry (reviewed in (Chang and Dutch, 2012). For example, human parainfluenza virus 1 preferentially recognises oligosaccharides containing terminal Neu5Acα2-3Gal sialic acids (Villar and Barroso, 2006), however Nipah virus uses Ephrin B2/B3 for cellular fusion and entry (Bonaparte et al., 2005, Negrete et al., 2006).

The N10 NA protein was shown by sequence analysis to be highly divergent from the other 9 IAV subtypes. Indeed, initial sequence analysis showed N10 shares only 24% sequence identity to other IAV NA subtypes, a similarity which is lower than that between influenza A and B virus NAs (Tong et al., 2012). Upon acquisition and analysis of N10 crystal structures, it was seen that this protein exhibits a similar overall structure to other IAV NAs, containing a canonical sialidase fold (Li et al., 2012a, Zhu et al., 2012). However N10 was subsequently found to have no sialidase activity due to an altered active site, in particular it lacks the usually conserved arginine triad (residues R118, R292 and R371) responsible for binding the carboxylate group of sialic acids (Li

et al., 2012a, Zhu et al., 2012). It is unclear if this neuraminidase would have a separate function in the life cycle of this virus, or it may be that these viruses do not require sialidase activity to propagate their release from host cells. Indeed, it seems more appropriate to designate N10 as an NA-like protein due to its non-canonical identity.

1.4.3 Potential for zoonotic transmission

Bat species are a well-characterised reservoir for zoonotic viruses. Bats are of the order *Chiroptera* with a worldwide representation of more than 1150 species, accounting for approximately one quarter of all mammalian species. Many important emerging infectious diseases have been found to use bat species as their natural reservoir, with such viruses capable of causing severe disease in humans such as filoviruses, for example Ebola (Leroy et al., 2005), coronaviruses, for example, severe acute respiratory syndrome (SARS) (Li et al., 2005) and lyssaviruses, for example Rabies (Lumio et al., 1986). Thus, the discovery of two novel influenza viruses in two species of bats represented concern for a new reservoir of IAVs with the potential to infect humans, causing either severe or pandemic disease. The understanding of these viruses is particularly essential due to the worldwide distribution, large population numbers and interactions with other mammalian species that are associated with bats, increasing the potential for cross-species virus transmission.

Investigating whether these viruses follow a canonical IAV-like replication cycle is essential for understanding the potential for reassortment with other IAVs, a facet often associated with the production of pandemic IAVs. Analysis of H17N10 polymerase complex (PB2, PB1, PA and NP) functionality has been investigated by minigenome reporter assay, and was demonstrated to efficiently transcribe RNA in human lung cells, showing that these bat virus components can function in human cells (Tong et al., 2012). Further investigation involved the N-terminal domain of the PA subunit (PAn) whose endonuclease activity is responsible for the production of small RNA primers essential for the initiation of viral transcription (Dias et al., 2009). It was shown that the H17N10 PAn domain does indeed harbour endonuclease activity (Tefsen et al., 2014). Interestingly, the PAn domain from H17N10 shares only a 71-72% sequence identity with four other IAV strains (two avian H5N1s, a human pH1N1 and a human H3N2), compared with between 93-98% identity shared among these four strains

(Tefsen et al., 2014). Analysis of the H17N10 PAn domain crystal structure showed no major differences between other IAV structures, with the active site containing the catalytically conserved residues H41, E80, D108, E119 and K134 (Tefsen et al., 2014). Furthermore, H17N10 PAn was demonstrated to possess endonuclease activity at a similar level to the other IAV domains tested. These data therefore indicate that the H17N10 virus at least possesses seemingly canonical replication machinery.

Efforts to rescue the full bat IAVs have been unsuccessful; however two separate studies have generated synthetic IAVs containing six out of the eight bat virus gene segments (Zhou et al., 2014, Juozapaitis et al., 2014). These studies sought to determine the host species range and reassortment capabilities of these novel viruses. The first generated a chimeric bat IAV using the HA and NA segments from a A/SC35M (H7N7) virus, along with the internal segments of the H17N10 virus (Juozapaitis et al., 2014). Infectious virus was only obtained when additional 97-114 nucleotides from the 5' and 3' ends of the H17 and N10-coding regions were added to the SC35M HA and NA coding regions; these coding regions contain specific packaging signals, suggesting that these signals are not compatible between bat IAVs and other IAVs (Juozapaitis et al., 2014). The chimeric viruses were shown to replicate well in mammalian cell lines including human and pig, though to lower titres than the control SC35M virus, but showed impeded growth kinetics in avian cell lines (Juozapaitis et al., 2014). However, further passage in avian DF-1 (chicken fibroblast) cells resulted in the acquisition of adaptive mutations in various gene segments including HA, PA, M1 and M2, which allowed higher titre replication in these cells (from 10^7 to 10^8 PFU per ml) (Juozapaitis et al., 2014). Importantly, there was no evidence for the ability of the bat chimeric viruses to reassort with IAVs, either artificially through reverse genetic approaches, or during co-infection of MDCK cells (Juozapaitis et al., 2014). These data suggest that the genomic segments of the H17N10 virus are not compatible with those of other IAVs, and that the differences in packaging signals may impede generation of reassortant bat viruses.

A further study was also able to generate a synthetic bat IAV containing the internal H17N10 gene segments with the HA and NA segments from either the H1N1 A/PR/8/34 (PR8) or the H3N2 A/swine/Texas/4199-2/1998 (Tx98) viruses (Zhou et al., 2014). Again, viral particles could only be generated with the putative packaging signals from H17 and N10 coding regions flanking the PR8 or Tx98 HA and NA

coding regions (Zhou et al., 2014). The PR8-based chimeric virus was able to infect and replicate in mice lungs with 75% mortality, compared to the 100% PR8 wild type virus mortality (Zhou et al., 2014). A second chimeric virus using the Tx98 (a virus non-lethal in mice) glycoproteins also replicated in mice lungs, but had 0% mortality rate suggesting that the pathology of the Bat09:PR8 virus was due to the presence of the mouse-adapted PR8 HA and NA (Zhou et al., 2014).

In agreement with a previous study, it was also confirmed that reassortment of the chimeric bat IAV with conventional IAVs does not readily occur. Mini-genome reporter assays have shown functional compatibility between the NP protein of the bat IAV and other IAVs (Juozapaitis et al., 2014, Zhou et al., 2014). However, substitution of individual components of the RNA polymerase complex (comprised of PA, PB1 and PB2) from different IAVs with the corresponding H17N10 components did not support efficient polymerase activity (Juozapaitis et al., 2014, Zhou et al., 2014). Interestingly, however, is the observation that the polymerase subunits from the H17N10 and H18N11 bat viruses are capable of reassortment in a mini-genome assay, and also during co-infection of chimeric viruses (using PR8 HA and NA glycoproteins) (Zhou et al., 2014). This is particularly intriguing due to the divergent nature of these two virus genomes as they harbour more genetic diversity between each other than found between all other IAVs (Tong et al., 2013).

1.4.4 Preliminary characterisation of the H17N10 NS1 protein

Analysis of the H17N10 NS1 protein revealed that it shares some similarities and differences with the well-characterised PR8 NS1. Firstly, the ability to antagonise the IFN-β response in human cells was found to be conserved (Zhou et al., 2014). The H17N10 NS1 was able to inhibit host cell IFN-β induction to a level comparable to PR8 NS1, with C-terminal NS1 truncations (i.e. deletion of the NS1 ED) attenuating this ability for both (Zhou et al., 2014). However, in the context of IAVs (either chimeric bat or wild type PR8), the truncated NS1 proteins showed differential consequences. Truncated H17N10 NS1-containing viruses replicated to titres corresponding to the wild type virus and remained lethal in mice, whereas the truncated PR8 NS1 virus had 100-1000 fold lower titres than the wild type (Zhou et al., 2014). These observations suggest that perhaps the bat NS1 ED does not function efficiently

in mice and the ability to antagonise the IFN-β response is due to the sequestering of dsRNA by the RBD.

It is therefore of interest to further characterise this protein, and indeed the other proteins expressed by the bat IAV genomes. It remains unclear if these viruses have the potential to present a future threat to the human population, and characterising them may help to address this whilst also providing new insights into the evolution and biology of IAVs.

1.5 Thesis aims

The objectives of this particular study were to characterise functions of the H17N10 and H18N11 NS1 proteins from the two novel bat IAV genomes. This characterisation would enable a comparison between the two bat IAV NS1 proteins themselves, and also involve a comparison with representatives from other human and avian IAV NS1 proteins. As discussed, the IAV NS1 protein is known to have a number of canonical and also strain-specific functional capabilities. It was therefore of interest to determine if the novel bat IAV NS1 proteins would maintain the canonical functions associated with IAV NS1 proteins, and additionally whether they would present novel strain-specific abilities.

The first aim of this study was to analyse the divergences of these bat NS1 proteins by conducting an amino acid sequence alignment analysis for comparison with one human and two avian IAV NS1 representatives. This would then enable a prediction of the functional capacities of the bat IAV NS1 proteins as many NS1 functions have been successfully mapped to certain residues involved in that particular function. Following this, the aim was to clone the two bat IAV NS1 cDNAs into expression vectors which would allow expression of the NS1 proteins with N-terminal V5 tags. The addition of a V5 tag facilitates the study of these NS1 proteins in assays where antibodies against the V5 tag can be utilised.

Functional capabilities of the two bat NS1 proteins that were to be tested included:

- The intracellular localisation in both human and bat cells.

- The ability to antagonise IFN-β induction in, firstly, human and bat cells, followed by testing this ability in a range of cell types from different species as a potential measure of the contribution to cross-species transmission.
- The ability to block general gene expression in human and bat cells with a prediction for the ability to interact with host CPSF30 based on sequence analysis.
- The ability to interact with the host cell factors RIG-I and Riplet as a mechanism for the antagonism of the IFN-β signalling pathway.
- The ability to interact with the p85β subunit of PI3K as an indication of potential activation of this pathway.

Chapter 2: MATERIALS AND METHODS

2.1 Cell culture

2.1.1 Cell lines used

Cell lines that were used in this study include the following:

293T: human embryonic kidney 293 cells containing the SV40 Large T-antigen which can bind to SV40 enhancers in expression vectors to increase expression (see Graham et al. (1977)).
MRC5-hTERT: human telomerase reverse transcriptase-immortalised human lung fibroblast cells (MRC5).
TB1-Lu: bat lung epithelial cells isolated from Mexican free-tailed bats.
NBL6: horse dermal fibroblast cells.
BF: mouse dermal fibroblast cells.
MDBK: bovine epithelial-like kidney cells.

2.1.2 Maintenance of cell lines

All cell lines were maintained as monolayers in 25cm^2 and 75cm^2 flasks in either DMEM (Dulbecco's modified Eagle's medium; Life Technologies, UK), or EMEM (Eagle's minimal essential medium; Life Technologies, UK) supplemented with 10% heat-inactivated FBS (foetal bovine serum; Life Technologies, UK) and 1µg/µl Penicillin/Streptomycin (Life Technologies, UK). Cells were incubated at 37°C and 5% CO_2. Cells were maintained by trypsinisation (Trypsin, Life Technologies, UK) upon confluency and subsequent seeding into new tissue culture flasks in dilutions between 1 in 3 and 1 in 15 depending on the rate of cell growth.

2.1.3 Cryopreservation and recovery of cell stocks

Confluent cell monolayers in 75cm^2 flasks were trypsinised, resuspended in 10ml of the appropriate growth medium, and subsequently pelleted at 1500 rpm for 5 minutes. Cell pellets were then resuspended in freezing medium comprised of 90% DMEM (supplemented with 10% FBS) and 10% DMSO (dimethyl sulfoxide). Resuspended cells were aliquoted into cryovials at 1ml per vial, and were frozen in a 'slow-freeze'

box for 2 days at -70°C before being transferred into liquid nitrogen for long-term storage.

Recovery of cells involved thawing of cryovials at room temperature followed by centrifugation at 1500 rpm. Cell pellets were resuspended in 5ml of the appropriate medium and seeded initially in 25cm^2 flasks. Growth media was changed the following day to remove any traces of DMSO or unattached, dead cells. When the cell monolayer reached confluency, the cells were trypsinised and transferred to 75cm^2 flasks.

2.2 Cloning

2.2.1 pLVX expression vector

Specific NS1 cDNAs were amplified for insertion into the multiple cloning site (MCS) of separate pLVX.V5.MCS.IRES.zsGreen vectors (a schematic of this vector is shown in **Figure 4**). This MCS encodes many restriction enzyme sites which can be used for the insertion of NS1 cDNA.

2.2.2 Polymerase chain reaction (PCR)

Amplification of DNA sequences for the cloning of NS1 cDNAs was achieved by polymerase chain reaction (PCR) to produce full-length NS1 cDNA. Individual PCR reactions, made up to a final volume of 50µl using sterile water, consisted of the following:

42.5 µl	ddH_20
5.0 µl	10X pfu buffer (Promega, UK)
0.5 µl	10 µM forward primer
0.5 µl	10 µM reverse primer
0.5 µl	10 mM dNTPs (Promega, UK)
0.5 µl	0.1 µg/µl DNA template
0.5 µl	pfu DNA polymerase (Promega, UK)
50 µl	

PCR reactions involved an initial denaturing step at 95°C for 2 minutes. This was followed by 30 cycles consisting of; a further denaturing step at 95°C for 30 seconds,

an annealing step at a primer-dependent temperature for 30 seconds, and an extension step at 72°C for 1 minute. A final extension step was performed at 72°C for 5 minutes.

Primers were designed for the amplification of H5N1 (A/Nigeria/OG10/2007), H7N9 (A/Shanghai/2/S1078/2013), H17N10 (A/little yellow shouldered bat/Guatemala/153/2009) and H18N11 (A/flat-faced bat/Peru/033/2010) NS1 cDNA, and are detailed in **Table 1.** These primers were designed to introduce a restriction enzyme site at the 5' and 3' positions of the NS1 cDNA, with particular restriction sites chosen when the appropriate coding sequence was absent from the NS1 cDNA. In the case of the H5N1 NS1, the restriction enzyme coding sequences for SpeI (5') and NotI (3') were inserted into the primer sequences. For the cloning of H7N9 NS1, the restriction sites for EcoRI (5') and NotI (3') were inserted at the given ends, whereas for the H17N10 and H18N11 the restriction sites EcoRI (5') and XbaI (3') were inserted. To allow for efficient cutting, 5 or 6 nucleotides were added to the 5' end of the restriction enzyme site. In the case of the 5' primers, an extra base was added between the restriction enzyme site and the NS1 coding region to allow in-frame expression of the NS1 cDNA after insertion into the vector. Finally, around 21 nucleotides from the NS1 coding sequence were included in the primers. **Table 1** also shows the melting temperatures for each primer, with the initial melting temperature given as that required for the sequence in black to anneal in the first reaction cycle. Subsequent annealing of the full-length primer sequence (including the region highlighted in red) would raise the melting temperatures as indicated.

2.2.3 4-primer overlap PCR for NS1 mutants

In order to create bat H17N10 NS1 proteins containing single amino acid substitutions, 4-primer overlap PCR consisting of two-steps was performed, the steps in which are highlighted in **Figure 5**. The first step produced two NS1 fragments (5' and 3') encoding the appropriate nucleotide mutations in an overlapping region. These fragments were obtained using primers designed with one or two nucleotide changes at the appropriate positions, plus either the 5' or 3' primer for amplification of full-length H17N10 NS1 cDNA (shown in **Table 2**). Specifically, the 5' full-length primer was used with the 3' mutation primer to produce the 5' NS1 fragment, with the 3' full-length and 5' mutation primers used to produce the 3' NS1 fragment. Altering more

than two nucleotides in the mutation primer sequences was avoided to assist with efficient annealing of the primers during the PCR reaction.

