VIRUS AND INTERFERON: A FIGHT FOR SUPREMACY: COMPARISON OF THE MECHANISMS OF INFLUENZA A VIRUSES AND PARAINFLUENZA VIRUS 5 IN COMBATTING A PRE-EXISTING IFN-INDUCED ANTIVIRAL STATE

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A Thesis Submitted for the Degree of PhD
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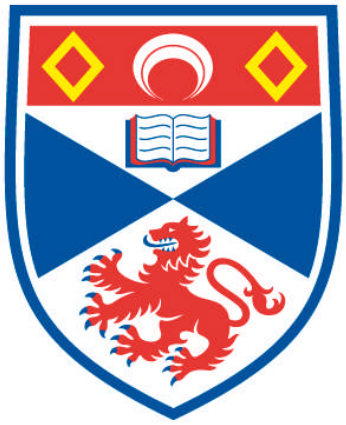

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Virus and Interferon: A fight for supremacy

Comparison of the mechanisms of Influenza A viruses and Parainfluenza Virus 5 in combatting a pre-existing IFN-induced antiviral state

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Abstract

The Interferon (IFN) family of cytokines are produced in direct response to virus infection and they constitute the first line of defence against virus infection by inducing hundreds of interferon stimulated genes (ISGs) which act in concert to establish the so called "antiviral state". Influenza A viruses and parainfluenza virus type 5 (PIV5) are both small negative strand RNA viruses that must circumvent their hosts' interferon (IFN) response for replication. However, the ways in which these viruses interact with the IFN system are very different. Although PIV5 replication is initially severely impaired in cells in a pre-existing IFN-induced antiviral state, it manages to overcome the antiviral state by targeting an essential component of type I IFN signalling, STAT1, for degradation. Thus the cells cannot maintain the antiviral state indefinitely without continuous signalling. Consequently, the virus resumes its normal replication pattern after 24-48 hours post-infection. In clear contrast, influenza virus fails to establish its replication in the majority of infected cells (90-95%) with a pre-existing IFN-induced antiviral state, although a few cells are still able to produce viral antigens.

To further investigate how influenza virus interacts with cells in a pre-existing IFN-induced antiviral state, I have used *in situ* hybridization to follow the fate of input and progeny genomes in cells that have, or have not, been treated with IFN prior to infection. Here I show for the first time that IFN pre-treatment blocks the nuclear import of influenza A virus genome, which prevents the establishment of virus replication, but this can be overcome by increasing multiplicities of infection. Of those IFN-induced antiviral molecules, human MxA is an essential component of the IFN-induced antiviral state in blocking influenza virus genome import, as this block can be abolished by lentivirus-mediated knockdown of MxA. I also show that in cells constitutively expressing MxA the viral genome still manages to be transported into the nucleus, indicating that MxA might require an unidentified IFN-induced factor to block nuclear import of the influenza virus genome.

These results reveal that IFN exerts its action at an early stage of virus infection by inducing MxA to interfere with the transport of viral genome into the nucleus, which is the factory for viral RNA production.

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I, **Han Xiao**, hereby certify that this thesis, which is approximately **44300** words in length, has been written by me, that it is the record of work carried out by me and that it has not been submitted in any previous application for a higher degree.

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Abbreviations

ADAR1	Adenosine deaminase.
ATF-2	Activating transcription factor 2.
bRSV	Bovine respiratory syncytial virus.
BSA	*Bovine* serum albumin.
BVDV	Bovine Diarrhoea Virus.
CARD	Caspase activation and recruitment domain.
CARDIF	CARD Adaptor inducing IFN-β
CDV	Canine distemper virus.
CHX	Cycloheximide.
CPSF	Cleavage and polyadenylation specific factor.
CPE	cytopathic effect.
CPSF30	Cleavage and polyadenylation specificity factor.
CRM1	Chromosome region maintenance 1 protein.
cRNA	Complementary RNA
DAPI	DNA-binding fluorochrome 4′,6′-diamidino-2-phenylindole.
DDB1	DNA-binding protein 1.
DIG	Digoxigenin.
DMEM	Dulbecco's modified Eagle's medium.
DMSO	Dimethyl sulfoxide.
DRBM	dsRNA binding motif.
dsRNA	Double strand RNA.
DTT	Dithiothreitol.
ECL	Enhanced luminol-based chemiluminescent.
EDTA	Ethylenediaminetetraacetic acid.
eIF2α	alpha subunit of eukaryotic initiation factor 2.
eIF4GI	Eukaryotic translation initiation factor 4GI.
EMCV	Encephalomyocarditis virus.
Epf	Estrogen-responsive finger protein.
ER	*Endoplasmic reticulum*.
FCS	Fetal calf serum.
FITC	Fluorescein isothiocyanate.
FLUAV	Influenza A virus.
FP	Fusion peptide.
GAS	Gamma-activation sequence.
GDP	*Guanosine* diphosphate.
GFP	Green fluorescent protein.
GTP	*Guanosine*-5'-triphosphate.
HA	hemagglutinin.
HCMV	human cytomegalovirus.
Herc5	interferon-induced HECT E3 enzyme.
HeV	Hendra virus.
HGM I(Y)	Non-histone high mobility group.
hMPV	Human metapneumovirus.
hPIV	Parainfluenza viruses.

HPV human papillomavirus.
HRP Horseradish peroxidase.
hRSV Human respiratory syncytial virus.
IFN Interferon.
IFNAR IFN-α/β receptor.
IFNGR IFN-γ receptor.
IPS-1 IFN-β promoter stimulator 1.
IRF3 Interferon regulatory factor 3.
IRFs Interferon regulatory factors.
ISGF3 ISG factor 3.
ISGs Interferon stimulated genes.
iSH2 inter-SH2.
ISRE IFN-stimulated response element.
Jaks Janus activated kinases.
JEV Japanese encephalitis virus.
LCMV lymphocytic choriomeningitis virus.
LGP2 Laboratory of genetics and physiology 2.
MAVS Mitochondrial antiviral signalling protein.
MDA-5 Melanoma differentiation-accociated gene 5.
MeV Measles virus.
MFSV Maize fine streak virus.
MOI Multiplicity of infection.
mRNA Messenger RNA.
MuV Mumps virus.
Mx1 Orthomyxovirus resistance gene 1.
MxA Myxovirus resistant protein A.
NA Neuraminidase.
NAT N-acetyl trypsin.
ND Newcastle disease.
NDV Newcastle disease virus.
NEP Nuclear export protein.
NES Nuclear export signal.
NF-κB nuclear factor kappa-light-chain-enhancer of activated B cells.
NiV Nipah virus.
NLRs Nucloetide oligomerization domain-like receptors.
NLSs Nuclear localization signals.
NMR *Nuclear* magnetic resonance
NOD nuclotide oligomerization domain.
NoLS Nucleolar localization signal.
NS1 non-structural protein 1.
NS1B Influenza B virus NS1.
NS2 non-structural protein 2.
OAS Oligoadenylate synthetase.
ORFs Open reading frames.
p.i. post-infection.
PABII poly(A)-binding protein II.
PAMPs Pathgen associated molecular patterns.
PBS Phosphate buffered saline.
PCR Polymerase chain reaction.
pfu Plaque-forming units.

PI3K	Phosphatidylinositol 3-kinase.
PIP3	Phosphatidylinositol-3,4,5-trisphosphate.
PKR	dsRNA-dependent protein kinase.
PRDs	Positive regulatory domains.
PRRs	pattern-recognition receptors.
PTEN	Phosphatase and tensin homolog.
PVDF	Polyvinylidene difluoride.
RBD	RNA-binding domain.
RIG-I	Retinoic acid inducible gene I.
RLRs	Retinoic acid inducible gene-like receptors.
RSV	Respiratory Syncytial Virus.
SDS	Sodium dodecyl sulfate.
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis.
SeV	Sendai virus.
SH2	Src homology 2.
SH3	Src homology 3.
shRNA	Small hairpin RNA or short hairpin RNA.
SSC	Saline-sodium citrate.
ssRNA	Single strand RNA.
STATs	Signal transducer and activator of transcription.
SUMO	Small ubiquitin-like modifier.
SV5	Simian virus 5.
TANK	TRAF-associated NF-κB activator.
TLRs	Toll-like receptors.
TM	transmembrane.
TMD	Transmembrane domains.
TNF	Tumor necrosis factor.
TPR	tetratricopeptide.
TRAF	Tumor necrosis factor receptor-associated factor.
TRIM	Tripartite motif
tRNAi	Initiator transfer RNA
ts	Temperature sensitive.
TYK2	Tyrosine kinase 2.
UBE1L	Ubiquitin-activating enzyme E1-like protein.
Udorn	Influenza A virus strain A/Udorn.
viperin	Virus inhibitory protein, ER-associated, interferon-inducible.
VISA	Virus-induced signalling adaptor
VRC	Vanadyl Ribonucloside Complex.
vRNP	Viral ribonucleocapsid.
VV	Vaccinia virus.
W3	PIV5 strain W3.
WHO	World health organization.
WSN	Influenza A virus strain A/WSN/33.
wt	wild-type.