A second step PCR was then performed, using the two NS1 cDNA fragments as the templates for amplification. Full-length NS1 cDNAs were produced using the primers designed for the full-length PCR amplification and encoding the restriction enzyme sites.

2.2.4 Agarose gel electrophoresis

PCR product DNA was analysed by gel electrophoresis in 80ml 1% agarose gels made up in 1X TAE buffer (40mM Tris, 20mM acetic acid, 1mM EDTA) and containing 30µg ethidium bromide (at 10 µg/µl) for the visualisation of DNA. To enable loading of DNA onto the gel, a 6X DNA loading dye was added to the PCR products at the appropriate dilution. Samples were run at 90V in TAE buffer until the dye front reached the end of the gel. A 1kb DNA ladder was run in parallel with the samples to determine the size of the PCR products. Visualisation of agarose gels was achieved using a long-wave ultraviolet (UV) transilluminator with excision of products of interest under the UV light. Excised bands were purified for DNA using a Gel Extraction Kit and following the manufacturer's instructions (QIAquick, QIAGEN Ltd, UK).

2.2.5 Restriction enzyme digest of PCR products and vector

Purified PCR products (insert) were digested at the appropriate restriction enzymes sites encoded into their 5' and 3' ends (detailed in Table 1). 16.5µl of the purified 30µl DNA elute was digested in a reaction containing 2µl of 10X buffer D/H (depending on the restriction enzymes used, Promega, UK), 0.5µl BSA (10 mg/ml), and 0.5µl each of the two restriction enzymes at 10 units per µl (Promega, UK) for a reaction of 20µl final volume. The reactions were incubated overnight at 37°C.

The plasmid backbone (vector) of pLVX.V5.MCS.IRES.zsG at 1µg/µl was digested with the same restriction enzymes depending on the nature of the insert. This reaction mixture contained 1µl of plasmid, 2µl 10X buffer D/H, 0.5µl BSA, 0.5µl each of the restriction enzymes and subsequently made up to a final volume of 20µl with 15.5µl of sterile water. Digestion of the plasmid required incubation at 37°C for 2.5 hours.

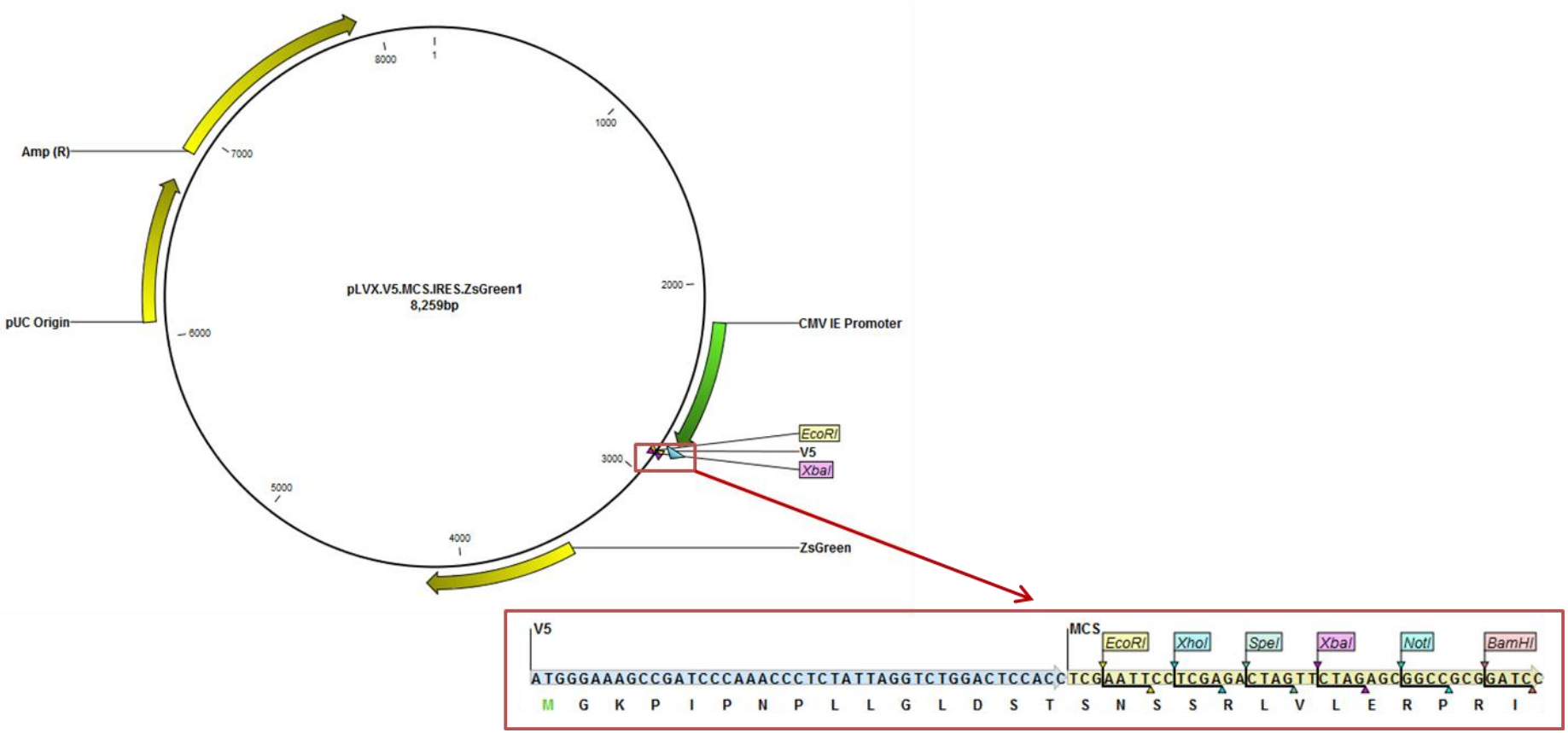


Figure 4. Schematic of the pLVX expression vector. The NS1 cDNAs were cloned into the MCS highlighted in the zoomed in view. H17N10 and H18N11 NS1 cDNA were inserted between the EcoRI and XbaI restriction sites, the H5N1 NS1 was inserted between the SpeI and NotI sites and the H7N9 NS1 was inserted between the EcoRI and NotI sites. This gave the proteins an N-terminal V5 tag upon expression (highlighted in blue in the zoomed in view). Also shown in this schematic is the presence of the coding region for zsGreen fluorescent protein which is produced from the bicistronic mRNA due to an IRES.

Forward primers	Reverse primers
5-SpeI-H5N1-NS1 5’ gcgaccactagtg ATGGACTCCAACACTGTGTCAAG 3’ (M_t = 55.3°C, increasing to 67.9°C)	**3-NotI-H5N1-NS1** 5’ gctaagcggccgc TCAAACTTTTGACTCAATTGTTCTC 3’ (M_t = 51.1°C, increasing to 66.6°C)
5-EcoRI-H7N9-NS1 5’ gctaccgaattcc ATGGATTCCAATACTGTGTCAAG 3’ (M_t = 51.7°C, increasing to 64.4°C)	**3-NotI-H7N9-NS1** 5’ gctaagcggccgc CTACTTTGTAGAGAGTGGAGATC 3’ (M_t = 53.5°C, increasing to 69°C)
5-EcoRI-H17N10-NS1 5’ ggtcagaattcg ATGGAACCAAACCCGACAACTATC 3’ (M_t = 63°C, increasing to 65.6°C)	**3-XbaI-H17N10-NS1** 5’ gcagtctaga CTATTCTGTTGAGCCATCTTGCGGTAG 3’ (M_t = 64.4°C, increasing to 66.7°C)
5-EcoRI-H18N10-NS1 5’ gctcagaattcc ATGGAATCGACCCCGACAACTATC 3’ (M_t = 64.4°C, increasing to 66.7°C)	**3-XbaI-H18N10-NS1** 5’ gcactctaga TCATTCTGCTGGCTCATTTTCCTG 3’ (M_t = 61.4°C, increasing to 64.4°C)

Table 1: **Forward and reverse primers designed for the amplification of H5N1, H7N9, H17N10 and H18N11 cDNA**. Primers encoded restriction enzyme sites to be present at the 5’ and 3’ ends of NS1 cDNA. These restriction sites are highlighted in red. Also shown are the melting temperatures for the 1st and subsequent PCR cycles, with the temperatures increasing upon incorporation of the restriction sites into the cDNA.

2.2.6 Ligation of digested insert and vector

Digested vector was purified by agarose gel electrophoresis followed by recovery using a gel extraction kit as described previously. Digested vector was run alongside an uncut plasmid on the gel to ensure successful digestion. Digested inserts were purified by a 'quick clean-up' with the addition of 5X binding buffer (PB buffer, QIAGEN, UK), binding to a gel extraction column with subsequent steps followed in the gel extraction protocols (QIAquick, QIAGEN Ltd., UK).

Purified insert and vector were then ligated together in a reaction mixture containing a 1:1 ratio of vector to insert. 1µl of insert was mixed with 1µl vector, 1µl 10X ligase buffer, 1µl T4 DNA ligase (Promega, UK) and made up to a final volume of 10µl with 6µl sterile water. A negative control for ligation contained 7µl sterile water instead of the insert. Ligation reactions were incubated for 5 hours at room temperature and stored at -20°C until transformation of bacterial cells as described below.

2.2.7 Heat-shock transformation of competent bacterial cells

5µl of the ligation reaction were added to highly competent DH5α *E. coli* cells (Invitrogen, UK) (thawed on ice in 50µl aliquots), with a positive control of 0.5µl of non-digested vector added to one tube. Bacterial cells were incubated on ice for 30 minutes with the ligation reactions, with occasional flicking of the tubes to mix. Heat-shock of cells lasted for 40 seconds at 42°C in a pre-heated water bath. Cells were recovered on ice for 2 minutes before addition of 300µl of super optimal broth medium (SOC medium, Sigma-Aldrich, UK) and shaking incubation at 37°C for 45 minutes to stimulate cell growth. 100µl of cells of the mix was then plated onto LB-agar plates supplemented with 1 µg/µl ampicillin for selective growth of the ampicillin-resistance gene-containing bacteria. Plates were incubated, inverted, at 37°C overnight.

2.2.8 Generation of plasmid stocks

For growth of DNA in bacterial cells for mini-prep scale preparations, single colonies from transformation plates were picked and grown in 5ml of LB broth containing 1 µg/µl ampicillin. Growth was overnight in a shaking incubator at 37°C. The next day, DNA was extracted from the cells using a DNA mini-prep kit (QIAGEN Ltd, UK) and according to the manufacturer's instructions. For the preparation of DNA stocks for

midi- or maxi-prep scale concentrations, bacterial cells were added in a 1:1000 dilution (from mini-culture stocks) to either 100ml (for midi-preps) or 200ml (for maxi-preps), again supplemented with 1 µg/µl ampicillin. These cultures were again grown overnight in a shaking incubator at 37°C. Again, DNA was extracted and purified using QIAGEN midi- or maxi-prep kits (QIAGEN Ltd, UK) following the manufacturer's instructions.

2.2.9 DNA spectrophotometry

Purified DNA preparations were quantified by a NanoDrop Spectrophotometer (Thermo Scientific, UK), with 2µl of plasmid DNA read against elution buffer/sterile water to determine the DNA concentration. The purity of the plasmid DNA was also calculated and indicated by the Abs_{260}/Abs_{280} ratio, with a ratio of 1.8 or greater considered to be DNA of an acceptable purity. An appropriate concentration of plasmid DNA preparations were then sent for sequencing using a primer for the CMV promoter region encoded in the pLVX plasmid to determine correct cloning of the appropriate insert (Dundee DNA Sequencing Services, UK).

2.3 Reporter assays

2.3.1 Interferon-β (IFN) induction assay

This assay was performed in a range of cell types including human 293T, bat TB1-Lu, horse NBL6, mouse BF and dog MDCK to analyse the activation of the IFN-β promoter. Cells were either transfected in suspension or seeded at an appropriate number for ~60% confluency the following day for transfection. Cells in a 24-well plate were transfected with 12.5ng of p125luc containing *Firefly* luciferase (FF-luc) under the control of an IFN-β promoter, 12.5ng of pRL-TK containing *Renilla* luciferase (Ren-luc) under the control of a constitutively active herpes simplex virus thymidine kinase (HSV-TK) promoter and 0.25µg pLVX plasmid expressing either GST or an NS1 (Hale et al., 2010). Transfection mixtures also contained 50µl of Opti-MEM (Life Technologies, UK) and FuGene at a 3 µg:1 µl DNA ratio (Promega, UK) to facilitate transfection. Cells were incubated at 37°C for 24 hours followed by infection with a 1 in 50 diluted Sendai virus (SeV) stock for a further 16 hours at 37°C. Cells were harvested and luciferase activity determined by Dual-Luciferase Reporter Assay (Promega, UK) by following the manufacturer's instructions. .

1st step PCR primers designed for single amino acid substitution NS1s	
Q95L 5’ CA GAG ATG ACT CTC GAG GAA ACA ATC AG 3’ 5’ CT GAT TGT TTC CTC GAG AGT CAT CTC TG 3’ M_t = 58°C, increasing to 59.9°C	**T98M** 5’ G ACT CAA GAG GAA ATG ATC AGA AAC TGG 3’ 5’ CCA GTT TCT GAT CAT TTC CTC TTG AGT C 3’ M_t = 56.4°C, increasing to 58.5°C
I99S 5’ CAA GAG GAA ACA AGC AGA AAC TGG GTG 3’ 5’ CAC CCA GTT TCT GCT TGT TTC CTC TTG 3’ M_t = 58°C, increasing to 59.5°C	**R143T** 5’ GGA AAA CTA GAA ACA CTT GTA TTA GCT AG 3’ 5’ CT AGC TAA TAC AAG TGT TTC TAG TTT TCC 3’ M_t = 53.7°C, increasing to 55.9°C
N161S 5’ GTG GGT GAA ATC AGC CCT CTG TCT TTT G 3’ 5’ C AAA AGA CAG AGG GCT GAT TTC ACC CAC 3’ M_t =58°C, increasing to 61.4°C	**S164P** 5’ C AAC CCT CTG CCT TTT GTT ACC GGA C 3’ 5’ G TCC GGT AAC AAA AGG CAG AGG GTT G 3’ M_t = 59.3°C, increasing to 61.1°C

Table 2: Primers designed for the 1st step PCR in a 4-primer overlap PCR for the generation of 6 single amino acid substitutions in NS1. The H17N10 NS1 was used as the background sequence for the substitutions Q95L, T98M, I99S, R143T, N161S and S164P achieved by the mutation of one or two nucleotides in the NS1 cDNAs. The primers were designed to introduce a mutation in an overlapping region of NS1 cDNA. Also shown in this table are the varying melting temperatures for the initial primer annealing steps where the nucleotides highlighted in red will not anneal. The melting temperatures increase for subsequent annealing steps where the full-length primers will bind to the template DNA.

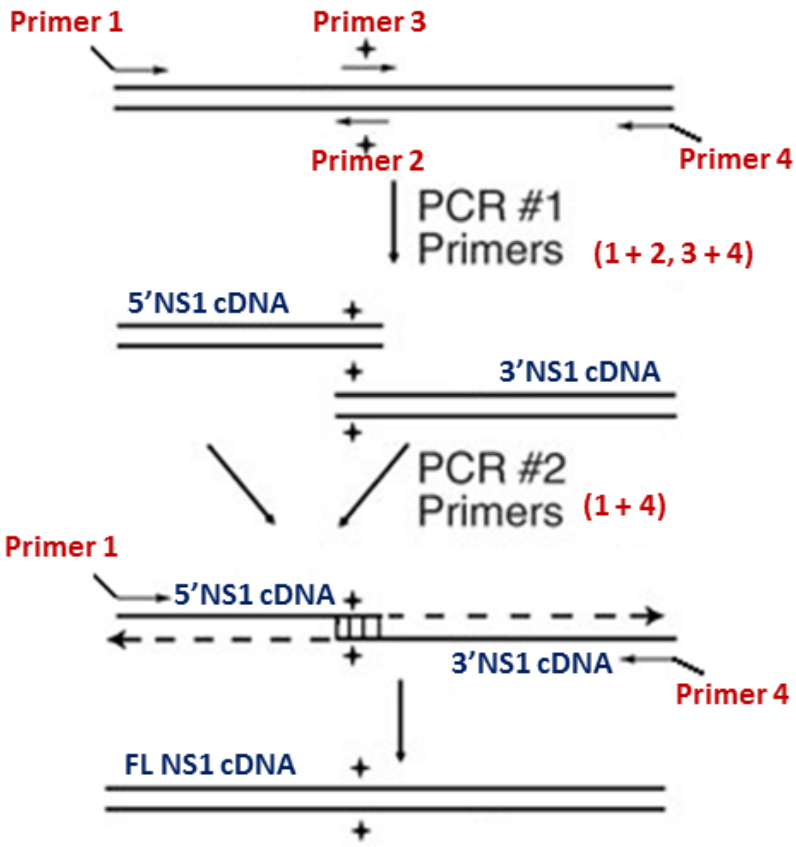


Figure 5. 4-primer overlap PCR method for the production of full-length NS1 cDNAs encoding a single amino acid substitution. A first round PCR reaction is performed using primers (1 + 2 and 3 + 4) that introduce a specific mutation in an overlapping region. Details of these primers used can be found in Table 2. This produces two NS1 cDNA fragments both encoding the mutation. A second round PCR uses these fragments as templates to amplify full-length (FL) NS1 cDNA using primers for the full-length construct (1 + 4). Diagram adapted from (Heckman and Pease, 2007).

Cells were lysed in 100µl passive lysis buffer for 15 minutes. FF-luc levels were measured by addition of Luciferase Assay Reagent (LAR II) followed by Ren-luc levels measured by addition of the Stop & Glo Reagent. Luciferase levels were determined by use of a luminometer. All transfection conditions were repeated in triplicate, with experiments also repeated independently twice.

2.3.2 General gene expression assay

To analyse the levels of general gene expression, human 293T and bat TB1-Lu cells in 24-well plates were co-transfected with 12.5ng of pRL-TK and 0.25µg of pLVX expressing GST or an NS1, using FuGene at a 3:1 ratio (Promega, UK). 24 hours post-transfection the activity of Ren-luc was determined by *Renilla* Luciferase Assay System (Promega, UK) by following the manufacturer's instructions. Cells were lysed in 100µl *Renilla* Luciferase Assay Lysis Buffer with Ren-luc activity measured using *Renilla* Luciferase Assay Reagent and read by luminometer. All transfection conditions were repeated in triplicate, with experiments also repeated twice independently.

2.4 Immunoprecipitations

293T cells were co-transfected in T25 flasks with 2µg of plasmid for the expression of the tagged host protein of interest, and 2µg of NS1 or GST using FuGene (Promega, UK). Cells were harvested 48 hours post-transfection by removing the medium and washing twice in ice-cold PBS. Cells were lysed for 10 minutes on ice in 1ml (per T25 flask) of immunoprecipitation (IP) buffer consisting of; 20mM Tris-HCl (pH 7.8), 5mM EDTA, 0.5% (v/v) IGEPAL/NP-40 and 650mM NaCl, supplemented with a fresh protease inhibitor cocktail tablet (1 tablet per 10ml IP buffer, Roche Diagnostics, Germany). Lysates were disrupted by syringing 3 times with a 1ml 25G syringe and a further 3 times with a 0.5ml 29G syringe. Lysates were then clarified into insoluble and soluble fractions by centrifugation at 14,000 rpm for 50 minutes at 4°C.

Following clarification, 50µl samples were taken from the soluble fraction and with addition of 2X urea disruption buffer (6M urea, 4% SDS, 1M β-mercaptoethanol, bromophenol blue) then represented 'input' samples. Immune complexes were formed by incubating the remaining soluble antigen supernatant with 2µl of α-V5 336 antibody

for 2 hours at 4°C in an 'end-over-end' tumbler. Immune complexes were precipitated by incubating with Protein G Sepharose Fast Flow beads (Sigma, Life Sciences, UK) overnight at 4°C in an 'end-over-end' tumbler. Beads bound with immune complexes were washed 6 times by centrifugation at 2,000 rpm for 1 minute to pellet the beads followed by removal of the supernatant and addition of 1ml fresh IP buffer to the beads. Following the last wash all supernatant was removed and samples lysed in 50µl 2X urea disruption buffer to represent 'pull-down' samples. Immune complexes were dissociated by heating at 100°C for 5 minutes and beads were pelleted by centrifugation at 2000 rpm for 1 minute. Samples were then analysed by SDS-PAGE and western blot as described below.

2.5 Protein analysis

2.5.1 SDS-PAGE and western blot

Cell media was removed and cells washed twice with 1X PBS before lysing in 2X urea disruption buffer (6M urea, 4% SDS, 2M β-mercaptoethanol with the addition of a small amount of bromophenol blue to colour appropriately). Cells were lysed in varying volumes of disruption buffer depending on the well-size i.e. 100µl for 24-well plates and 150µl for 12-well plates. Harvested samples were passed through a 0.5ml 29G syringe 3 times in order to shear DNA, followed by boiling at 100°C for 5 minutes. Proteins were separated and analysed by sodium dodecyl sulphate – polyacrylamide gel electrophoresis (SDS-PAGE) using 4-12% NuPAGE polyacrylamide gradient gels (Novex, Life Technologies, UK). Gels were run at 180V in 3-(N-morpholino)propanesulfonic acid (MOPS) buffer (Novex, Life Technologies, UK) until the dye front reached the bottom of the gel. Samples were run alongside a protein molecular weight marker to determine their size (Bio-Rad Laboratories, USA).

Following separation by SDS-PAGE, proteins were then transferred to either a polyvinylidene difluoride (PVDF) membrane (requires activation in methanol) or a nitrocellulose blotting membrane (GE Healthcare, Life Sciences, UK). Membrane transference took place in XCell II Blot Modules (Invitrogen, UK) in NuPAGE Transfer Buffer (Novex, Life Technologies, UK) for 75 minutes at 28V. Following transfer, membranes were blocked in 5% milk (5% skimmed milk powder in 1X PBS with 0.1% Tween-20, PBS-T) for 1 hour. Blocking buffer was removed by washing

the membranes 3 times in PBS-T before addition of primary antibody diluted appropriately in 5% milk/PBS-T. Incubation in primary antibody occurred overnight at 4°C, or for 1 hour at room temperature. Antibody was removed by washing 3 times initially in PBS-T, followed by 6 further washes over 30 minutes. Secondary antibodies of horseradish peroxidase (HRP) conjugated anti-mouse or anti-rabbit IgG were also diluted appropriately in 5% milk/PBS-T and incubated on the membranes for 1 hour at room temperature. Final washes consisted of 3 times initially in PBS-T, a further 4 times in PBS-T followed by 2 times in either 1X PBS or distilled water (to remove the Tween-20, or in the case of distilled water to remove any background fluorescence from the presence of PBS on the membrane), over the course of 30 minutes.

Protein bands were visualised by one of two methods; by x-ray films or digital images. The first requires application of an enhanced chemiluminescence substrate, Western Lightning Plus-ECL (PerkinElmer, Inc., USA) to detect the HRP-conjugated antibodies, followed by exposure using KODAK Medical X-ray Film (Carestream Health, France). The second uses an Odyssey CLx Infrared Imaging System (LI-COR, Biosciences, Ltd, UK) to detect the fluorescent antibody and transform it into a digital signal.

2.5.2 Indirect Immunofluorescence

For indirect immunofluorescence analysis, cells were grown on 10mm coverslips in 24-well plates for 24 hours post-transfection with NS1 expressing plasmids. Cells were subsequently washed twice with 1X PBS before fixing for 15 minutes in 5% formaldehyde (in 1X PBS). Cells were washed twice in 1X PBS to remove the formaldehyde before permeablisation using 0.5% Triton-X-100 (in 1X PBS) for 5 minutes at room temperature. Coverslips were washed 3 times in 1X PBS and then blocked for 30 minutes with 1X PBS supplemented with 2% FBS. Per coverslip, 35µl of α-V5 336 primary antibody (diluted in 1X PBS supplemented with 2% FBS) was added and incubated for 1 hour at room temperature. Following 3 washes in 1X PBS, 35µl of the secondary antibody, Alexa Fluor 555 Donkey anti-mouse IgG (Life Technologies, UK), was added (diluted in 1X PBS supplemented with 2% FBS) and incubated for 1 hour at room temperature. Coverslips were kept in the dark for this incubation period to avoid bleaching of the fluorescence. Incubation was followed by

3 washes using 1X PBS followed by 2 washes with sterile water. Coverslips were then mounted on glass slides using 8µl VECTASHIELD HardSet Mounting Medium (Vector Laboratories Ltd., UK) incorporating DAPI for nuclei staining. Immunofluorescence was visualised using a Zeiss LSM 710 Confocal Microscope.

2.5.3 Antibodies

Antibodies were used for immunoprecipitation (IP), western blot (WB) and immunofluorescence (IF) studies. Primary and secondary (HRP-conjugated) antibodies used in the duration of this study are detailed in **Table 3,** indicating the application in which the antibodies were used, the dilution they were used at, the species in which they were raised and from where they were obtained, including the catalogue number.

2.5.4 Amino acid sequence alignment

Multiple sequence alignment of various NS1 protein amino acid sequences was conducted using the Clustal Omega web service (EMBL-EBI, UK).

Primary antibody	Application	Species	Dilution	Source
V5 336	WB, IP and IF	Mouse	1:2000 for WB 1:1000 for IF	Kindly provided by Prof. Rick Randall (University of St. Andrews)
V5 680 conjugate (Dylight)	WB	Mouse	1:2000	Thermo Fisher Scientific
GFP	WB	Rabbit	1:2000	Abcam, UK
HA-tag	WB	Mouse	1:2000	Cell Signalling, UK
FLAG-tag	WB	Rabbit	1:2000	Sigma, UK
β-actin	WB	Rabbit	1:3000	Sigma, UK
Secondary antibody	**Application**	**Species**	**Dilution**	**Source**
V5-HRP	WB	Mouse	1:10,000	AbD Serotec, Bio-Rad, UK
Anti-mouse IgG	WB	Mouse	1:4000	Sigma, UK
Anti-rabbit IgG	WB	Rabbit	1:4000	Sigma, UK
Dylight 800 conjugates	WB	Rabbit	1:10,000	Fisher Scientific
Alexa-555 anti-mouse	IF	Donkey	1:1000	Life Technologies, UK

Table 3. A list of the primary and secondary antibodies used for western blot, indirect immunofluorescence and immunoprecipitation analyses. Detailed are the applications in which the indicated antibodies were used in, the species in which the antibodies were raised, the experimental dilution the antibodies were used at, and the source from which the antibodies were obtained (including the catalogue number shown in brackets).

Chapter 3: RESULTS

Characterisation of the HA and NA glycoproteins from the two novel bat IAVs revealed that neither has the canonical functions associated with these proteins in other IAVs, in particular, the H17 and H18 HA proteins cannot bind sialic acids, and the N10 and N11 NA proteins cannot cleave them (Li et al., 2012a, Zhu et al., 2013, Zhu et al., 2012, Sun et al., 2013). It is intriguing to determine if the other proteins encoded by these bat viral gene segments have non-canonical functions also. Indeed, a study from Zhou et al. presented preliminary characterisation of the H17N10 NS1 protein, compared with PR8 NS1, in the context of a synthetic virus (Zhou et al., 2014). Deletion of the NS1 ED highlighted functional differences between these NS1 proteins during infection of a mouse model; mainly that truncation of the bat IAV NS1 had less of an effect, in comparison to PR8 NS1, on viral replication or the antagonism of the host IFN response (Zhou et al., 2014). These findings suggest that the H17N10 NS1 ED differs in its functional abilities, or perhaps may not function efficiently in mice. This study therefore sought to further characterise the H17N10 NS1, alongside the H18N11 NS1, for a range of functional abilities in a variety of cell types from different species.

3.1 Bat IAV NS1 proteins have divergent sequences compared to other IAV NS1 proteins

3.1.1 Sequence alignment of a panel of IAV NS1 proteins highlights differences

An initial sequence alignment analysis was conducted with the two bat IAV NS1 proteins alongside representatives from human (H1N1) and avian (H5N1 and H7N9) viruses for comparison of amino-acid level conservation (**Figure 6)**. This alignment highlighted the high variability of the two bat IAV NS1 proteins in comparison to the other IAV NS1 proteins. The H17N10 and H18N11 NS1 proteins are both 221 amino acids in length, in comparison to PR8 NS1 which is 230 amino acids long, H5N1 NS1 which is 225 amino acids long and finally H7N9 NS1 which is 217 amino acids long. The difference in length is mainly due to variable C-terminal tail regions, with both the bat IAV NS1 proteins and the H7N9 NS1 featuring truncated tail regions. Also observed are two amino acid insertions in the bat NS1 sequences, featured at positions

3 and 115 in comparison to the PR8 NS1. Therefore, for the remainder of this thesis, PR8 NS1 residue numbering will be referred to, with the corresponding bat IAV NS1 numbering referred to in brackets, for example W187 in PR8 NS1 corresponds to W189 in the bat IAV NS1 proteins.

Initial BLAST analysis gave insight into the divergence of NS1 proteins from different IAV strains. Human PR8 NS1 was found to share only 45 and 49% protein level sequence similarity to the H17N10 and H18N11 bat NS1 proteins respectively. Furthermore, the avian H7N9 NS1 shares only 46 and 50% similarity to the bat H17N10 and H18N11 NS1s, but has a much closer similarity (86%) to the human PR8 NS1. Within species differences were also compared, with the two avian NS1 representatives, H5N1 and H7N9, found to share 85% sequence identity. The bat H17N10 and H18N11 NS1 proteins were found to share only a slightly lower 82% similarity at the protein level, showing that inter-species differences are much greater. As NS1 proteins are divided into two alleles based on sequence diversity (A and B), a further analysis using an NS1 representative from an allele B (A/Mallard/Alberta/827/78, H8N4) was conducted. It was found that the H17N10 and H18N11 NS1 proteins share 49% identity with the H8N4 NS1, whereas the allele A NS1 proteins from PR8 and H7N9 share 67% with the H8N4 NS1. As the bat IAV NS1 proteins share comparable sequence identities with both allele A and B NS1 representatives, it is difficult to place the bat IAV NS1 proteins into either classification; indeed it may be that they require a separate allele grouping.

3.1.2 Prediction of functional abilities of bat IAV NS1 proteins based on sequence analysis

Analysis of the multiple NS1 protein alignment allows some predictions of the functional capabilities of the two bat NS1 proteins. Residues implicated in certain NS1 functional properties are highlighted in the sequence alignment shown in **Figure 6** and further summarised in **Table 4.** Conservation of the NLS essential residues at R35, R38 and K41 (R36, R39 and K42) suggest that the two bat NS1 proteins will exhibit a nuclear localisation pattern (Melen et al., 2007). Furthermore, conservation of R38 and K41 (R39 and K42) in both H17N10 and H18N11 NS1 proteins suggest that both RBDs of these proteins will have RNA-binding capabilities (Wang et al., 1999).