Contents

1.1 The Interferon system

The interferons (IFN) were initially discovered more than 50 years ago by Isaacs and Lindenmann as a molecule induced by heat-inactivated influenza virus, which were found to interfere with virus replication in previously uninfected cells (Isaacs and Lindenmann, 1957; Lindenmann, Burke, and Isaacs, 1957). Half the century has passed since this discovery, and a lot more has been learned about IFN, including the discovery of cytoplasmic sensors of viruses infection leading to IFN induction, the IFN induced antiviral molecules and their mode of actions, as well as the underlying strategies evolved by viruses to coutermeasure the IFN system.

The IFN superfamily are grouped according to their amino acid sequences, mode of induction and biological activities into three classes: type I, II and III IFNs. Type I IFNs are also known as viral IFNs and constitute a multi-gene family of 13 closely related IFNα genes, and a single IFN-β gene. Type I IFNs are synthesized by many cell types after infection by diverse group of viruses, and they bind to a common type I IFN receptor complex, IFNAR1 and IFNAR2.

Type II IFN is also known as immune IFN and consists of a single gene encode IFN-γ that binds the IFNγ receptor complex (IFNGR). In contrast to type I IFNs, IFN-γ is not directly induced by viral infection but is produced later by immune cells, including natural killer (NK) cells, CD4 Th1 cells, CD8 cytotoxic suppressor cells and has an important role in the adaptive immune response.

Novel antiviral cytokines IFN-λ1, IFN-λ2, and IFN-λ3 (also referred to as IL-29, IL-28A and IL- 28B, respectively) are classified as type III IFNs. These cytokines are also induced in direct response to virus infection and have been shown to share many functional characteristics with type I IFNs. However, type III IFNs use a distinct set of receptors from type I or type II IFNs, comprising IL-28Rα and the IL-10Rβ [reviewed in (Ank and Paludan, 2009; Ank, West, and Paludan, 2006; Uze and Monneron, 2007)].

1.1.1 Mechanisms of type I IFN induction

1.1.1.1 Type I IFN induction

Induction of type I IFN begins with recognition of pathogen associated molecular patterns (PAMPs), foreign to host cells, by host pattern-recognition receptors (PRRs), which could distinguish self from non-self (Akira, Uematsu, and Takeuchi, 2006; Janeway and Medzhitov, 2002). A schematic representation of recognition of RNA viruses by cellular sensors is shown in **Fig. 1.1**. A major component of PAMPs are viral nucleic acids, typically double-strand or single-strand RNA and DNA, that can be recognized by three class of PRRs: cytoplasmic retinoic acid inducible gene (RIG-I)-like receptors (RLRs), endosomal Toll-like receptors (TLRs) that recognize PAMPs at the cell surface or inside endosomes, and nucleotide oligomerization domain (NOD)-like receptors (NLRs). The RLR family consists of three members, RIG-I (Yoneyama et al., 2004), melanoma differentiation-associated gene 5 (MDA-5) (Andrejeva et al., 2004; Kang et al., 2002; Yoneyama et al., 2005) and laboratory of genetics and physiology 2 (LGP2) (Rothenfusser et al., 2005; Yoneyama et al., 2005). RIG-I and MDA-5 share

structural homologies: they both contain two tandem caspase activation and recruitment domains (CARD) at their N-terminus, and a DExD/H Box helicase domain at their C-terminus. However, LGP2 only contains a DexD/H Box helicase domain. RIG-I has been shown to recognize ssRNA bearing a 5'-triphosphate (Hornung et al., 2006; Pichlmair et al., 2006) and short double strand RNAs, whilst MDA-5 recognizes long dsRNAs (Gitlin et al., 2006) [For recent reviews, see (Meylan, Tschopp, and Karin, 2006; Takeuchi and Akira, 2009; Wilkins and Gale, 2010)].

A common downstream signalling molecule shared by RIG-I and MDA-5 is MAVS (also called IPS-1, VISA and Cardif) [(Hiscott et al., 2006; Kawai et al., 2005; Meylan et al., 2005; Seth et al., 2005; Xu et al., 2005), reviewed by (Hiscott et al., 2006)]. MAVS is localized at the outer mitochondria membrane by its C-terminal transmembrane domain, and its N-terminal CARD domain is essential for mediating the interaction of RIG-I with MAVS [reviewed in (Meylan, Tschopp, and Karin, 2006)]. The recruitment of MAVS by RIG-I/MDA-5 leads to the transmembrane (TM)-dependent dimerization of MAVS and subsequent recruitment and activation of the tumor necrosis factor (TNF) receptor-associated factor (TRAF) family members, leading to signal transduction cascade and the activation of transcription factors NF-κB and IRF-3, which are both essential components for type I IFN induction (Baril et al., 2009).

1.1.1.2 IFN-β promoter

Induction of type I IFN requires the assembly of a higher order multi-component transcription factor enhancer complex, termed the enhanceosome [reviewed by (Thanos,

1996)]. The IFN-β promoter consists of a set of *cis* regulatory elements designated positive regulatory domains (PRDs). PRDIV, PRD I/III and PRD II are recognized by activating transcription factor 2 (ATF-2)/C-Jun, IFN regulatory factors (IRFs), and NF-κB respectively. These *trans* elements, together with the architectural protein non-histone high mobility group HGM I(Y), form the enhanceosome (Falvo, Thanos, and Maniatis, 1995; Thanos and Maniatis, 1995). The cooperative assembly of the enhanceosome not only facilitates the binding of activators to promoter regions, but also stabilizes the interaction with basal transcription machinery to activate IFN-β gene transcription (**Fig. 1.2**).

All of these *trans* activators need to be phosphorylated by cellular kinases from the signal transmitted from RLRs/MAVS signalling pathway. NF-κB is normally held in a quiescent state in the cytoplasm by association with an inhibitory molecule IκB. The associated IκB masks the NF-κB nuclear localization signal (NLS) and thereby inhibits its nuclear translocation. Upon stimulation of a varity of stress signals, IκB is phosphorylated and targeted by proteasomal degradation thus leading to NF-κB nuclear translocation [reviewed in (Israel, 2000)]. IFN regulatory factor 3 (IRF-3) is constitutively expressed inside the cells and mainly localized in the cytoplasm. Upon virus infection, IRF-3 is hyperphosphorylated on a number of serine and threonine residues at its C-terminus, leading to its dimerization and translocation into the nucleus (Kumar et al., 2000).

1.1.2 Mechanisms of IFN signalling

IFNs are cytokines secreted by cells in response to virus infection, and induce the expression of IFN stimulated genes (ISGs) via the classical JAK-STAT signalling pathway. This pathway was originally discovered in the 1990s (Fu et al., 1992; Schindler et al., 1992; Silvennoinen et al., 1993), and has been well studied by many experimental approaches [reviewed by (Platanias, 2005; Randall and Goodbourn, 2008b)]. Key players in this pathway are protein tyrosine kinases of the Janus activated kinase (Jaks), activated by ligands binding to cell surface receptors and the signal transducer and activator of transcription (STATs) [reviewed by (Murray, 2007)]. JAK-STAT signalling pathway allows signal to be transmitted from cell surface to the nucleus, leading to the induction of hundreds of ISGs (de Veer et al., 2001; Der et al., 1998).