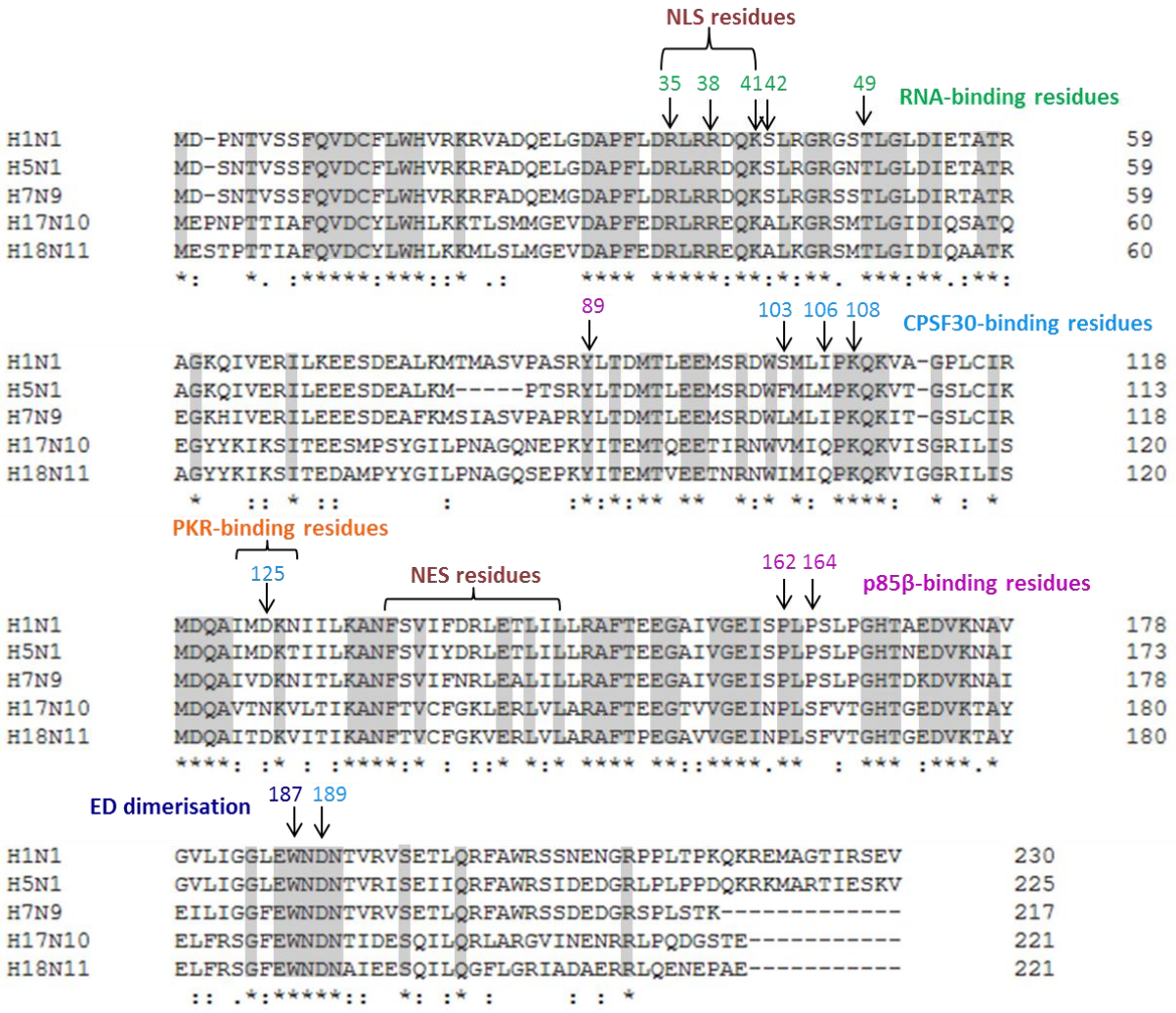


Figure 6. Amino acid sequence alignment of NS1 protein representatives from human, avian and bat IAVs. The bat H17N10 (A/little yellow shouldered bat/Guatemala/153/2009) and H18N11 (A/flat-faced bat/Peru/033/2010) NS1 protein sequences are compared with the human H1N1 (A/Puerto Rico/8/34), the avian H5N1 (A/Nigeria/OG10/2007) and the avian H7N9 (A/Shanghai/2/S1078/2013) NS1 representatives. Highlighted in grey are the conserved residues across the NS1 sequences shown. The residues highlighted in green are those critical for the binding of dsRNA by the NS1 RNA-binding domain, residues highlighted in brown show the nuclear localisation and export signals (NLS and NES), residues highlighted in pink are those critical for binding of the p85β subunit of PI3K, residues in light blue indicate those essential for CPSF30 interaction, in orange are the residues associated with the binding of PKR, and finally the residue highlighted in dark blue is known to mediate the dimerisation of the NS1 effector domain. This multiple sequence alignment was produced using Clustal Omega.

The W187 residue (W189) is also conserved, again suggesting efficient RNA-binding as this residue is essential for ED dimerisation and contributes to effective dsRNA binding (Ayllon et al., 2012b, Kerry et al., 2011). However, there is a difference in the S42 residue normally found in PR8 which occurs as A43 in both bat IAV NS1 proteins. This particular residue substitution has been shown previously to decrease RNA-binding by approximately 10-fold in association with a loss of phosphorylation at the serine (Hsiang et al., 2012).

Predictions can also be made about the binding capabilities of the bat IAV NS1 proteins with cellular factors known to bind NS1 in a strain-specific manner. Certain residues have been associated with the interaction between NS1 and CPSF30, namely at positions 103, 106, 108, 125 and 189 (Das et al., 2008, Hale et al., 2010). Differences occur at residues 103, 106 and 125 in the H17N10 and H18N11 NS1 proteins, suggesting that these proteins may not form interactions with CPSF30 and would therefore be unable to block general host gene expression. Due to this prediction an NS1 protein capable of interacting with CPSF30 (H5N1 NS1) was included in this study for the purposes of a comparative gene expression assay.

Three other residues have been documented to be necessary for the interaction between NS1 and the p85β subunit of PI3K; Y89, P162 and P164. Y89 is seen to be conserved in the bat IAV NS1 proteins, with P162 also conserved, but not P164. This makes it unclear if these NS1 proteins would be able to bind p85β, particularly as more residues are likely to play a role in this interaction. Finally, NS1 can interact with host PKR, involving residues 123 to 127 on NS1 (Min et al., 2007, Li et al., 2006). There are several differences in the bat NS1 proteins in this PKR-binding motif, suggesting that neither may have the ability to interact with PKR.

Residues of interest	Corresponding residues in H17N10 NS1	Predicted functional ability in bat NS1s	References
NLS1 (R35, R38, K41) **NLS2/NoLS** (219-232)	NLS1: R36, R39, K41 NLS2/NoLS: not present	Likely to have a nuclear but not nucleolar localisation pattern as NLS2 is only found in H3N2 NS1 proteins.	(Melen et al., 2007, Greenspan et al., 1988)
RNA-binding (R38, K41, S42)	R39, K42, A43	RNA binding capability may be reduced by the presence of A43	(Wang et al., 1999, Cheng et al., 2009)
ED dimerisation (W187)	W189	Dimerisation of ED should promote RNA-binding	(Ayllon et al., 2012b, Kerry et al., 2011)
CPSF30 binding (F103, M106, K108, D125, D189)	V103, Q106, K108, N125, D189	Unlikely to bind CPSF30 due to several polymorphisms at interaction site	(Das et al., 2008, Hale et al., 2010)
PI3K binding (Y89, P162, P164)	Y89, P162, S164	Cannot predict interaction with p85β subunit of PI3K	(Hale et al., 2008b)
PKR binding (residues 123-127: IMDKN)	123-127: VTNKV (H17) ITDKV (H18)	May be unable to interact with PKR	(Li et al., 2006, Min et al., 2007)

Table 4. Predicting the functional capabilities of the H17N10 NS1 protein by sequence analysis. The sequence alignment of a panel of IAV NS1 proteins with the H17N10 bat IAV NS1 protein highlighted residues that were conserved and those with polymorphisms present. Functional abilities of the H17N10 NS1 could be predicted based on these residue observations. This table displays certain known residue requirements for nuclear/nucleolar localisation signals (NLS1/2, NoLS), RNA-binding, ED dimerisation, CPSF30, PKR and PI3K-binding capabilities in NS1 proteins. The corresponding residues that are present in the H17N10 NS1 are also shown. Any polymorphisms in the H17N10 NS1 are highlighted in red here with the predicted effect on its functional abilities also commented on.

3.2 Construction of NS1 expression vectors

3.2.1 Cloning strategy for NS1 cDNA

In order to characterise the two bat IAV NS1 proteins, a cloning strategy was developed for the generation of a panel of IAV NS1 expression vectors. Three IAV NS1 proteins were chosen for comparison with the two bat IAV NS1 proteins in this study; H1N1, H5N1 and H7N9 NS1 proteins. A human representative (H1N1) from the A/Puerto Rico/8/34 virus was chosen as this NS1 is a well-studied, lab-adapted example and a good basis for comparative studies. The avian NS1 (H7N9) from the A/Shanghai/2/S1078/2013 virus was chosen as it was found by preliminary BLAST analysis to be one of the closest related avian IAV NS1 proteins to the bat IAV NS1 proteins. The H5N1 NS1 protein from the A/Nigeria/OG10/2007 virus was chosen as an example of an NS1 protein that can interact with CPSF30 in order to suppress host gene expression (Kainov et al., 2011).

The H17N10, H18N11, H5N1 and H7N9 NS1 cDNAs were cloned into separate pLVX.V5.MCS.IRES.zsGreen vectors, with the PR8 H1N1 NS1 in the same vector already available in the lab. The NS1 cDNAs were cloned into the MCS present in the vector (shown in **Figure 4**). All NS1 cDNAs encoded silent mutations in the splice acceptor site to prevent expression of NEP (described in (Basler et al., 2001)). The H17N10 and H18N11 NS1 cDNAs were restriction enzyme digested and ligated into the vector between the EcoRI and XbaI restriction site, the H5N1 NS1 cDNA was between the SpeI and NotI sites, whereas H7N9 NS1 cDNA was cloned using the EcoRI and NotI sites. Use of these sites depended on their absence in the NS1 cDNA.

Present in the vector is an internal ribosome entry site (IRES), designating the mRNA bicistronic as it produces the NS1 protein of interest along with a zsGreen fluorescent protein. Presence of the zsGreen protein was useful for determining the transfection efficiency of the NS1 expression plasmids during these studies. A further feature of this vector is a stretch of nucleotides encoding the 14-amino acid V5 tag located at the 5’ end of each NS1 cDNA. Thus the NS1 proteins were expression with the V5-tag present at the N-terminus as the NS1 C-terminus is variable and can be responsible for a number of functions in which interference is avoided.

3.2.2 Testing expression of NS1 proteins from the pLVX vector constructs

To determine if the pLVX expression plasmids constructed were able to express the two bat NS1 proteins encoded, 293T cells were transfected with pLVX vectors encoding the PR8, H17N10, or H18N11 NS1 cDNAs. 48 hours post-transfection cells were lysed in UDB and analysed by SDS-PAGE and western blot using α-V5-336 antibody to determine expression levels. The total NS1 expression levels are shown in **Figure 7** where the bat NS1 proteins, and the PR8 NS1, can be seen to express equally.

3.3 Bat IAV NS1 proteins have nuclear localisation patterns in human and bat cells

During the early stages of infection the IAV NS1 protein is known to localise to the nucleus, with this targeting predominantly mediated by the presence of an N-terminal NLS1, involving the residues R35, R38 and K41 (Melen et al., 2007, Greenspan et al., 1988). As these residues are conserved in the two bat NS1 proteins it was predicted that they would also exhibit nuclear localisation patterns. There is also the presence of a second NLS (NLS2) in certain avian IAV NS1 proteins with an elongated length of 237 amino acids, positioned between residues 219-232 (Melen et al., 2007). However, as the H17N10 and H18N11 NS1 proteins are only 221 amino acids long this is not likely to be present or play a role in nuclear localisation.

In order to determine the intracellular localisation of the two bat IAV NS1 proteins, and in particular compare between human and bat cells, human lung fibroblast (MRC5-hTERT) and bat lung epithelial (TB1-Lu) cells were transfected with the V5-NS1 expression plasmids for 24 hours. Fixing of the cells was followed by immunofluorescence using α-V5 antibody, and intracellular localisation was observed by confocal microscopy. Localisation patterns of the H1N1, H5N1, H7N9, H17N10 and H18N11 NS1 proteins were seen to be all predominantly nuclear in both human and bat cells (shown in **Figures 8A and B)**. All NS1 proteins tested were absent from the nucleolus. As a nucleolar location signal (NoLS) has been reported to be found in NS1 proteins in the NLS2 region (between residues 219 and 237), it was expected that none of the NS1 proteins tested would exhibit nucleolar localisation due to their truncated lengths (Melen et al., 2007).

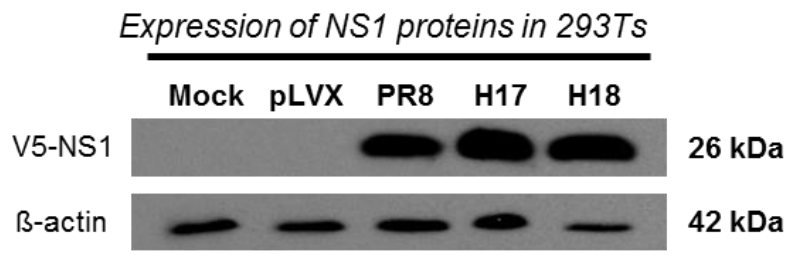


Figure 7. Testing the expression of NS1 proteins from the constructed pLVX expression vectors. 293T cells were transfected with either a pLVX expression plasmid with no NS1 encoded, or pLVX vectors encoding the PR8 (H1N1), H17N10 or H18N11 NS1 cDNAs. Cells were harvested after 48 hours with total lysates analysed by SDS-PAGE and western blot using α-V5 antibody to show total levels of NS1 present. Also shown is a further negative control (mock) where no DNA was transfected. Levels of NS1 expression were seen to be similar for the PR8, H17N10 and H18N10 NS1 proteins. Levels of NS1 expression were compared to that of β-actin as a loading control for the amount of protein.

Furthermore, there is perhaps a greater cytoplasmic presence in the bat cells for the two bat IAV NS1 proteins compared with the other IAV NS1 proteins which remain predominantly nuclear. This could potentially be governed by differences seen in the NES residues in the bat IAV NS1 proteins which are located at positions 134 to 147 (and highlighted in the sequence alignment shown in **Figure 6**). Nuclear export requires the alleviation of inhibitory signals located 14 residues downstream, thus there may be differences in the functioning of these inhibitory signals amongst the NS1 proteins (Li et al., 1998). However, it may be that there are differences in the expression levels of the NS1 proteins and that the two bat IAV NS1 proteins are simply expressed at a higher level in these bat cells.