1.1.2.1 Type I IFN signalling

The large family of type I IFNs bind a common cell-surface hetero-dimeric receptor, which is known as the type I IFN receptor, composed of at least two distinct subunits: IFNAR1 and IFNAR2 [reviewed by (Mogensen et al., 1999)]. Each of these receptor subunits interacts with a member of the JAK family. IFNAR1 subunit is constitutively associated with tyrosine kinase 2 (TYK2), whereas IFNAR2 is associated with JAK1. Type I IFNs binding induce type I IFN receptor to oligomerize and brings the Jaks close enough for them to cross-phosphorylate each other. The resulting phosphorylation of IFNAR1 by Tyk2, allows the docking of STAT2, and phosphorylation of IFNAR2 by

Jak1, allows the docking of STAT1. After STATs dock on specific phosphotyrosines on the receptor, the Jaks phosphorylate them, phosphorylated STATs dissociated from receptor and form heterodimer via their Src Homology 2 (SH2) domain. The STAT1-STAT2 complex associates with a monomer of IRF-9 to form the ISG factor 3 (ISGF3) heterotrimer complex, translocates to the nucleus, and binds to IFN-stimulated response element (ISRE) located in the promoter region of most ISGs, thereby initiating their transcription (**Fig. 1.3**).

1.1.2.2 Type II IFN signalling

Signalling in response to IFN-γ follows very similar features to that in response to type I IFN. The IFN-γ receptor is also a heterodimeric glycoprotein, comprising at least two major subunits, IFNGR1 and IFNGR2 [reviewed by (Bach, Aguet, and Schreiber, 1997)]. In contrast to the type I receptor, the cytoplasmic domains of IFNGR1 and IFNGR2 are associated with a different set of JAKs, Jak1 and Jak2, respectively. Dimeric IFN-γ binding to the receptor subunits triggers receptor dimerization and activation of receptor associated Jak1 and Jak2, which subsequently phosphorylate a tyrosine residue (Y440) on IFNGR1 that serves as a docking site for STAT1 [Reviewed in (Bach, Aguet, and Schreiber, 1997)]. STAT1 is then activated by phosphorylation at tyrosine 701, resulting in the homo-dimerization of STAT1 and dissociation from the receptor. Dimerized STAT1s translocate to the nucleus and bind to gamma-activation sequence (GAS) that is present in the promoter region of certain ISGs (**Fig. 1.3**).

Of the hundreds of known ISGs, some have either a GAS or ISRE elements in their

promoters, whilst others have both elements; thus the expression of certain ISGs are regulated by different sets of STATs. Indeed, there is some redundancy in IFN-γ and IFN-α/β signalling (Vilcek, 2003).

1.1.3 IFN-induced antiviral state

Type I IFNs are produced in direct response to virus infection and lead to the induction of the expression of a number of antiviral proteins, which are involved in the establishment of the "antiviral state" in infected cells and neighbouring cells, and thus to limit the extent of virus spread (van Boxel-Dezaire, Rani, and Stark, 2006). However, not all the IFN-induced ISGs are involved in the establishment of the antiviral state, and probably different subset of genes are required to limit different type of viruses replication. Among them, three families of IFN-inducible genes have been extensively studied with respect to their antiviral activities. These genes encode the dsRNA-activated protein kinase (PKR) (Clemens and Elia, 1997), the 2',5'-oligoadenylate synthetases (OAS), and the Mx protein(s) (Haller et al., 1980; Haller, Staeheli, and Kochs, 2007). Further more, recently other ISGs have been reported to be involved in IFN-induced antiviral responses (Discussed later in this session).

1.1.3.1 dsRNA-dependent protein kinase (PKR)

One of the best-characterized IFN-induced antiviral molecules is PKR. PKR is a ubiquitously expressed serine/threonine protein kinase that consists of two dsRNA binding motifs (dsRBM) at its N terminus and a conserved kinase domain at its C-terminus (Meurs et al., 1990). PKR can be induced by IFN and activated by a variety of

signals such as dsRNA, pro-inflammatory stimuli, cytokines, growth factors and oxidative stress signals. PKR normally exists in a latent inactive form due to the auto-inhibitory effect of its dsRBM. Upon binding to dsRNA, PKR undergoes conformational change to form homodimer (Dey et al., 2005) and autophosphorylate itself (Galabru and Hovanessian, 1987), leading to its activation.

PKR was discovered from experiments showing that IFN pretreated cells infected with vaccinia virus (VV) have a translational inhibition of viral mRNAs. The cell extracts prepared from these IFN-treated VV-infected cells showed restricted translation of viral and cellular mRNAs (Friedman et al., 1972; Metz and Esteban, 1972). Later on it was shown that in rabbit reticulocyte system, protein translation is inhibited by phosphorylation of the alpha subunit of eukaryotic initiation factor 2 (eIF2α) in the presence of hemin or low concentrations of dsRNA (Farrell et al., 1977). eIF2α is one of the best-known PKR substrates. The eIF2 complex, consisting of three subunits (α, β, γ), is involved in the recruitment of Met-tRNAi to the 40S ribosome subunit for initiation of protein synthesis [reviewed in (Hershey, 1991)]. eIF2 binds the Met-tRNAi in a GTP-dependent manner to form a ternary complex Met-tRNAi/GTP/eIF2, which associates with the 40S subunit. After Met-tRNAi is delivered, hydrolysis of GTP to GDP promoted by eIF5, eIF2/GDP is released from the 40S subunit. Inactive eIF2/GDP complexes are reactivated by the GDP-to-GTP exchange, catalyzed by eIF2B, thus allowing active eIF2/GTP to bind to Met-tRNA. However, PKR phosphorylation of eIF2α at S51 increases the affinity of eIF2 and eIF2B (Sudhakar et al., 2000), leading to the PKR-mediated down-regulation of translation initiation.

In addition to phosphorylating eIF2α and the inhibition of translation initiation, PKR also

actively regulates diverse cellular processes in transcription, apoptosis, cell growth, differentiation by phosphorylation of downstream effectors such as NF-κB, IRF-1 (Kumar et al., 1997), p53 (Cuddihy et al., 1999a; Cuddihy et al., 1999b), STATs (Deb et al., 2001; Wong et al., 1997), and ATF-3 (Guerra et al., 2006) [for reviews, see (Garcia, Meurs, and Esteban, 2007; Williams, 2001)].

1.1.3.2 2'-5'OAS/RNase L system

2'-5' oligoadenylate synthetases are a group of enzymes that are induced by IFNs in mammalian cells that catalyse the formation of 5'-triphosphorylated, 2'5'-phosphodiester-linked oligoadenylates (2-5A) from ATP (Player and Torrence, 1998). 2-5A is relatively unstable and is degraded by the concerted action of phosphodiesterase and phosphatase shortly after formation. 2-5A is found in cells in the trimeric form (Knight et al., 1980), and the only well established function of 2-5A is activation of endoribonuclease RNase L [(Zhou, Hassel, and Silverman, 1993), reviewed by (Bisbal and Silverman, 2007)]. RNase L is expressed constitutively in most cells types and is normally hold in a quiescent state, but it is rapidly activated by the association of 2-5A. Activated RNase L, causes the degradation of single-stranded RNA as well as 28S ribosomal RNA, therefore leading to protein synthesis inhibition. Thus the level of 2-5A is believed to be a major factor that controls RNase L activity (Minks et al., 1979).

2'-5'A/RNase L system has been suggested to have an important role in antiviral and antitumor functions [reviewed in (Hovanessian and Justesen, 2007)]. Overexpression of 2′-5′OAS is sufficient to protect cells against infection with EMCV, Mengo viruses, and

HIV (Chebath et al., 1987; Coccia et al., 1990; Maitra and Silverman, 1998; Williams and Kerr, 1978). Despite the fact that RNase L could degrade cellular mRNA, it preferentially targets viral mRNA as a result of local activation of 2'-5'OAS by viral replication and transcription (Nilsen and Baglioni, 1979).