3.4 Bat IAV NS1 proteins antagonise IFN-β induction in human and bat cells

The ability to antagonise the IFN-β response is perhaps the major function of NS1 during IAV infection (Garcia-Sastre et al., 1998) therefore this capability was tested for the two bat IAV NS1 proteins. A previous study has shown the H17N10 NS1 to inhibit IFN-β induction to a level comparable to that of PR8 NS1 in human HEK-293T cells (Zhou et al., 2014). This study sought to expand on these findings to include the H18N11 NS1 and to investigate the ability of these NS1 proteins to inhibit IFN induction in a range of cell types from various species.

To determine the ability of the two bat IAV NS1 proteins to antagonise the induction of IFN-β in human and bat cells, a dual luciferase assay was conducted for the panel of IAV NS1 proteins. Human 293T cells and bat TB1-Lu cells were co-transfected with a pLVX plasmid expressing the NS1 of interest, or GST as a negative control, plus two luciferase reporter plasmids; a *Firefly* luciferase (FF-luc) expressing plasmid under the control of the IFN-β promoter, and a *Renilla* luciferase (Ren-luc) expressing plasmid under the control of a constitutively expressed HSV-TK promoter. FF-luc expression therefore is an indicator of the level of IFN-β activation, whereas the Ren-luc expression is used as an internal control for transfection efficiency.

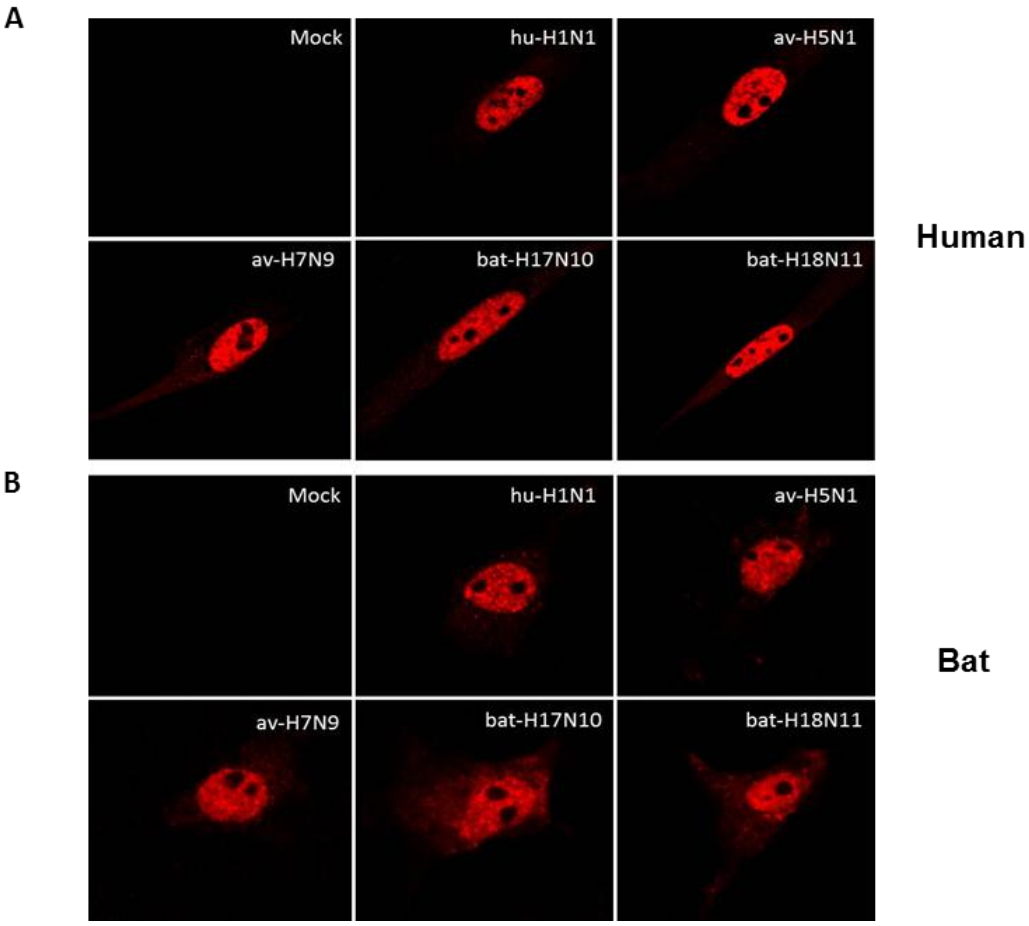


Figure 8. Intracellular localisation of human, avian and bat IAV NS1 proteins in (A) human and (B) bat cells. Human lung fibroblast (MRC5-hTert) and bat lung epithelial (TB1-Lu) cells were transfected with pLVX plasmids expressing V5-tagged NS1 proteins or an empty pLVX plasmid as a negative control. Coverslips were fixed after 24 hours and immunostained with α-V5 antibody followed by an anti-mouse fluorescent antibody (Alexa-555). Cells were visualised using confocal microscopy. Images shown represent observations from two independent experiments.

24 hours post-transfection the cells were infected with Sendai virus (SeV) for 16 hours, leading to induction of the IFN-β promoter and expression of FF-luc. Luciferase levels were then determined by dual luciferase assay and read by luminometer. Relative FF-luc production was calculated as the ratio between the FF-luc and Ren-luc levels. Values were then converted to a relative percentage compared to that for GST transfected cells infected with SeV, which was designated as the maximum induction achieved, at 100%. A further well of cells for each transfection condition was lysed to determine the levels of NS1 expression by SDS-PAGE and western blot analysis using α-V5-336 antibody.

In order to establish the dual luciferase assay in new cell types (including a panel of cell types from different species later described), transfected cells were first tested for the ability of SeV to induce IFN-β reporter activity. This was achieved by infecting replicate wells of reporter transfected cells with 10-fold dilutions of SeV, and the levels of FF-luc production were compared to that of non-infected cells. Cell lines in which induction of the IFN promoter by SeV was successful included human, bat, horse, mouse and dog cells (raw data, not including dog cells, is provided in **Table 5A**). However the chicken, bovine and porcine cell lines were not found to support IFN-β reporter induction after SeV infection (raw data for the chicken and bovine cells is provided in **Table 5B**). This could be due to issues with transfection efficiency of the luciferase plasmids, or indeed that SeV cannot competently infect these cell types.

In human cells, in comparison to GST, the PR8 NS1 was found to antagonise IFN-β reporter activity by approximately 90% (shown in **Figure 9A**). This was comparable to the two avian IAV NS1 proteins, H5N1 and H7N9, which inhibited induction by approximately 85 and 90%. Additionally, the two bat IAV NS1 proteins, H17N10 and H18N11, were also able to inhibit IFN-β reporter activity by over 80 and 85% in human cells, therefore at a level comparable to the other IAV NS1 proteins tested.

In bat cells, SeV infection also induced robust of the IFN-β reporter activity (shown in **Figure 9B**). However, the human and avian IAV NS1 proteins were only able to antagonise induction by less than 50% compared to the GST control. Similar abilities were again observed for the two bat IAV NS1 proteins, with an ability to limit IFN-β promoter induction to approximately 45% on average. A lesser ability of the panel of NS1 proteins to antagonise IFN-β reporter activity in the bat cells may be due to the

NS1 proteins having less of an ability to interact with the bat cell specific factors involved in the IFN induction cascade, for example RIG-I or Riplet. The remaining ability to limit IFN-β promoter activity could be solely due to the sequestering of dsRNA.

Also shown in **Figures 9A** and **B** are the expression levels of each NS1 protein in human and bat cells. In human cells these expression levels are seen to be comparable amongst the NS1 proteins, with the exception of the H5N1 NS1 which expressed at relatively low levels. This may be due to its ability to bind CPSF30 thus affecting general gene expression and therefore its own expression. In the bat cells, there is a noticeable decrease in H1N1 NS1 expression in comparison to the other NS1 proteins (aside from H5N1 NS1). This finding is surprising given that PR8 NS1 is known to express well, and it may be that there were experimental issues with this particular plasmid stock having a lower concentration than thought.

3.5 Bat IAV NS1 proteins antagonise IFN-β induction in a range of cell lines from different species

The ability of the bat IAV NS1 proteins to limit the induction of IFN in cells from a range of species was tested to assess the potential contribution to the zoonotic ability of these bat viruses. Studies involving the IFN-antagonist factors from other viruses have established that these viral proteins can play a role in determining host range. The V protein of parainfluenza virus 5 (PIV5) circumvents the human innate immune response by STAT2-mediated STAT1 degradation, however due to the divergent STAT2 protein found in mice, the PIV5 V protein is unable to antagonise IFN signalling in mouse cells (Park et al., 1999, Didcock et al., 1999). Another study established that the V protein of Newcastle disease virus (NDV) is a host range determinant due to species-specific effects on IFN-antagonism and apoptosis prevention (Park et al., 2003).

A

Cell type	SeV dilution	FF	Ren	Ratio	Fold over mock
Human (293T)	*Mock*	17,277	458,914	0.04	0
	1:10	7,015,355	457,738	15.32	380
	1:100	2,881,533	475,871	6.0553	150
Bat (TB1-Lu)	*Mock*	1,703	2,007	0.85	0
	Neat	138,822	2,118	65.57	77
	1:10	162,883	3,348	48.65	57
	1:100	39,931	1,538	25.96	31
Horse (NBL6)	*Mock*	270	1,225	0.22	0
	Neat	1,044	182	5.73	26
	1:10	16,111	272	59.14	269
	1:100	2,028	196	10.32	47
Mouse (BF)	*Mock*	66,707	42,595	1.57	0
	Neat	312,187	21,534	14.50	9
	1:10	276,271	18,611	14.84	9.5
	1:100	256,560	19,399	13.23	8

B

Cell type	SeV dilution	FF	Ren	Ratio	Fold over mock
Chicken (DF-1)	*Mock*	1,676,649	1,969,866	0.85	0
	Neat	1,496,705	1,366,523	1.10	1.3
	1:10	1,486,409	1,631,255	0.91	1
	1:100	1,588,767	1,746,477	0.91	1
Bovine (MDBK)	*Mock*	173	2,719	0.064	0
	Neat	204	1,748	0.12	1.9
	1:10	262	2,294	0.11	1.7
	1:100	330	2,406	0.14	2

Table 5. Raw data for the induction of the IFN-β reporter activity by Sendai virus in various cell types (A) Cell lines in which induction was successful. To establish the dual luciferase IFN-β induction assay in various cell types, cells were infected with different dilutions of SeV 24 hours post-transfection with the FF-luc and Ren-luc plasmids. Dilutions of SeV were 10-fold from mock (non-infected), neat SeV, a 1 in 10 dilution and a 1 in 100 dilution. After 16 hours of IFN-β promoter stimulation by SeV, cells were lysed and luciferase levels read by luminometer. Cell lines in which IFN-β promoter induction was successful include human 293T, bat TB1-Lu, horse NBL6, mouse BF and dog MDCK cells, though data for MDCK cell induction is not shown here. An increase in the ratio between FF and Ren-luc readings following SeV infection denoted successful IFN-β promoter induction. Values highlighted in red indicate successful induction of the IFN-β promoter by SeV. **(B) Cell lines in which induction was not successful.** Certain cell lines in which induction of the IFN-β promoter by various concentrations of SeV infection was not successful included chicken DF-1, bovine MDBK and porcine PK15 cells, however raw data for the PK15 cells is not shown here. Any increases in FF-luc:Ren-luc ratios were not deemed great enough to represent successful induction in these cells.

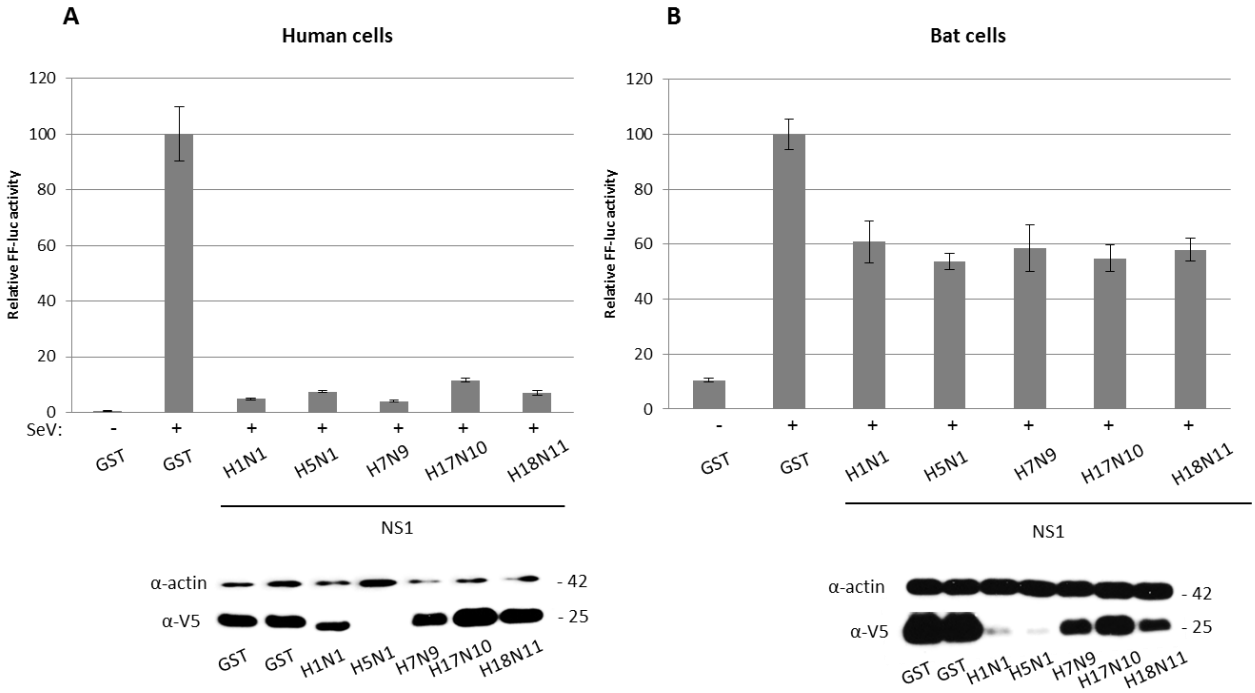


Figure 9. Limitation of IFN-β reporter activity by various IAV NS1 proteins in (A) human and (B) bat cells. Human 293T and bat TB1-Lu cells were co-transfected for 24 hours with a pLVX plasmid expressing NS1 or GST, plus two luciferase reporter plasmids; an IFN-β promoter driven *Firefly luciferase* expressing plasmid (p125luc) and an HSV-TK promoter driven *Renilla luciferase* constitutively expressing plasmid (pRL-TK). Cells were infected with SeV 16 hours post-transfection to stimulate the IFN-β promoter. Following this, dual luciferase assay and luminometer readings determined the levels of luciferase produced. Relative FF-luc activity was determined as the ratio between the two luciferase readings. Results were normalised to GST plus SeV as this was deemed the greatest level of induction possible. Bars represent mean values for triplicate repeats and error bars represent the standard deviation for these repeats. Results are also representative of two independent experiments. Also shown are western blot results for the expression of the GST/NS1 proteins for each condition.

To determine if the bat IAV NS1 proteins would have the ability to antagonise IFN induction in different species, a panel of mammalian and avian cell types were tested for this ability. A panel of swine, bovine, horse, chicken, dog and mouse cells were chosen as these species are known host species for IAVs (aside from mice which are a laboratory model) or would potentially be in close contact with bats. However, attempts at establishing this assay in swine, bovine and chicken cells were unsuccessful (data shown in **Table 5B**).

Thus, the dual luciferase assay as described previously was performed successfully in only horse (NBL6), dog (MDCK) and mouse (BF) cells. Cells were co-transfected with pLVX plasmids expressing NS1 or GST, and the two luciferase reporter plasmids (FF-luc and Ren-luc). After 24 hours the cells were infected with SeV to stimulate the IFN-β promoter. 16 hours later cells were lysed and levels of FF- and Ren-luc determined by luminometer readings. Relative FF-luc levels were converted to percentages and compared to GST-transfected cells infected with SeV as the positive control set at 100% reporter activity.

Data presented here (shown in **Figure 10**) shows IFN-β reporter antagonism by the panel of IAV NS1 proteins in horse, mouse and dog cells. For all three of these cell lines, the two bat NS1 proteins are able to antagonise induction of the IFN-β reporter to similar levels as the human and avian IAV NS1 proteins. In the horse cells, all the NS1 proteins antagonise IFN-β reporter induction around 80% compared to the GST control. This antagonism was below the high background level seen in the GST control not infected with SeV for the horse cells, and it may be that the IFN response is highly active in these cells. Again, in the dog cells the panel of NS1 proteins were able to antagonise IFN-reporter activity, this time to around 50% on average when compared to the GST control.

In the mouse cells, there was more variation seen amongst the antagonism abilities of the NS1 proteins. The H1N1, H7N9, H17N10 and H18N11 NS1 proteins were capable of limiting induction of the IFN-β reporter to approximately 70% on average compared to the GST control. However, in this particular assay it seems that the H5N1 NS1 protein was unable to antagonise FF-luc production. It may be that due to this NS1 blocking general gene expression (discussed later), and the fact that this data is normalised to the levels of Ren-luc (reading general gene expression) that for the

H5N1 the effect on specific FF-luc antagonism is 'masked'. This is a caveat to this particular assay when NS1 proteins that dramatically affect gene expression are included.

3.6 Bat IAV NS1 proteins do not block general gene expression

A known strain-specific function of the IAV NS1 is the ability to interact with host cell CPSF30. This interaction limits the cellular processing of mRNAs, thus suppressing general gene expression (Das et al., 2008, Nemeroff et al., 1998). This ability has been mapped to specific NS1 residues and includes the residues found at positions 103 and 106, where the presence of F103 and M106 is the consensus for CPSF30 binding (Hale et al., 2010, Kochs et al., 2007). Interaction with CPSF30 is conserved in circulating IAVs that infect humans and in the H5N1 NS1 that was used in this study (Twu et al., 2007). However PR8 NS1 and the avian H7N9 NS1 are known to be unable to bind CPSF30 due to polymorphisms at residues 103 and 106, thus cannot block general host gene expression (Kochs et al., 2007, Ayllon et al., 2014).

To determine the ability of the bat IAV NS1 proteins to block general gene expression in human and bat cells; 293T and TB1-Lu cells were co-transfected with a pLVX plasmid expressing the NS1 of interest, or GST, alongside a constitutively expressed Ren-luc reporter plasmid as described previously. The total level of Ren-luc production was measured 24 hours later by luciferase assay and values were made relative to the GST control which was set to 100%.

In human cells, PR8 NS1 was unable to block general gene expression whereas the H5N1 NS1 was able to do so, reducing the level of luciferase production approximately 70% compared to the GST control (**Figure 11A**). As H5N1 NS1 has the consensus residues (F103 and M106) for CPSF30 binding, whereas PR8 NS1 does not (S103 and I106), this was to be expected. The H7N9 NS1 was also unable to block general gene expression in human cells, which is expected as it features the residues L103 and I106, and therefore is unable to bind CPSF30. It was also found that the two bat IAV NS1 proteins are also unable to block gene expression in human cells. These NS1 proteins are also predicted to not interact with CPSF30 due to polymorphisms at residues 103 (V/I) and 106 (Q).

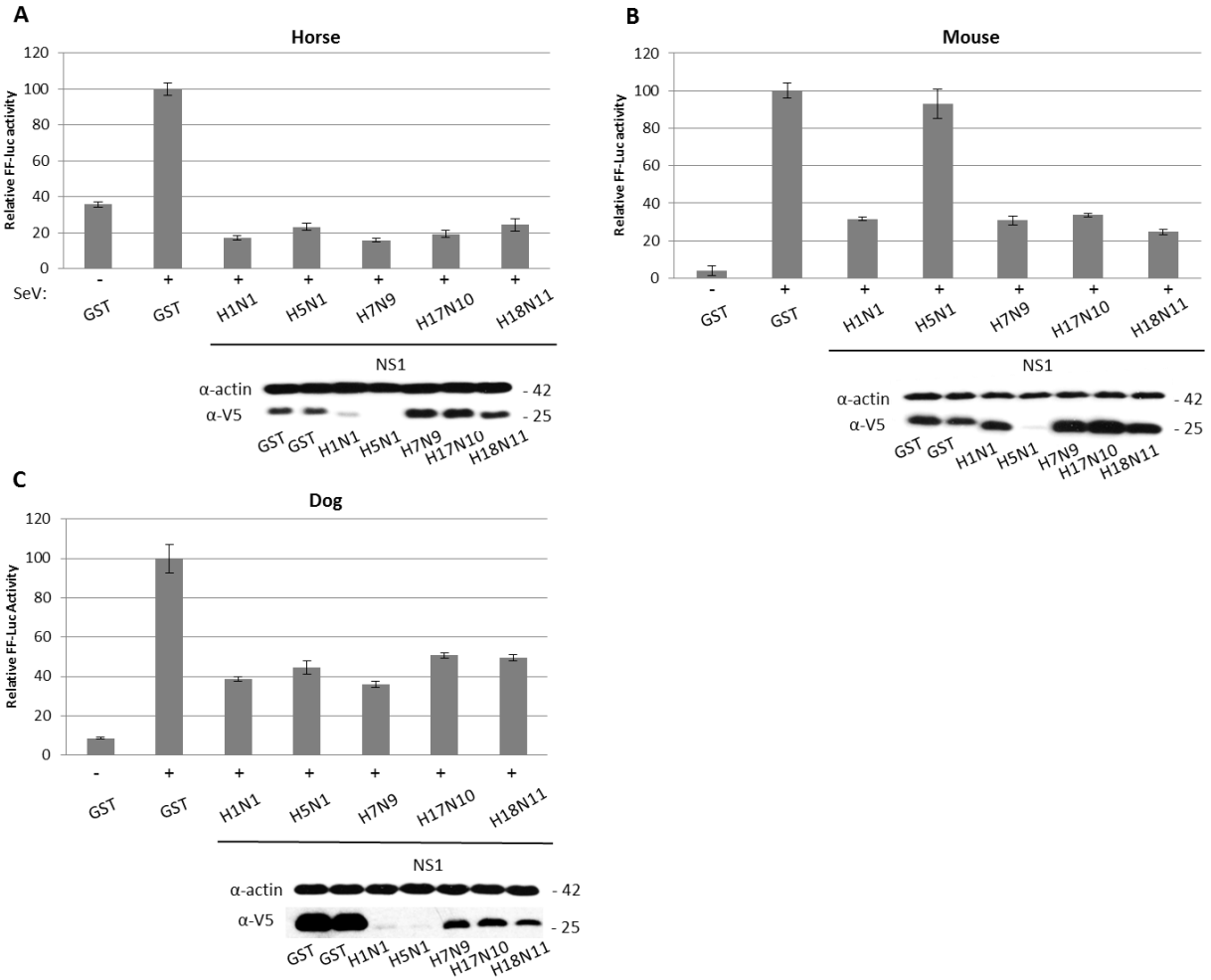


Figure 10. Limitation of IFN-β reporter activity in different species: (A) horse, (B) mouse and (C) dog cells. Horse NBL6, mouse BF and dog MDCK cells were co-transfected for 24 hours with a pLVX plasmid expressing NS1/GST, plus two luciferase reporter plasmids; an IFN-β promoter driven *Firefly luciferase* expressing plasmid (p125luc) and an HSV-TK promoter driven *Renilla luciferase* constitutively expressing plasmid (pRL-TK). Cells were infected with SeV 16 hours post-transfection to stimulate the IFN-β promoter. Following this, dual luciferase assay and luminometer readings determined the levels of luciferase produced. Relative FF-luc activity was determined as the ratio between the two luciferase readings. Results were normalised to GST plus SeV. Bars represent mean values for triplicate repeats and error bars represent the standard deviation for these repeats. Results are representative of two independent experiments. Western blot results are also shown which indicate the levels of GST/NS1 expression in each of the experimental conditions.

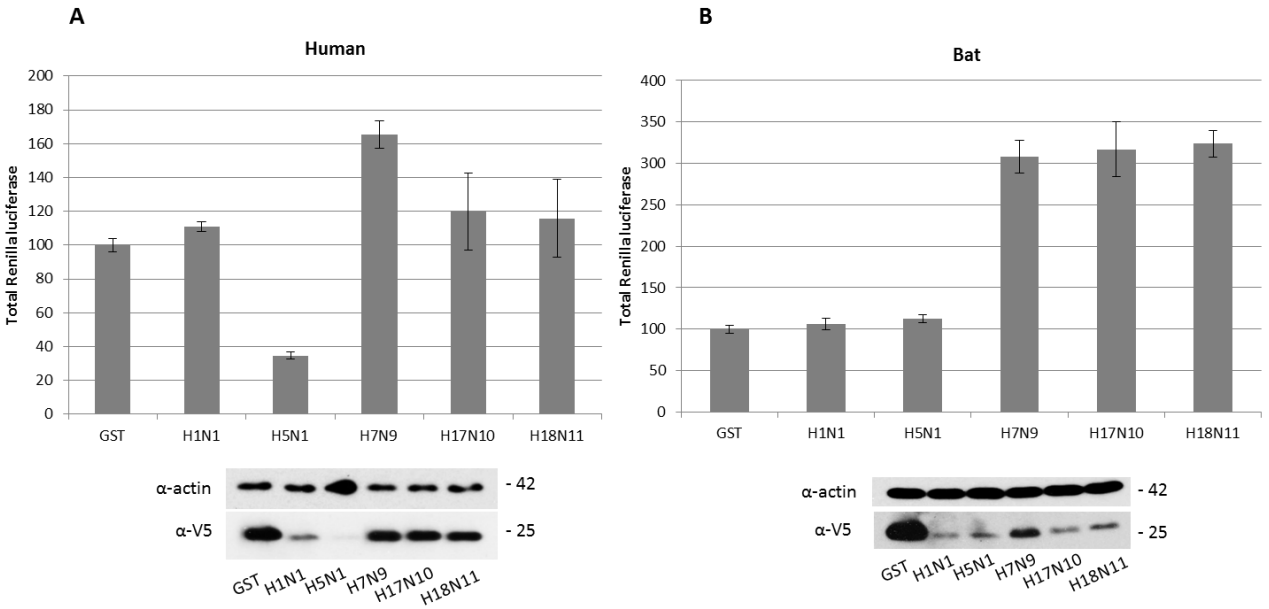


Figure 11. Ability of the various NS1 proteins to block general host gene expression in (A) human and (B) bat cells. Human 293T and bat Tb1-Lu cells were co-transfected with a pLVX plasmid expressing a GST or NS1 and a constitutively expressed *Renilla luciferase* encoding plasmid (pRL-TK). Total *Renilla luciferase* expression levels were measured 24 hours later by luciferase assay and luminometer readings. Values obtained were converted to a percentage and made relative to the GST control which was designated 100%. Bars represent mean values for triplicate repeats and error bars represent the standard deviation for these repeats. Results are representative of two independent experiments. Western blot results are also shown for the expression levels of GST/NS1 for each experimental condition.

In bat cells, it was also observed that the PR8 and H7N9 NS1 proteins cannot block general gene expression (**11B**). However, conversely to the observation in human cells, the H5N1 NS1 was also unable to block gene expression in the bat cells. It may be that this NS1 is unable to bind the bat specific CPSF30. Furthermore, it was observed that the two bat NS1 proteins (alongside the H7N9 NS1) actually enhance gene expression to over 300% in comparison with the GST control. The mechanism for this enhancement is unknown, however a previous study has reported PR8 NS1 to be an enhancer of reporter plasmid expression at the post-transcriptional level, in a non-specific manner (Salvatore et al., 2002). Further work would be required to determine if it is at this level that the bat IAV NS1 proteins are enhancing gene expression.

3.7 Investigating interactions with host cell factors

3.7.1 H17N10 NS1 co-precipitates human RIG-I

It has been previously reported that NS1 interacts with the pattern-recognition receptor RIG-I to prevent the pre-transcriptional activation and production of IFN (Guo et al., 2007). In order to assess the RIG-I binding ability of the two bat IAV NS1 proteins, an immunoprecipitation study was conducted using a FLAG-tagged RIG-I construct. 293T cells were co-transfected with a pLVX vector expressing an NS1 or GST (or an empty vector as a negative control), plus a vector expressing FLAG-tagged RIG-I. Cells were harvested after 48 hours and soluble lysates precipitated with an α-V5 antibody to pull-down the V5-tagged NS1 proteins, and any associated proteins. Soluble 'input' and the 'pull-down' fractions were analysed by SDS-PAGE and western blot, probing with α-FLAG and α-V5 antibodies to visualise the RIG-I and GST/NS1 proteins, respectively.

Western blot analysis showed that there is a low level of non-specific RIG-I pull-down by GST (**Figure 12A**), however there is an enhanced pull-down of RIG-I for the PR8 NS1 as expected. This is also observed for the avian H7N9 and bat H17N10 NS1 proteins, suggesting specific interactions with RIG-I. The H18N11 NS1 protein is however unable to co-precipitate RIG-I to a level greater than the non-specific level of GST. Therefore it cannot be concluded that this particular NS1 interacts with RIG-I. Furthermore, the H5N1 NS1 does not show co-precipitation of RIG-I, suggesting this

NS1 cannot interact with RIG-I. Indeed, an H5N1 NS1 protein with the F103 and M106 residues which are the consensus for CPSF30 interaction showed an inability to bind RIG-I (Dankar et al., 2013). However, the ability of the H5N1 NS1 to block general gene expression, and therefore its own expression may also explain a lack of noticeable co-precipitation. A caveat of this system is expressing this NS1 and host factor in the same cell, whereas in the Dankar et al. study, separate bacterial expression vectors were employed.

3.7.2 H17N10 NS1 co-precipitates human Riplet

Another strain-specific NS1 capability is the interaction with the ubiquitin E3 ligase Riplet as a further means to prevent the pre-transcriptional production of IFN (Rajsbaum et al., 2012). To determine if the bat IAV NS1 proteins could interact with human Riplet, a further immunoprecipitation was performed. 293T cells were co-transfected with a pLVX vector expressing a V5-tagged NS1/GST and an HA-tagged Riplet. Following harvesting 48 hours later, soluble cell lysates were precipitated with α-V5 336 antibody to pull-down V5-tagged NS1/GST and any associated proteins. Input and pull-down samples were analysed using SDS-PAGE and western blot, probing with α-HA and α-V5 antibodies to detect HA-tagged Riplet and V5-tagged GST/NS1 proteins.

Again, western blot analysis shows that there is a non-specific interaction of HA-Riplet with the GST control; however co-precipitation levels of Riplet by the PR8 NS1 are much enhanced (**12B**). This enhanced pull-down is also observed for the bat H17N10 NS1, but not for the H18N11 NS1, whose co-precipitation levels of Riplet do not exceed that of GST. Thus, specific interactions between H18N11 NS1 and Riplet cannot be concluded, but most likely can for the H17N10 NS1.