1.1.3.3 Mx GTPases

Mx proteins are key components of the antiviral state induced by type I IFN [reviewed in (Haller, Staeheli, and Kochs, 2007)]. In 1962, Lindenmann discovered that the inbred mouse strain A2G showed an unusually high degree of resistance towards infection with influenza A virus (FLUAV) (Lindenmann, 1962). Later on, it was found that the innate resistance depends on a single gene, named Mx1 (for orthomyxovirus resistance gene 1), which was the major mediator of innate immunity against influenza A virus in mice (Arnheiter et al., 1990; Horisberger, Staeheli, and Haller, 1983; Staeheli et al., 1986), whilst most inbred strains of mice carry defective Mx1 alleles and are highly susceptible to pathogenic influenza A virus strains (Staeheli et al., 1988). It was intriguing for the existence of an influenza virus resistance gene in mice because mice are not natural hosts for influenza viruses. However, subsequently it became clear that Mx1 is the first member of Mx gene family which exist in a variety of organisms, including yeast, fish, chicken, ducks, dogs, pigs, cows, sheep, horses, and humans (Horisberger, 1992; Horisberger and Gunst, 1991; Nakayama and Ishihama, 1992; Nakayama et al., 1991; Pitossi et al., 1993; Samuel, 1991; Staeheli, 1990), and inhibit a broad spectrum of viruses from different taxonomic groups [reviewed by (Haller, Staeheli, and Kochs,

2007)].

Mx1 gene expression is normally undetectable, but is rapidly upregulated by type I or type III IFNs (IFNs) (Haller et al., 1980; Holzinger et al., 2007). Mx proteins belong to the dynamin family of large GTPase (Pavlovic et al., 1993; Staeheli, Pitossi, and Pavlovic, 1993), which function in endocytosis, vesicle trafficking between intracellular membrane compartments, maintenance of mitochondria morphology and viral resistance (Danino and Hinshaw, 2001; Staeheli and Haller, 1985; van der Bliek, 1999). Mx proteins of human and mice can self-assemble into ring-structures and associate with intracellular membranes in solution (Hinshaw and Schmid, 1995; Kochs et al., 2005). Mx contains a highly conserved tripartite GTP binding motif within the N-terminal G domain. The less conserved C-terminal effector domain, which contains leucine zipper motifs, is involved in homo-oligomerization and association with other molecules.

MxA, a human homolog of Mx1, accumulates in the cytoplasm and interferes with multiplication of orthomyxoviruses (Haller et al., 1995; Marschall et al., 2000; Pavlovic, Haller, and Staeheli, 1992), paramyxoviruses (Zhao et al., 1996), bunyaviruses (Frese et al., 1996), picornaviruses (Pavlovic et al., 1990a), hepatitis B virus (Netherton et al., 2009), and Semliki Forest virus (Landis et al., 1998). Current evidence suggests that MxA exerts its antiviral activity by interacting with viral ribonucleoproteins. Human MxA inhibits Thogoto virus replication by recognizing and preventing the nuclear import of viral nucleocapsids, thereby inhibiting transcription of the viral genome (Kochs and Haller, 1999a) and inhibits La Crosse virus replication by binding and sequestering viral nucleoprotein in the perinuclear region, forming an elongated tubular structure resulting

in cytoplasmic bodies, thus preventing genome amplification, budding, and release (Frese et al., 1996; Kochs et al., 2002; Reichelt et al., 2004).

1.1.3.4 ISG15

ISG15, first identified more than 20 years ago, is a small ubiquitin-like protein (Blomstrom et al., 1986; Haas et al., 1987; Korant et al., 1984). It is expressed as a 17kDa precursor protein, and maturation involves cleavage at its C-terminus to generate a 15kDa protein, revealing di-glycine residues in the form of a LRLRGG motif (Potter et al., 1999), which is essential for ISG15 conjugation to target proteins (ISGylation). ISGylation is accomplished through the concerted action of IFN-inducible conjugation cascade, includes an E1 (UBE1L), E2 (UbcH8, UbcH6), and two E3 ligases Efp (estrogen-responsive finger protein) and Herc5 (Dastur et al., 2006; Kim et al., 2004; Yuan and Krug, 2001a; Zou and Zhang, 2006). Efp is the ISG15 E3 ligase for 14-3-3σ protein, Herc5 is involved in the global regulation of ISGylation. USP18/UBP43 is the deconjugating enzyme, removing ISG15 from its target proteins (Malakhov et al., 2002).

Recent publications have shown that ISG15 targets nearly 200 cellular proteins found in many important cellular pathways (Giannakopoulos et al., 2005; Zhao et al., 2005). Most targets are constitutively expressed and function in diverse cellular pathways, while a few targets are induced by IFN, including RIG-I, STAT1, PKR, MxA. Thus it has been speculated ISGylation plays an import role in IFN-induced antiviral immunity. Indeed, current evidence suggests that ISG15 alone, and/or its conjugation, has a broad-spectrum of antiviral activity against HIV-1(Bach, Aguet, and Schreiber, 1997), Ebola virus (Okumura, Pitha, and Harty, 2008), herpes virus, Sindbis virus and influenza virus

(Hsiang, Zhao, and Krug, 2009; Lai et al., 2009; Lenschow et al., 2007).

1.1.3.5 Viperin

Viperin (virus inhibitory protein, endoplasmic reticulum-associated, IFN-inducible), also known as *RSAD2,* or *cig5* in humans, is an IFN-inducible protein that is evolutionarily conserved. Viperin was initially discovered in human cytomegalovirus (HCMV) infected cells (Chin and Cresswell, 2001), and has a broad range of antiviral activities (Chin and Cresswell, 2001; Helbig et al., 2005; Rivieccio et al., 2006; Wang, Hinson, and Cresswell, 2007). siRNA-mediated knockdown of viperin enhances the replication of alphaviruses, Sendai virus, Sindbis virus (SIN) and HIV-1 (Chan et al., 2008; Chin and Cresswell, 2001; Jiang et al., 2008; Zhang et al., 2007).

Viperin is localized to the ER through an N-terminal amphipathic α-helix (Brass et al., 2002; Elazar et al., 2004; Hinson and Cresswell, 2009a; Hinson and Cresswell, 2009b), and interacts with the lipid biosynthesis enzyme farnesyl-diphosphate synthase, resulting in the disruption of lipid raft microdomains and inhibition of influenza virus budding from plasma membrane (Wang, Hinson, and Cresswell, 2007). Viperin is highly induced in neutrophils and macrophages by lymphocytic choriomeningitis virus infection (LCMV), and may contribute to the antimicrobial activity of neutrophils (Hinson et al., 2010). It was also shown that it is targeted by Japanese encephalitis virus (JEV) for degradation through a proteasome-dependent mechanism (Chan et al., 2008).

1.1.3.6 ISG56

The ISG56 family of proteins are induced strongly in response to virus infection, IFNs and dsRNA. In humans, this family comprises four members: ISG54, ISG56, ISG58 and ISG60. In mice, this family comprises three members: ISG49, ISG54 and ISG56 (de Veer et al., 1998; Lee et al., 1994; Levy et al., 1986). All of these proteins contain multiple tetratricopeptide (TPR) motifs that are known to mediate protein-protein interactions (Lamb, Tugendreich, and Hieter, 1995). ISG56 C-terminal region is responsible for interaction with eIF3e subunit to impair the ability of eIF3 to stabilize the eIF2·GTP·Met-$tRNA_i$ ternary complex (Guo et al., 2000; Hui et al., 2003a; Terenzi et al., 2006). Mouse ISG54 and ISG56, as well as human ISG54, bind to eIF3c and eIF3e subunits to inhibit protein translation (Hui et al., 2005; Terenzi et al., 2006; Terenzi, Pal, and Sen, 2005).

ISG56 was recently been reported to be an important antiviral molecule in IFN-induced antiviral state, it was reported to inhibit hepatitis C virus (Sumpter et al., 2005), Sindbis virus (Zhang et al., 2007), West Nile virus, lymphocytic choriomeningitis virus (LCMV) (Wacher et al., 2007) and human papillomaviruses (HPV) (Terenzi, Saikia, and Sen, 2008). ISG56 exert its antiviral action against HPV by binding and translocating the DNA replication origin-binding protein E1 of HPV from the nucleus to the cytoplasm, thus mediating the inhibitory action of IFN on HPV DNA replication. Mutational studies showed that the interaction is mediated by TRP repeat 2 (TRP2) of ISG56 and the C-terminal region of E1 (Terenzi, Saikia, and Sen, 2008).

1.1.4 Virus encoded IFN antagonists

In order for viruses to survive the antiviral IFN response, viruses developed diverse strategies to counteract the IFN system. It is now becoming clear viral antagonistic strategies target virtually all aspect of IFN system. Viruses were found to interfere with induction of IFN production, IFN signalling, to suppress the activities of IFN-induced antiviral effector molecules, or a broad shut down of host protein synthesis [See extensive reviews in (Bonjardim, 2005; Goodbourn, Didcock, and Randall, 2000; Levy and Garcia-Sastre, 2001; Randall and Goodbourn, 2008a; Weber, Kochs, and Haller, 2004)]. Viruses often encode one or more IFN antagonists and they are often multifunctional proteins to allow efficient evasion of host antiviral mechanims.

1.1.4.1 Vaccinia virus E3L

A typical IFN antagonist encoded by vaccinia virus is E3L. It blocks IFN induction and action by sequestering dsRNA and inhibiting IRF-3/7 activation (Xiang et al., 2002), and it has also recently been shown to inhibit the 5′-ppp-ssRNA- and dsDNA-induced activation of IFNβ even though it does not interact with these nucleic acids (Marq et al., 2009). In addition, it was also reported to inhibit IFN-induced ISGs. It has been shown to bind and sequester viral dsRNA thus blocking the activation of PKR and 2'-5' OAS/RNaseL pathway, inhibits IFN inducible RNA-specific adenosine deaminase (ADAR1) activity (Liu et al., 2001), and disables ISG15 function by direct binding (Guerra et al., 2008).

1.1.4.2 Influenza A virus NS1

FLUAV encodes NS1 that is a multifunctional protein. It targets various points of IFN pathway. It was described in details in section ***1.3.4.3***.

1.1.4.3 Influenza B virus NS1

Influenza B virus NS1 protein (NS1B) contains 281 amino acids. NS1B forms homodimers and binds to single- and double-stranded RNAs *in vitro* (Wang and Krug, 1996). It consists of an N-terminal RNA binding domain and a C-terminal effector domain, sharing less than 20% sequence identity with the influenza A virus NS1 protein. It accumulates in nuclear speckles domains and disrupts their normal functions, and this activity is independent of other viral proteins (Schneider et al., 2009).

It has previously been shown to inhibit antiviral responses by blocking the induction of type I IFNs (IFN) (Dauber, Heins, and Wolff, 2004; Dauber, Schneider, and Wolff, 2006; Donelan et al., 2004), preventing the activation of PKR (Dauber, Heins, and Wolff, 2004; Dauber, Schneider, and Wolff, 2006; Donelan et al., 2004), blocking ISG15 conjugation (ISGylation) to its target proteins (Yuan and Krug, 2001b). Recently it was shown that the antagonistic activity against ISGylation of NS1B is species-specific, it prevents human but not mice or canine ISGylation (Sridharan, Zhao, and Krug, 2010; Versteeg et al., 2010).

1.1.5 Virus shut-off of host protein synthesis

Virus infection of susceptible cells leads to a general inhibition of cellular protein synthesis, a phenomenon known as host shut-off. Different viruses employ very different mechanisms to shut-off host protein synthesis. The host shut-off can happen at different points in the pathway of eukayotic gene expression, such as transcription initiation, RNA maturation, nuclear-cytoplasmic transport, translation initiation and protein synthesis [Reviewed in (Aranda and Maule, 1998; Lyles, 2000)]. Adenoviruses block nucleo-cytoplasmic transport of cellular mRNA. Herpes viruses degrade pre-existing cellular mRNAs. Piconaviruses inhibit host transcription by disruption of transcription initiation and translation by cleavage of translation initiation factor eIF4G. Poliovirus, a prototype of piconavirus, utilizes Cap-independent translation of viral mRNA that is of major importance shut-off of host protein synthesis after infection. VSV inhibits host translation by inhibiting eIF2.

1.2 The Paramyxoviridae

1.2.1 Introduction of paramyxoviruses

The family of Paramyxoviridae (from Greek para- beyond; -myxo-, mucus) are single-stranded, non-segmented, negative sense enveloped RNA viruses and are responsible for a number of human and animal diseases including measles, mumps, Newcastle disease and some emerging diseases caused by newly discovered Paramyxoviruses.

Measles virus (MeV) belongs to the family of paramyxovirus and is one of the most contagious viruses known to human. It was targeted for eradication by the World Health Organization (WHO). However, it is still one of the leading causes of death among young children globally, even though a safe and cost-effective vaccine is available. An estimated 164, 000 people died from measles in 2008 (WHO, 2008). **Human Parainfluenza viruses** (hPIV) commonly cause a range of disease to children, from mild influenza-like symptom to bronchitis and pneumonia. **Mumps virus** (MuV) has been noted in history for a long time, with the description of parotitis and orchitis by Hippocrates dated back to the 5th century BC. Today, MuV still imposes a great impact on public health and zoonotic diseases. It is responsible for a very common childhood illness, with characteristic symptoms including fever, and hallmark symptom of parotid gland swelling prior to immunization. Occasionally, MuV infection results in serious complications including aseptic meningitis and infertility (Hviid, Rubin, and Muhlemann, 2008).

Among paramyxoviruses that infect animal species, **Newcastle disease** (ND) is a viral disease affecting many species of birds and causing severe economic losses in the poultry

sector. The aetiological agent is **Newcastle disease virus** (NDV). The severity of avian disease depends on the pathotype of the NDV strain ranging from mild or asymptomatic infections (caused by lentogenic strains) to mesogenic strains of intermediate severity, and velogenic strains causing systemic infections with high mortality.

Over the past few years, an increasing number of new paramyxoviruses have been discovered. Among those emerging paramyxoviruses, **Hendra** (HeV) and **Nipah** (NiV) viruses cause highly lethal central nervous system diseases.

1.2.2 Classification of Paramyxoviruses

The family Paramyxoviridae is part of the virus order Mononegavirales, and it is classified into two subfamilies: the *Paramyxovirinae* and the *Pneumovirinae* based on virion morphology, the organization of genome, activity of encoded proteins and relatedness of gene sequence. The two subfamilies are further sub-divided into different genera. Examples of the members of the Paramyxoviridae are shown in **Table 1.1**.

Table 1.1. Classification of the *Paramyxoviridae* family into subfamilies and genera [adapted from (Kolakofsky and Lamb, 2001)].

Family *Paramyxoviridae*

Subfamily *Paramyxovirinae*

- Genus *Rubulavirus*
  - Mumps virus (Mu V)
  - Parainfluenza virus 5 [simian virus 5 (SV5)]
  - Human parainfluenza virus type 2, type 4a and 4b (hPIV2/4a/4b)
  - Porcine rubulavirus
  - Mapuera virus
- Genus *Avulavirus*
  - Newcastle disease virus (avian paramyxovirus 1) (NDV)
- Genus *Respirovirus*
  - Sendai virus (mouse parainfluenza virus type 1) (SeV)
  - Human parainfluenza virus type 1 and type 3 (hPIV1/3)
  - Bovine Parainfluenza virus type 3 (bPIV3)
- Genus *Henipaviruses*
  - Hendra virus (HeV)
  - Nipah virus (NiV)
- Genus *Morbillivirus*
  - Measles virus (MeV)
  - Rinderpest virus
  - Cetacean morbillivirus
  - Canine distemper virus (CDV)
  - Phocine distemper virus
  - Peste-des-petits-ruminants virus

Subfamily *Pneumovirinae*

- Genus *Pneumovirus*
  - Human respiratory syncytial virus A2, B1, S2 (hRSV)
  - Bovine respiratory syncytial virus (bRSV)
  - Pneumonia virus of mice (PVM)
- Genus *Metapueumovirus*
  - Human metapneumovirus (hMPV)
  - Avian metapneumovirus

1.2.3 Virion structure

The virions of *Paramyxoviridae* are pleiomorphic, having typical spherical forms ranging from 150-350nm in diameter (see **Fig. 1.4**). The virions have a lipid bilayer envelope derived from the plasma membrane of the host cell. Inserted into the envelope are the attachment (HN, H or G) and fusion (F) glycoproteins that extend 8-12 nm from the surface of the membrane. The fusion genes are relatively well conserved among members of the *Paramyxoviridae,* whilst the attachment glycoproteins are more diverse. Members of the *Respirovirus* and *Rubulavirus* genera have an attachment glycoprotein with both haemagglutinin and neuraminidase activities (HN protein), while those of the *Morbillivirus* genus have a attachment protein with only haemagglutinin activity (H protein), and those of the *Henipavirus* genus and *Pneumovirinae* subfamily have attachment protein with neither activities (G protein).