Clear from these immunoprecipitation results is the difference in binding ability between the two bat IAV NS1 proteins. The H17N10 NS1 is able to co-precipitate human RIG-I and Riplet to comparable levels to the human H1N1 NS1 protein. However, the H18N11 NS1, if it is able, does so to a much lesser extent. This may highlight differences present between the two bat IAV NS1 proteins themselves, and could be an indication of the diversity of these bat viruses

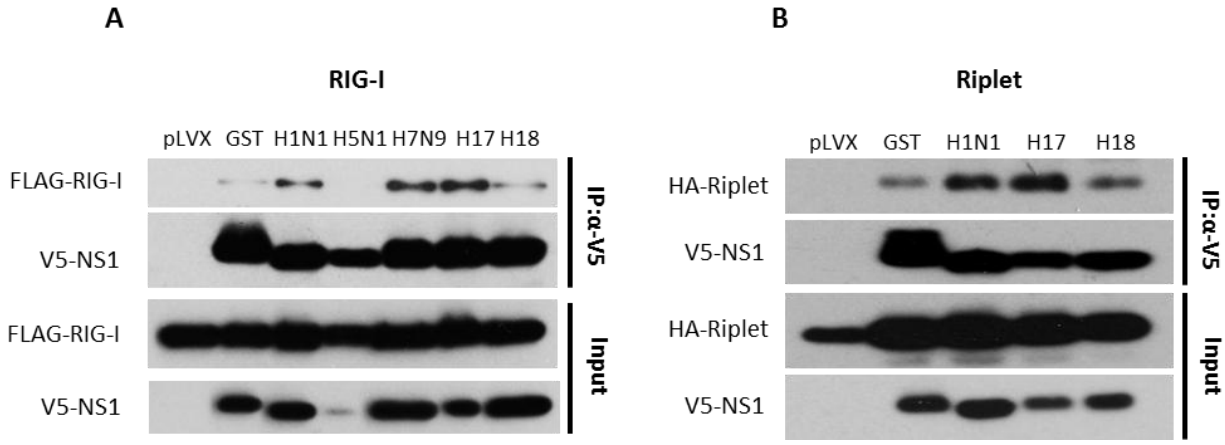


Figure 12. Immunoprecipitation studies with various NS1 proteins and known host interactors (A) RIG-I and (B) Riplet. Human 293T cells were co-transfected with a plasmid expressing a tagged host protein of interest (FLAG-tagged RIG-I or HA-tagged Riplet) plus either an empty pLVX plasmid or one expressing V5-tagged GST/NS1. Cells were harvested 48 hours post-transfection. Soluble lysates were immunoprecipitated using α-V5 antibody. The soluble 'input' samples and IP 'pull-down' samples were analysed by western blot. α-V5 HRP antibody was used to probe for GST/NS1, with α-FLAG and α-HA antibodies used to probe for tagged RIG-I and Riplet, respectively.

3.7.3 H17N10 and H18N11 NS1 proteins do not interact with human p85β

A known function of NS1 proteins during IAV infection is the binding and activation of the PI3K signalling pathway (Hale et al., 2006). This interaction occurs specifically between the NS1 ED and the p85β subunit of PI3K; however no major downstream consequences resulting from the IAV activation of this pathway have currently been elucidated. Whilst all IAV NS1 proteins are known to bind p85β, it has been shown that a specific H5N1 NS1, from A/Chicken/Guangdong/1/05, whilst retaining p85β binding capabilities, is unable to activate the PI3K signalling pathway (Li et al., 2012b). Furthermore, there have been strain-specific consequences due to the activation of this pathway, with viral growth kinetics affected in only certain strains (Hale et al., 2006, Ayllon et al., 2012a). It was therefore initially tested, using an immunoprecipitation assay, if the two bat IAV NS1 proteins could interact with the human p85β subunit.

In order to assess the binding ability of the two bat IAV NS1 proteins to the p85β subunit of PI3K, co-transfection of 293T cells with EYC-tagged p85β and V5-tagged NS1/GST was conducted. Soluble cell lysates were immune-precipitated for NS1 (and any co-precipitates) and analysed by SDS-PAGE and western blot, using an α-GFP antibody to detect p85β and a α-V5 antibody to detect GST or NS1. As expected, the human PR8 and avian H5N1 and H7N9 NS1 proteins were able to co-precipitate p85β (**Figure 13A**). However, the two bat IAV NS1 proteins were unable to co-precipitate human p85β. Therefore, these bat IAV NS1 proteins represent the first naturally occurring IAV NS1 proteins that are unable to interact with p85β.

3.7.4 Residues that may be responsible for lack of p85β binding

The observation that the two bat IAV NS1 proteins do not bind human p85β presented two hypotheses for the lack of binding; firstly that the bat IAV NS1 proteins have evolved to preferentially bind a different factor, or secondly that the bat-specific p85β subunits have divergent sequences, with the bat IAV NS1 proteins interacting with their host-specific factor.

It was intriguing that the two bat IAV NS1 proteins did not interact with p85β, given that they both possess the Y89 and P164 residues previously seen to play an important role in this interaction (Hale et al., 2006). However in a parallel study a fellow student

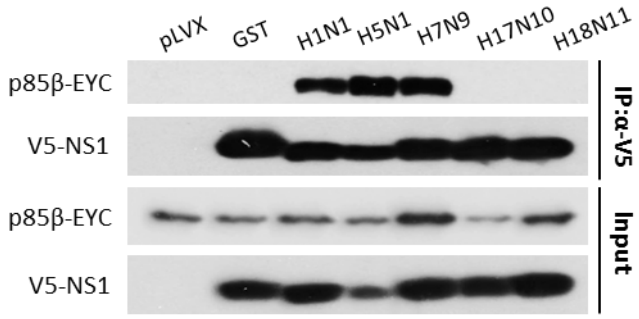
has analysed the NS1-p85β interaction surface by alanine scanning, identifying many more important residues important for the binding of PR8 NS1 to p85β. The three residues found to be most critical included L95, M98 and I145; however the NS1-p85β interaction site involves 20 residues on NS1. After analysis of the H17N10 NS1 sequence, six particular residues that differ in the bat IAV NS1 sequence from that of PR8 NS1 were chosen for single amino acid substitution experiments (highlighted in **Figure 13B**).

These particular six residues were chosen to be substituted to the corresponding residues found in PR8 NS1 to assess by immunoprecipitation if any has an effect on the ability to bind p85β. These residue substitutions included Q95L, T98M, I99S, R143T, N161S and S164P (PR8 NS1 numbering). Three of the residues chosen were shown to be important in the alanine scan performed in the parallel study; these include positions 95, 98 and 164. The other three residues to be substituted were found to be less important for binding but were included to fully assess all potential residue contributions. These residues include positions 99, 143 and 161. Amino acid substitutions were generated in the H17N10 NS1 background, as opposed to the H18N11 background, as this NS1 was found to have more functional similarities to PR8 NS1.

3.7.5 Single amino acid substitutions are unable to restore p85β-binding

To determine the effect of single amino acid substitutions on p85β binding, an immunoprecipitation study was conducted as described previously for transfected EYC-tagged p85β and V5-tagged GST or NS1 proteins. **Figure 14** shows the immunoprecipitation results for the H17N10 NS1 mutants, compared with GST, PR8 NS1 and wild-type H17N10 NS1. As seen previously, the PR8 NS1 co-precipitates p85β, whereas the H17N10 NS1 is unable to co-precipitate p85β. Strikingly, none of the six single amino acid H17N10 NS1 substitutions had a substantial effect on the ability of this NS1 to bind p85β. It may be necessary to substitute multiple residues in this interaction surface of NS1 in order to restore p85β binding.

A



B

	89	95	98	99	143	161	164
H1N1	Y	L	M	S	T	S	P
H7N9	Y	L	M	S	A	S	P
H17N10	Y	Q	T	I	R	N	S

Figure 13. Investigating the interaction between the novel bat IAV NS1 proteins and the p85β subunit of PI3K. (A) Immunoprecipitation with various NS1 proteins and p85β. Human 293T cells were co-transfected with an empty pLVX expression plasmid or V5-tagged GST/NS1 alongside an EYC-tagged p85β. Cells were harvested 48 hours post-transfection. Soluble lysates were immunoprecipitated with α-V5 antibody. The soluble 'input' fractions and the IP 'pull-down' fractions were analysed by western blot using α-V5 HRP antibody for GST/NS1 detection and α-GFP antibody for p85β detection. **(B) Table showing residues of NS1 that interact with p85β with H17N10 polymorphisms highlighted.** Shown are the residues previously seen (unpublished data) to be either key for the NS1-p85β interaction (residues 89, 95, 98 and 164) or to be of a significant structural change between the H1N1 and H17N10 sequence (residues 99, 143 and 161). Highlighted in yellow are the residues at these positions that differ from the H1N1 consensus sequence, which is known to interact strongly with p85β.

3.7.6 Alignment of p85β sequences from various species reveals that NS1- interacting region is highly conserved

The broader question for these NS1 proteins is however if they are specifically able to bind the p85β subunits found in the bat species from which the bat IAV genomes were discovered. A sequence alignment of the NS1-interacting region of p85β (residues 556 to 591) from a range of species was therefore performed. These species included the four bat species that have been sequenced, plus a wider range of species.

Figure 15A shows the multiple sequence alignment for residues 556-591 of the p85β sequences for 14 different species of animals. This alignment shows that this region of p85β is essentially conserved across the mammalian species observed, with any polymorphisms occurring in more divergent species, such as the zebra fish. Conservation is also almost 100% across the bat species shown. Residue positions with polymorphisms observed in this alignment (at positions 562, 567, 580, 583) are highlighted in red in **15A** and are also shown and highlighted in red in the structure shown in **15B.** This structure represents the NS1 ED (shown in grey) and its p85β interaction surface (highlighted in yellow). Binding of the p85β molecule (shown in blue) occurs in this cleft. Highlighted in red are the residues of p85β that were shown in the alignment to vary in certain species. The structural representation in **15B** illustrates that the side chains of these varying residues do not form interactions with the surface of the NS1 ED, except for that of residue 562. However, this residue was only seen to vary in the zebra fish and not mammalian species.

The major caveat of this analysis is however that the two species of bat in which the virus genomes were discovered (little yellow shouldered and flat-faced bats) are not included here. Additionally, the bat species included in the analysis have not been shown to be infected with IAVs. It therefore cannot be concluded that all bat species have the same conserved p85β that should dictate NS1 interactions and it may be that these particular species have divergent p85β subunits.

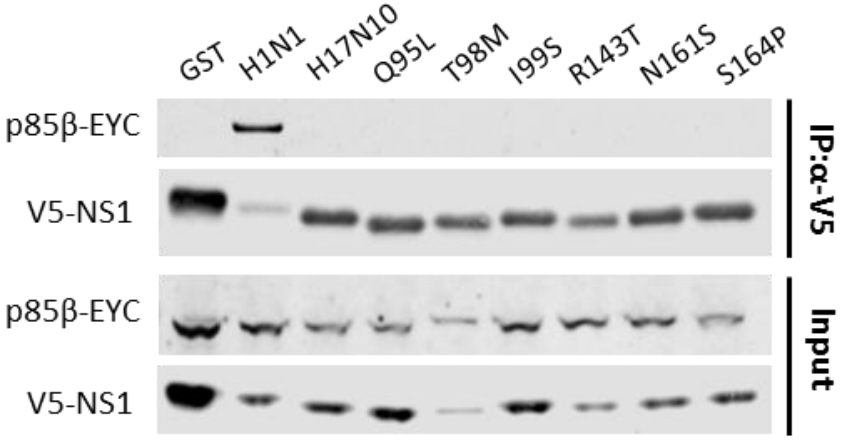


Figure 14. Immunoprecipitation studies of various H17N10 NS1 single amino acid substitutions and p85β. Six single amino acid substitutions were made in the H17N10 NS1 background at residues deemed potentially important for NS1 binding p85β. Substitutions were made in the H17N10 NS1 to the PR8 NS1 sequence at these positions. Cells were co-transfected with either PR8 H1N1 NS1, wt H17N10 NS1 or one of the 6 H17N10 NS1 mutants (Q95L, T98M, I99S, R143T, N161S and S164P) and EYC-tagged p85β. Cells were harvested 48 hours post-transfection. Soluble lysates were immunoprecipitated with α-V5 antibody. The soluble 'input' fractions and the IP 'pull-down' fractions were analysed by western blot using α-V5 HRP antibody for NS1 detection and α-GFP antibody for p85β detection.

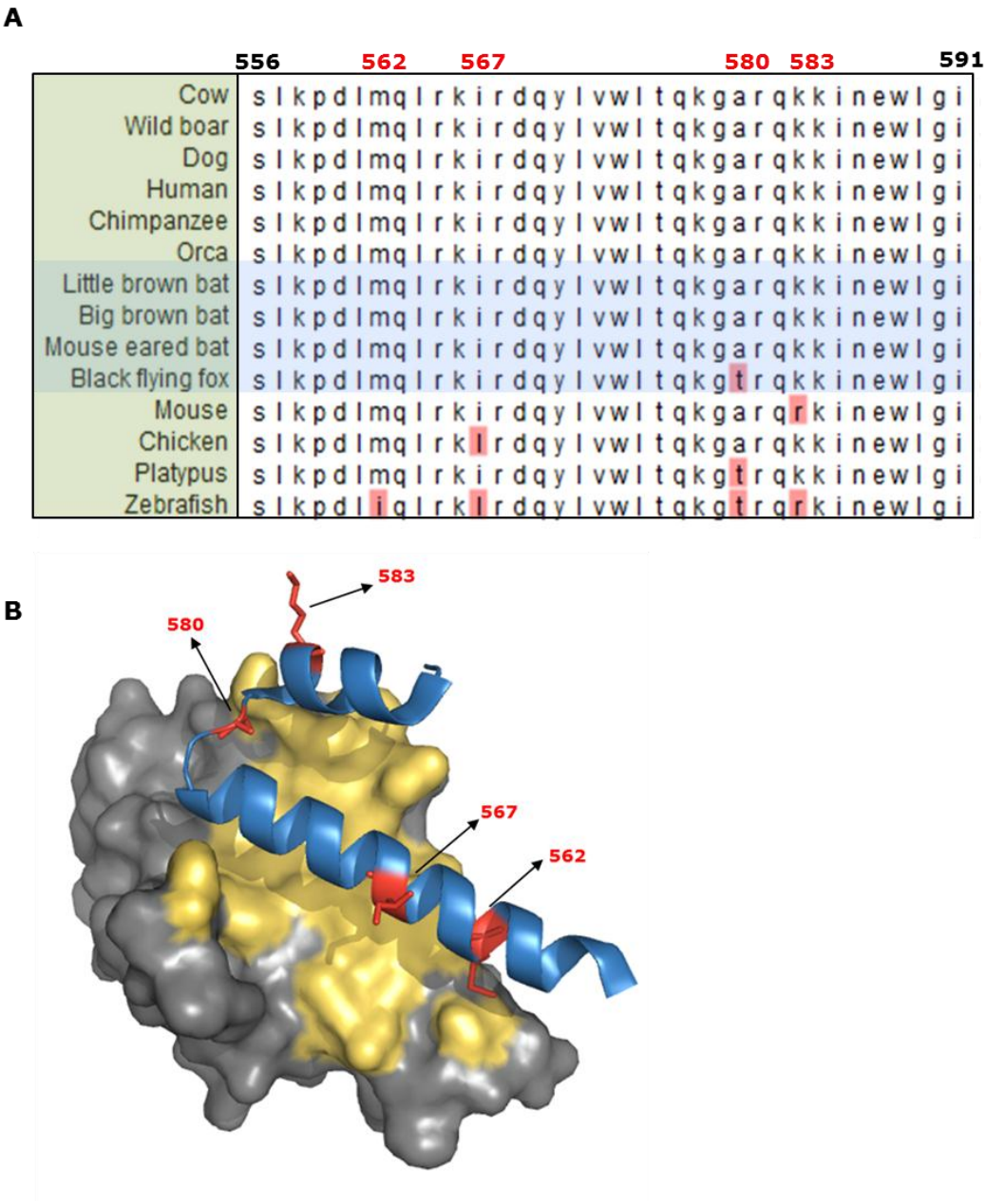


Figure 15. Conservation of the NS1 binding site in p85β subunits from different species (A) Multiple sequence alignment of the NS1-binding region of p85β from various species. The region of p85β that interacts with the NS1 ED encompasses residues 556-591. Shown are the alignments for these regions represented by 14 distinct species including 4 species of bats highlighted in blue. Any polymorphisms are highlighted in red. **(B) Structure of the NS1 ED interacting with the iSH2 domain of p85β.** The NS1 ED is depicted in grey with the region that p85β interacts with shown in yellow. p85β is shown in blue with any residues seen to diverge in certain species from the alignment (residues 562, 567, 580 and 583) highlighted in red. Only residue 562 has a side chain that forms interactions with the NS1 surface. This structure was generated in PyMOL using the published structure of the PDB ID: 3L4Q.