Underlying the envelope is the matrix (M) protein, playing an important role in maintaining the integrity and organization of the virus particle. Inside the viral membrane is the helical nucleocapsid core that contains the single-standed RNA genome. The viral genome is encapsidated by the nucleoprotein (N or NP), which is associated with the RNA-dependent RNA polymerase complex of phosphoprotein (P) and large (L) proteins.

1.2.4 Genome organization

The genome of paramyxoviruses is single-stranded, non-segmented, negative-sense RNA ranging in length from 15 kb to 19 kb (see **Fig. 1.5**). Flanking the genome is a 3' extracistronic region of ~ 50 nucleotides known as the leader, and 5' extracistronic region of 50 to 161 nucleotides known as the trailer. These regions are essential for virus transcription and replication. The genome contains 6 to 10 tandem linked genes, depending on species. At the beginning and end of each gene are conserved transcriptional control sequences that are transcribed into mRNA. These genes are separated by intergenic regions that vary in length depending on virus species.

Paramyxovirus helical nucleocapsids, rather than the free RNA genome, are the template for all RNA synthesis. The genomic RNA complexed with the NP (or N), P and L proteins (Holonucleocapsids) are thought to have the capacity to transcribe mRNAs *in vitro*. A major characteristic used to help classify paramyxoviruses into different subfamilies and genera is the structure of P genes. In certain paramyxoviruses, P gene encodes more than one protein by overlapping open reading frames (ORFs) through "RNA editing" or pseudotemplated addition of nucleotides (Kolakofsky and Lamb, 2001). In the genus *Rubulavirus*, PIV5, mumps virus P gene encodes both the P and V genes. The P and V proteins have a common N-terminal domain (1-164 aa), but after the editing site, viral polymerase could insert two additional non-templated G nucleotides into the mRNA that shift the translational reading frames (ORF) (Vidal, Curran, and Kolakofsky, 1990a; Vidal, Curran, and Kolakofsky, 1990b). As a result, the C-terminus

of P and V proteins are unique.

1.2.5 Paramyxoviridae genes and encoded proteins

1.2.5.1 The Nucleocapsid protein

The nucleocapsid protein (NP) is encoded by the first gene in the viral genome for all *Paramyxoviruses*, except the *Pneumoviruses*, and ranges in size from 489 to 553 amino acids. The NP protein serves several functions in the virus life cycle, including encapsidation of viral genome, interaction with the viral polymerase complex during viral RNA synthesis and interaction with the M protein during virus assembly. It was predicted that every copy of NP protein is associated with precisely six nucleotides of viral genome. Nevertheless, the protein does not seem to be a classical RNA-binding protein, as it does not contain any previously recognized RNA-binding motifs. Experimental data suggests that NP has two domains, a gobular amino-terminal body that represents 80% of the protein, and a highly variable tail-like carboxy-terminal domain that represents 20% of the protein [reviewed in (Portela and Digard, 2002a)].

1.2.5.2 The Phosphoprotein

The phosphoprotein (P) is essential for viral RNA synthesis, and it is a core component of the viral polymerase complex. As indicated by its name, this protein is heavily phosphorylated at serine and threonine residues. The P protein has no enzymatic activity, but it acts as a transactivator of L, which is the catalytic subunit of the RNA polymerase. P protein is encoded by the P gene that always produces more than one polypeptide

species. The expression of P/V/C proteins involves "RNA editing" that alters the ORFs by insertion of G residues at a specific position in the mRNA. In the case of PIV5 P protein, it is the translation product from mRNA that is generated by the addition of two non-templated G residues at the editing site of the V/P gene that shift the ORFs at the site of insertion.

The P protein C-terminal polymerase cofactor module is relatively well conserved, and is well studied for its role in viral RNA synthesis. P protein functions as a homo-oligomer, and the multimerization of P protein is mediated by its C-terminus domain. P protein also functions as a bridge to link L polymerase with the NP-RNA template. The C-terminal domain of P protein contains binding motif for interaction with L protein and for binding to the N-RNA complex (Bowman, Smallwood, and Moyer, 1999; Curran, Pelet, and Kolakofsky, 1994; Ryan, Morgan, and Portner, 1991).

1.2.5.3 The V protein

The V protein is encoded by the P/V gene and shares an N-terminal domain with the P protein but has a distinct C-terminal domain. The C-terminal domain of V protein is highly conserved and there is high sequence similarity among different paramyxoviruses. It contains a zinc-binding domain that binds two zinc molecules per V protein (Fukuhara et al., 2002; Li et al., 2006a). It has been proposed that the "Zinc-finger" domain is perhaps involved in interaction of V protein with other proteins. PIV5 V protein is a structural protein that is incorporated into the virion (approximately 350 copies of V protein per virion) and is associated with the nucleocapsids, whilst for respiroviruses and

morbilliviruses V protein is a nonstructural protein and it is absent from the virion.

The V protein of PIV5 is a multifunctional protein and plays an essential role in viral pathogenesis. V protein limits IFN production by interacting with MDA-5 (Andrejeva et al., 2004), and this property is shared among all of paramyxoviruses (Childs et al., 2007; Childs et al., 2009). In addition, paramyxovirus V proteins can also block IFN signalling by targeting STAT1 for polyubiquitylation and proteasomal degradation (Didcock et al., 1999; Young et al., 2000a). The 127 kDa cellular damage-specific DNA-binding protein 1 (DDB1) (Andrejeva et al., 2002; Lin et al., 1998), and Cullin 4A, members of the Cullin family of ubiquitin ligase subunits, are essential for targeting STAT1 for degradation (Ulane and Horvath, 2002). In addition to commonly targeting STAT1 for degradation, paramyxoviruses differ in their specificity to target other STATs. PIV5 can only target STAT1 for degradation, PIV2 targets STAT2 (Parisien et al., 2001), and mumps virus V protein targets both STAT1 (Nishio et al., 2002; Ulane et al., 2003) and STAT3 (Parisien et al., 2001; Ulane et al., 2003). STAT2 act as a host range determinant for the virus and is required for STAT1 destruction (Parisien, Lau, and Horvath, 2002; Parisien et al., 2002).

1.2.5.4 The Matrix Protein

The paramyxovirus matrix (M) protein is the most abundant protein in the virion. The M proteins contain 341-377 amino acids with predicted molecular weight between 38kDa to 43kDa. The M protein is quite basic and relatively hydrophobic, it is peripherally associated with membranes but it is not considered to be a membrane protein (Hirano et

al., 1992; Riedl et al., 2002). M protein also interacts with the cytoplasmic tails of the H and F glycoproteins (Cathomen et al., 1998; Cathomen, Naim, and Cattaneo, 1998; Spielhofer et al., 1998). M protein also interacts with the nucleocapsid (NP) protein components of RNPs (Hirano et al., 1993; Suryanarayana et al., 1994). Thus M protein plays a key role in virion assembly in bridging these elements (integral membrane proteins, lipid envelope and nucleocapsids) together and concentrating them at the plasma membrane for virion assembly and release [reviewed in (Takimoto and Portner, 2004)].

1.2.5.5 The Attachment protein

The *Respirovirus* and *Rubulavirus* attachment protein HN is a surface glycoprotein that contains both haemagglutinin and neuraminadase activities. The haemagglutinin activity of HN mediates virus attachment to sialic acid residues on cell surfaces, and the neuraminidase activity cleaves sialic acid from the surface of virions and the surface of infected cells to prevent self-aggregation of virus particles during budding at the plasma membrane, so that the assembled virus particles are efficiently released. In contrast, *morbilliviruses* attachment protein only contains haemagglutinin activity (H), whilst the *henipaviruses* and *pneumoviruses* have an attachment protein with neither activity (G).

The HN protein ranges in size from 565 to 582 amino acids. HN is a type II integral membrane protein that spans the membrane once. It contains an N-terminal cytoplasmic tail, a single N-terminal transmembrane (TM) domain, a membrane-proximal stalk domain, and a large C-terminal globular head domain that contains the receptor binding and enzymatic activity. In addition to receptor binding, HN also promotes the fusion

activity of fusion proteins (F proteins). Co-expression of HN and F protein is required for virus-cell fusion [reviewed in (Lamb and Kolakofsky, 2001)]. Interaction between HN and F has been reported in several studies (Deng et al., 1999; Malvoisin and Wild, 1993; Stone-Hulslander and Morrison, 1997; Yao, Hu, and Compans, 1997).

1.2.5.6 The Fusion protein

Paramyxovirus penetration into host cells requires fusion of viral envelope with host plasma membrane, and this process is mediated by the fusion (F) protein. F protein expressed at the plasma membrane can also mediate fusion with neighboring cells to form syncytia late in infection, which may facilitate virus spread. F protein is synthesized as an class I inactive precursor protein (F_0), which forms a trimer, and is proteolytically cleaved into its biologically active form F_1 and F_2 in a manner similar to other class I viral fusion proteins, such as influenza virus hemagglutinin (HA), HIV gp160, Ebola GP, and SARS CoV Spike protein (Colman and Lawrence, 2003; Dutch, Jardetzky, and Lamb, 2000; Earp et al., 2005; Jardetzky and Lamb, 2004). F1 contains the N-terminal fusion peptide (FP), located at the new N-terminus after cleavage, and the hydrophobic stop-transfer TM domain, and two heptad helical repeat regions, designated HRA and HRB.

F protein initially folds as a metastable prefusion form. The fusion activity of F protein requires a receptor binding protein (HN, H, or G). Interaction of HN with F protein triggers the HRA to undergo a major conformational change into the pre-hairpin intermediate form that extends the fusion peptide outward, so that it is inserted into membranes (Crennell et al., 2000). HRB adjacent to the TM domain then binds to HRA,

forming the postfusion hairpin conformation. The structures of prefusion PIV5 F (Yin et al., 2006) and postfusion NDV and human PIV3 F proteins have been solved (Chen et al., 2001; Yin et al., 2005).

The mechanism for HN binding to its receptor to trigger F protein refolding is not known (Lamb, Paterson, and Jardetzky, 2006; Morrison, 2003). HN may serve as a clamp that retains the F protein in its prefusion state. After HN binds the target cell, F is released and undergoes conformational change to activate fusion (the "clamp model"). Alternatively, HN itself may undergo a conformational change after receptor binding that destabilizes the metastable F protein and causes F to trigger (the "provocateur model").

1.3 The Orthomyxoviridae

1.3.1 Influenza A viruses

Influenza A viruses are significant human pathogens as they are highly contagious with often fatal outcomes. They are the causative agents of seasonal flu and due to their zoonotic characteristics can cause occasionally pandemic outbreaks such as those reported in 1918, 1957 and 1968 (Holmes et al., 2005). The current 2009 swine-origin H1N1 pandemic viruses have demonstrated how these viruses have great impact on public health worldwide and pose significant challenges to the scientific communities.

Influenza A viruses (FLUAV) belong to the family Orthomyxoviridae that is defined by viruses which have a negative sense, segmented, single-strand RNA genome. There are five different genera in the family of Orthomyxovidae: the influenza A viruses, influenza B viruses, influenza C viruses, Thogotoviruses and Isavirus.

FLUAV infect a variety of animal species, including humans, birds, pigs, horses, seals, dogs and cats, whilst influenza B and influenza C viruses have only been isolated from humans. All influenza A and B viruses have eight RNA segments, whilst influenza C viruses only have seven RNA segments. On the basis of the antigenicity of their haemagglutinin (HA) and neuraminidase (NA) molecules, influenza A viruses are classified into 16 HA subtypes (H1–H16) and 9 NA subtypes (N1–N9). The nomenclature given to each individual influenza A virus gives references to the host of origin (omitted if human), geographic location of isolation, strain number, year of isolation as well as the HA and NA subtype. For example, A/Udorn/307/1972(H3N2) is a type A influenza virus strain first isolated from humans in Russia in 1972. Of the 16

antigenically distinct HA subtypes and 9 NA subtypes, this virus has H3 and N2.

1.3.2 Virion Structure

Influenza A virus particles have a typical diameter of 80-160 nm. Each virion consists of three major sub-viral components: (i) on the outer most of the particle is the lipid bilayer derived from host cell membrane with three transmemberane proteins: HA, NA and M2; (ii) underneath the envelope is an intermediate layer of matrix protein (M1), and (iii) innermost within M1 shell is the viral ribonucleocapsid (vRNP) core formed by 8 segmented genome wrapped-up with nucleocapsid proteins, and are associated with three viral polymerases, PB2, PB1 and PA, responsible for viral transcription and replication. A diagram represent the influenza A virus particle, together with viral genome and encoded proteins, is shown in **Fig. 1.6**.

1.3.3 Virus life cycle

Virus replication inside the cells is a complicated process, requires not only viral proteins, but also a numbers of host factors to produce a large number of progeny viruses. A brief diagram illustrating the major steps of influenza virus life cycle is shown in **Fig. 1.7**.

1.3.3.1 Virus attachment and entry

The initial step in the replication cycle of influenza virus begins with the attachment of virion HA to sialic acid residues that are present on either glycoproteins or glycolipids (Skehel and Wiley, 2000). The specific conformation of the sialic acid (α-2,3 vs α-2,6) has been established to control species tropism of the virus, with human viruses preferentially interacting with an α2,6 linkage (SAα2,6 Gal) whereas avian viruses mostly bind to N-acetylsialic acid attached to the galactose with an α2,3 linkage (Martin et al., 1998; Suzuki et al., 2000).

Hemagglutinin (HA) is the major surface glycoprotein. Besides receptor binding, it also mediates fusion between viral envelope and the endosomal membrane. In order for the fusion to occur, precursor HA must be cleaved into HA_1 and HA_2 subunits inside the endosome to expose the N-terminal hydrophobic region to bring the endosomal membrane in close contact with viral envelope. Most of the influenza strains have a single arginine at the HA cleavage site and HA is cleaved by trypsin-like proteases, which are restricted to certain tissues and cell types (Klenk et al., 1975; Lazarowitz and Choppin, 1975), whilst the highly pathogenic avian virus subtypes H5 and H7 contain multiple

basic amino acids sequence R-X-R/K-R at the junction of HA_1 and HA_2 that can be cleaved by an intracellular subtilisin-type enzyme, such as furin or PC6 (Horimoto et al., 1994; Kawaoka and Webster, 1988; Walker et al., 1994; Walker et al., 1992). Thus under experimental conditions trypsin usually needs to be added to the culture medium of influenza virus infected cells in order to make the virus infectious.

1.3.3.2 vRNP nuclear import

Influenza virus is one of the few RNA viruses that rely on the cell nucleus for viral transcription and replication (Herz et al., 1981b; Jackson et al., 1982). Inside the nucleus, cellular pre-mRNAs donate their 5'-caps and prime viral RNA transcription and only those mRNAs synthesized after infection are used as primers (Herz et al., 1981b). In the virion and in isolated ribonucleoproteins, viral genomes are never found in naked RNA form but exist in the form of a helix associated with viral nucleoprotein (Pistorius et al.). Three viral polymerases subunits PB2, PB1 and PA are also part of the RNP and localized at the partially complementary ends of viral RNA, creating a "panhandle" structure (Hsu et al., 1987) (**Fig. 1.8**, panel B). Individual RNPs are approximately 15nm in diameter and between 50 and 130 nm long, depending on the length of the RNA (Compans, Content, and Duesberg, 1972; Heggeness et al., 1982; Jennings et al., 1983), and their diameters are close to the limit for movement through the nuclear pore complex, and thus vRNP import into the nucleus is a tightly regulated active transport process.

NP has multiple independent NLSs, one is localized at the N-terminus of the protein (Wang, Palese, and O'Neill, 1997), the other is identified at the amino acid position 198-

216 (Weber et al., 1998), having been shown to interact with nuclear import machinery, which is sufficient and necessary for the import of vRNP (Cros, Garcia-Sastre, and Palese, 2005; O'Neill et al., 1995). Nuclear import of RNPs is regulated by the viral M1 protein, which must be disassociated from RNPs during the uncoating process to allow RNP nuclear import (Martin and Helenius, 1991). In addition, it is also suggested that phosphorylation of NP protein at a serine residue may have a regulatory role in NP nuclear import (Arrese and Portela, 1996).

Although all the proteins of the vRNP complex (NP and polymerase subunits PB2, PB1 and PA) contain nuclear localization signals (NLSs) capable of directing their own entry into the nucleus (Mukaigawa and Nayak, 1991; Nath and Nayak, 1990; Nieto et al., 1994), NP was shown to be sufficient to mediate the nuclear import of viral RNAs (Cros, Garcia-Sastre, and Palese, 2005; O'Neill et al., 1995). The mechanism of nuclear import and assembly of the polymerase subunits has been controversial. However, recent publications suggest that newly synthesized PB1 and PA form a dimer in the cytoplasm, which is imported into the nucleus separately from PB2. Once in the nucleus, the PB1/PA dimer associates with PB2 to form the trimeric polymerase complex (Fodor and Smith, 2004; Huet et al., 2010).