Chapter 4: DISCUSSION

4.1 Bat IAV NS1 proteins have conserved functions

Immediately following the discoveries of two new subtypes of IAV in South American bats it was pertinent to begin characterisation of the viral proteins encoded by the novel gene segments. This characterisation would aid understanding of the threat presented by these new viruses and the capability for cross-species transmission and/or reassortment with other IAVs. Separate studies have revealed that the H17N10 and H18N11 HA and NA surface glycoproteins are highly divergent in terms of sequence identity and functional ability compared to other canonical IAVs: neither exhibited the functions expected during typical IAV replication, with the HA protein unable to bind sialic acids and the NA protein lacking sialidase activity (Zhu et al., 2013, Li et al., 2012a, Zhu et al., 2012, Sun et al., 2013). Thus, it was of particular interest to determine if the H17N10 and H18N11 NS1 proteins would also exhibit functional divergences from their canonical IAV NS1 counterparts, or indeed if some functions are conserved.

Following initial sequence analysis of these NS1 proteins it was clear that they exhibited divergent sequence identities. In particular, the two bat IAV NS1 proteins share only approximately 45 and 49% sequence identity with the well-characterised PR8 NS1. Therefore, it could be expected that these NS1 proteins would not exhibit the canonical functions associated with this virulence factor during the IAV replication cycle. Closer inspection of the specific residues involved in certain NS1 functional abilities allowed further prediction of whether the bat NS1 proteins would possess these capabilities. For example, it was observed that the NLS1 is conserved in the two bat NS1 proteins, but not the NLS2 with the incorporated NoLS (Melen et al., 2007). This suggested that these NS1 proteins would localise to the nucleus of a host cell upon expression, but not to the nucleolus. Following expression of the NS1 proteins in human and bat cells this intracellular localisation pattern was indeed observed by immunofluorescence and confocal microscopy. These predictions and observations highlight the value of the sequence analysis of novel viral proteins as a measure of potential functional capabilities.

As the main function of the NS1 protein during IAV infection of a host cell is attributed to be antagonism of the IFN-β response (Garcia-Sastre et al., 1998), this ability was first tested for the two bat IAV NS1 proteins. It was determined that both the H17N10 and

H18N11 NS1 proteins are capable of strongly inhibiting induction of IFN-β reporter activity in human and bat cells, with a much greater antagonism in human cells than in the bat cells. There are however limitations to this assay, in particular it is not clear at which stage of the IFN response these NS1 proteins are affecting. Further assays sought to determine at which stage in the IFN-induction pathway the bat IAV NS1 proteins are antagonising IFN-β reporter activity. The ability to bind the intracellular receptor of the RIG-I induction cascade, RIG-I, and the E3 ubiquitin ligase, Riplet, were confirmed for the H17N10 NS1 protein, but not for the H18N11 NS1 protein. This highlights the strain-specific differences between the two bat NS1 proteins themselves. Indeed, there are known strain-specific differences in the ability of NS1 proteins to bind species particular E3 ubiquitin ligases, in order to prevent activation of the RIG-I signalling pathway for IFN induction. Specifically, a study revealed NS1 proteins from human, swine, avian and mouse IAVs are able to interact with human TRIM25, but not mouse TRIM25, however the human NS1 protein could preferentially bind mouse Riplet as an alternative means to antagonise RIG-I signalling (Rajsbaum et al., 2012). It would be interesting to determine if the H18N11 NS1 is able to preferentially bind TRIM25 over Riplet, or indeed if this NS1 is only able to interact with the bat-specific versions of these factors. Furthermore, the inability of the H18N11 NS1 to bind these factors presents the question of how this NS1 is able to antagonise the IFN response if it cannot interact with either the RIG-I signalling protein or the ubiquitin ligase Riplet. It may be that this NS1 relies simply on sequestering dsRNA to limit the IFN response.

The ability of the bat IAV NS1 proteins to antagonise induction of the IFN-β promoter was also confirmed in a preliminary panel of cell lines from different species including horse, mouse and dog. This IFN antagonist ability in various species is a potential measure of the contribution to the zoonotic capability of the bat IAVs. There have been documented examples of viral IFN-antagonists from other viruses playing roles in determining host range due to differing abilities to interfere with species-specific host cell innate immune responses. For example, the V proteins of PIV5 and NDV act as host range determinants due to their varying abilities to antagonise IFN signalling in different species (Park et al., 1999), (Park et al., 2003). The ability of the two bat IAV NS1 proteins to antagonise the IFN response in these species therefore would not present a barrier to bat IAV infection, however there are other barriers that need to be considered including entry of IAV into a host cell and the ability of the viral polymerase to transcribe and replicate the viral genome (Reperant et al., 2012). The IAV polymerase complex is a crucial determinant for host-

switching and in fact a single amino acid mutation at position 626 in the PB2 subunit, from the glutamic acid found in avian IAVs to a lysine found in human IAVs (E627K), allows avian IAV polymerase complexes to function efficiently in mammalian cells (Subbarao et al., 1993, de Wit et al., 2008). Interestingly, the same position in the bat IAV PB2 subunits is uniquely a serine, however these polymerase complexes have been seen to function efficiently in both human and avian cells (Tong et al., 2012, Tong et al., 2013, Juozapaitis et al., 2014). Furthermore, the bat H17N10 NP has been shown to have functional complementarity with the remaining polymerase complex subunits from various human and avian IAVs (H1N1, H3N2 and H5N1) (Juozapaitis et al., 2014). Thus, the bat IAV polymerase does not appear to present a barrier for zoonotic transmission. The most significant barrier for the bat IAVs to cross species barriers lies in the unknown identity of the host cell receptor, with neither the H17 or H18 HA able to recognise typical IAV sialic acid receptors (Sun et al., 2013, Zhu et al., 2013).

4.2 Bat IAV NS1 proteins exhibit certain strain-specific functions

The NS1 protein of IAV is well-documented as being a highly strain-specific virulence factor. Different NS1 proteins from different strains of IAV are known to have varying functional abilities. This includes the ability to block general gene expression, namely as a result of the ability to interact with host cell CPSF30 (Hale et al., 2010). Here, the residues F103 and M106 in the NS1 ED are the consensus for CPSF30 binding, though other residues in NS1 do play a role (Hale et al., 2010, Twu et al., 2007). Analysis of the H17N10 and H18N11 NS1 amino acid sequences showed that they possess residue polymorphisms of V or I103 and Q106, allowing the prediction that these NS1 proteins would not possess the ability to bind CPSF30 and would therefore not block general gene expression. It was therefore to be expected that the bat IAV NS1 proteins were unable to block general gene expression in either human or bat cells, due to this inability to interact with CPSF30. Whilst NS1 binding of CPSF30 is known to vary amongst NS1 proteins, attenuation in virus replication is associated with a lack of interaction has been previously documented. A single amino acid substitution of I106M in the avian IAV H7N9 NS1 can restore CPSF30 binding and thus a block in general gene expression which was shown to increase virus replication and virulence *in vivo* (Ayllon et al., 2014). Furthermore, when CPSF30 binding was restored to the 1997 H5N1 NS1, via substitution of residues 103 and 106 to the F and M consensus for this interaction, there was a substantial 300-fold increase of lethality of the virus in mice (Spesock et al., 2011). It could therefore be hypothesised

that if the bat IAVs were able to infect humans, or indeed other animals, that their virulence would perhaps be low due to an inability to block general gene expression, but could be dramatically increased if mutations occurred to enable CPSF30 binding.

Of further note however is the intriguing ability of these NS1 proteins to instead enhance general gene expression, in both the human and bat cells, though more extensively in the bat cells. It has been previously reported that the PR8 NS1 is capable of enhancing gene expression, thought to be at the level of translation due to the inhibition of PKR which is then unable to phosphorylate and inactivate the translation factor eIF-2α (Salvatore et al., 2002). The particular mechanism by which the bat IAV NS1 proteins could enhance gene expression was not determined, therefore it would be of interest to determine if these NS1 proteins are able to also enhance at this level of translation.

4.3 Bat IAV NS1 proteins do not co-precipitate human p85β

Of particular interest in this study was the elucidation that neither of the bat IAV NS1 proteins was able to co-precipitate p85β, representing the first naturally occurring NS1 proteins not to do so. This inability was potentially mapped by sequence analysis to a number of residue polymorphisms in those positions considered to be important for the NS1-p85β interaction. As the normally conserved binding of the NS1 ED to the p85β subunit of PI3K has been associated with effects on viral replication and virulence, the inability of the bat NS1 proteins to interact with human p85β was intriguing. Whilst these NS1 proteins are the first reported examples to not bind p85β, there has been a report of an NS1 protein from the avian H5N1 A/Chicken/Guangdong/1/05 that whilst being able to efficiently bind p85β is unable to activate the PI3K/Akt pathway (Li et al., 2012b). This suggests that the activation of the PI3K signalling pathway during IAV infection may not always be beneficial for the virus. Therefore, two hypotheses for the lack of bat NS1-p85β binding have been presented; including the possibility that the bat IAVs do not require activation of the PI3K signalling pathway to promote viral replication or pathogenicity, and thus their NS1 proteins may have evolved to preferentially bind an alternate host cell factor. Secondly, as binding was only investigated with a human p85β, it was hypothesised that the bat IAV NS1 proteins may have evolved to interact with their species-specific p85β subunits.

Preliminary sequence alignment of the NS1 interacting regions of various p85β subunits from 14 different mammalian, avian and fish species revealed that this section of the iSH2 p85β subunit is highly conserved amongst all these species. This sequence analysis included the four bat species that have been previously sequenced, and again, the region of p85β with which the IAV NS1 protein interacts was found to be essentially conserved. A polymorphism in the p85β subunit from a black flying fox was found at residue 580 which was observed in crystal structure analysis to probably not play a role in the interaction with the NS1 ED. With the observation that the NS1 interacting regions of p85β subunits from divergent species are essentially conserved, it could be predicted that the bat IAV NS1 proteins have simply lost this binding ability and may preferentially interact with novel cellular factors. However, as the p85β subunits from the bat species in which the novel IAV genomes were discovered have not yet been sequenced, it is not possible to make any conclusions regarding the nature of this specific interaction. It may be that these particular p85β subunits are divergent and thus the bat IAV NS1 proteins could be specifically interacting with their species-specific subunit. Clearly, further work is required to clarify the potential of the H17N10 and H18N11 NS1 proteins to interact with the p85β subunits from the species from which they were isolated. Additionally, it would be of interest to also determine if these novel NS1 proteins are also, or alternatively, interacting with host cellular factors that have not been described previously.

4.4 Conclusions

In conclusion, the two bat IAV NS1 proteins from the novel H17N10 and H18N11 IAV genomes have revealed some striking conservations of typical NS1 functional abilities, including the ability to antagonise the IFN-β response in a range of cell types, despite their highly divergent sequences. Despite these particular conservations there were certain strain specific observations, for example the ability of the two bat IAV NS1 proteins to enhance general gene expression in human and bat cells and the inability to interact with the human p85β subunit of PI3K. Additionally, there were differences observed between the H17N10 and H18N11 NS1 proteins, with the H17N10 NS1 able to interact with the human factors RIG-I and Riplet, but the H18N11 NS1 unable to do so. Further investigations are needed to fully characterise these novel NS1 proteins and to potentially identify any unique functional abilities not yet described for this IAV virulence factor.

4.5 Future work

This study has revealed certain functional characteristics of the H17N10 and H18N11 bat IAV NS1 proteins, however there is still further characterisation needed. In particular, more work is needed on establishing IFN-β reporter assays in cell types from species in which IFN-β induction was not achieved. These included swine, bovine and chicken cells. Gathering a data panel for the ability of the two bat IAV NS1 proteins to antagonise the IFN response in a range of relevant species for IAV infection could indicate the potential of these novel viruses to infect other species.

Ongoing work in collaboration with the University of St Andrews has determined the crystal structure of the H17N10 and H18N10 NS1 RBDs (data not shown); however crystal structures of the EDs have not yet been elucidated. Following observations in the crystal structure, future work should further characterise the ability of the H17N10 and H18N11 NS1 proteins to bind dsRNA. As mentioned in this study, the critical residues for this ability, R38 (R39) and K41 (K42), are conserved in the H17N10 NS1 protein, however a polymorphism of A43 (usually S42 for effective RNA binding) could potentially reduce the dsRNA interaction. Therefore, further experiments would include performing a dsRNA binding assay with the wild type bat IAV NS1 proteins along with any mutants that would be predicted to reduce binding ability or perhaps increase binding ability (i.e. R39A, K42A and A43S). Indeed, as the H18N11 NS1 was reported not to bind human RIG-I and Riplet it is predicted that the major mechanism by which it can antagonise IFN-β induction is through dsRNA binding.

Of particular interest following from this study was the observation that the two bat IAV NS1 proteins could not interact with the human p85β subunit of PI3K. Six amino acid substitutions of the H17N10 NS1 in the NS1-p85β interaction region were found to have no effect on the p85β binding ability. Therefore, future work should extend these substitution studies to explore more NS1 residues present in the p85β binding interface that may play a role in this interaction. In addition, multiple substitutions will also be investigated, as it is more likely that changing more than one NS1 residue at the same time will show an effect on p85β binding.

Despite the elucidation that the p85β subunits of many different species are essentially conserved, it will be of great interest to determine if the p85β subunits from the bat species

in which the novel IAV genomes were discovered have polymorphisms. This would determine if the bat IAV NS1 proteins have evolved to preferentially interact with their species-specific p85β. On the other hand, it may be that these bat NS1 proteins have evolved to preferentially bind a different cellular factor. Therefore, it is also of interest for future work to investigate any novel host cell factors that these NS1 proteins may bind; achieved by transfection of the bat IAV NS1 proteins into cells, followed by immunoprecipitation and proteomics studies on any co-precipitated cellular factors.

Furthermore, an additional experiment of interest would be a NS1 complementation assay to determine if the bat IAV NS1 proteins can functionally complement a human NS1 protein (e.g. PR8 NS1) in the context of a human IAV. This work would involve creating lentiviruses for the stable expression of NS1 proteins in various cell lines. These cell lines could then be infected with a ΔNS1 virus (or a specific NS1 mutant lacking a known NS1 function) and measurement of either viral titres or quantification via plaque assay could determine if the bat IAV NS1 proteins can complement the mutant NS1, or whether the bat IAV NS1 proteins possess the known function being tested.

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