1.3.3.3 Transcription

Once the vRNPs are imported into the nucleus, viral mRNA synthesis begins. Initiation of viral mRNA synthesis requires 5'-cap structures generated by cleavage of capped cellular pre-mRNAs, which is known as "cap snatching". The initiation of transcription

begins with PB1 binding to the 5' end of vRNA, which induces conformational change of PB2, and activates its cap-binding activity that binds the 5' cap of host pre-mRNAs, produced by cellular RNA polymerase II. This in turn causes PB1 to interact with 3' end of vRNA, which is also stabilized by the base pairing between 5' and 3' end sequences. This event stimulates vial polymerase endonuclease activity, which cleaves 10-13 nucleotides after cellular pre-mRNA cap structure (Blaas, Patzelt, and Kuechler, 1982; Li, Rao, and Krug, 2001; Nakagawa et al., 1995; Ulmanen, Broni, and Krug, 1981). Viral endonuclease activity has long been thought to reside in the PB1 subunit. However, recent biochemical and structural studies showed that PA subunit contains the active endonuclease site (Dias et al., 2009). After endonuclease cleavage, the short-capped oligonucleotide is used by the viral polymerase as a primer for transcription initiation by PB1, with addition of a "G" residue to the primer, directed by the penultimate "C" nucleotide at the 3' end of vRNP template (Plotch et al., 1981). As a result, the first nucleotide of the template is not transcribed.

Elongation of nascent viral mRNA is catalyzed by the polymerase function of PB1 until a stretch of uridine residues are met near the 5' end of the vRNA. Synthesis of viral mRNA terminates by polyadenylation at an uninterrupted stretch of 5-7 U residues located 15-22 nucleotides before the 5' end of the vRNA (Robertson, Schubert, and Lazzarini, 1981). Initially it was proposed that the base-pairing region of 5' and 3' end vRNA causes a physical block so that viral polymerase cannot proceed across, resulting in reiterative copying of the U stretch (Luo et al., 1991; Robertson, 1979). However, the current model proposes that steric hindrance of polymerase causes it to stutter on the stretch of uridines,

which it reiteratively copies to produce a poly (A) tail (Poon et al., 1998; Pritlove et al., 1998). A diagram illustrating major steps of virus transcription is shown in **Fig. 1.8A**.

1.3.3.4 Replication

Replication involves generation of full-length complementary positive sense copies (cRNA) of the genomic RNA, which are subsequently used as a template to direct vRNA synthesis [reviewed in (Elton et al., 2006; Portela and Digard, 2002b)]. cRNA is different from mRNA: firstly, it does not contains a 5' cap derived from host mRNA by cap-snatching nor a polyadenylated 3' end; secondly, it is full-length complementary copy of vRNA, whilst mRNA is truncated at its 3' end where polyadenylation occurs. vRNA serves as a template for synthesis of both mRNA and cRNA. Thus there must be a switch from a cap-dependent, endonuclease dependant mRNA synthesis to cRNA synthesis by viral polymerase. However, the mechanism of switch from mRNA transcription to cRNA synthesis is not well understood.

Early studies suggest that viral replication requires *de novo* protein synthesis. If protein synthesis is blocked, vRNP is only capable of directing viral mRNA transcription but not genome replication (Hay et al., 1977). Unlike mRNA, much less cRNA is synthesized and constitutes only about 5-10% of the total positive sense viral RNA in infected cells (Barrett, Wolstenholme, and Mahy, 1979; Hay et al., 1977; Herz et al., 1981b). Additionally, in much the same way as vRNA, newly synthesized cRNAs need to be encapsidated by free NP proteins, synthesized from primary transcription and re-entering the nucleus and associate and stabilize cRNA (Shapiro and Krug, 1988). In the second

stage of replication, full-length positive sense antigenomic cRNA serves as a template for progeny vRNA synthesis. vRNA synthesis is also primer-independent, and generates full-length copies progeny vRNAs.

NP has been shown to be essential for virus replication and its potential role for switching from mRNA transcription to genome replication has drawn much attention. Several NP temperature sensitive (ts) mutants have been isolated that are defective for replication at the non-permissive temperature (Krug, Ueda, and Palese, 1975; Scholtissek, 1978; Thierry and Danos, 1982). Depletion of free NP by antibody in infected cells inhibits cRNA synthesis in vitro (Beaton and Krug, 1984; Shapiro and Krug, 1988). Thus NP is essential for virus replication. Currently there are three models that have been proposed for the role of NP in the switch between mRNA and cRNA synthesis. The encapsidation model proposes that NP may simply acts to coat the nascent cRNA molecules (Shapiro and Krug, 1988); the template modification hypothesis proposes that the interaction of NP with vRNA template alters its structure and therefore the mode of transcription initiation and termination (Hagen et al., 1994; Tiley et al., 1994); a third hypothesis proposes that NP modifies polymerase function to favor a replication polymerase over a transcription polymerase by directly binding to PB2 and PB1 (Biswas, Boutz, and Nayak, 1998; Mena et al., 1999). A diagram illustrating major steps of virus replication is shown in **Fig. 1.8A**.

1.3.3.5 Nuclear export

Parental RNPs must be imported into nucleus to serve as template for transcription and

replication, whilst progeny RNPs must be exported out of the nucleus for progeny viruses assembly and budding. Therefore, premature exit from the nucleus is detrimental to viral gene expression. In fact, nuclear export of RNPs is a tightly regulated active process. M1 and NEP (nuclear export protein), also known as non-structural protein 2 (NS2) (O'Neill, Talon, and Palese, 1998), were shown to be essential in the RNP nuclear export process. Both M1 and NEP are synthesized at late time during virus infection, M1 enters the nucleus through its own NLS (Ye, Robinson, and Wagner, 1995), binds to RNPs and inhibits their transcriptional activity (Perez and Donis, 1998; Zvonarjev and Ghendon, 1980). NEP binds directly to M1 protein (Ward et al., 1995; Yasuda et al., 1993). It also contains a functional nuclear export signal (NES) and interacts with components of nuclear pore complex. Chromosome region maintenance 1 protein (CRM1) is a nuclear export receptor and was shown to interact directly with NEP (Neumann, Hughes, and Kawaoka, 2000). Thus it was proposed that NEP acts as an adapter molecule that links "M1-RNP cargo" to the nucleo-cytoplasmic transport machinery, and mediates the nuclear export of vRNPs.

1.3.3.6 Virion assembly

Virion assembly and budding is a tightly controlled process and requires a number of steps: firstly, all viral components (eight vRNPs and structural proteins, HA, NA, M2, M1) must be directed to the site of the assembly: apical plasma membrane of polarized cells or plasma membrane in non-polarized cells [reviewed in (Schmitt and Lamb, 2005)]. Secondly, all viral components must interact in an orderly fashion to be packed

into infectious virion. Finally, concentration of virion components must induce the curvature of plasma membrane, and the separation of virions from the host cells.

Following synthesis, each viral integral membrane protein, HA, NA and M2 is translocated across the membrane into the lumen of the endoplasmic reticulum (ER), where they are correctly folded and glycosylated. They are subsequently transported to the Golgi network where the furin cleavage of HA_0 with the multi-basic cleavage site into HA_1 and HA_2 may occur. However, for HA molecules with only a single arginine residue at the cleavage site, HA cleavage can only occur in response to extra-cellular proteases, thus to ensure the production of infectious viral particles.

These integral membrane proteins are directed to the apical surface of polarized cells via their apical sorting signals. The sorting signals for HA and NA have been studied in detail and were shown to reside in their transmembrane domains (TMD), yet the apical sorting signal for M2 needs to be defined [reviewed in (Barman et al., 2001)].

Evidence indicates that HA and NA, but not M2, are enriched in lipid rafts, which are non-ionic detergent-resistant lipid microdomains within the plasma membrane that are enriched in sphingolipids and cholesterol. Lipid rafts have been shown to play critical roles in many aspects of the virus life cycle, including viral entry, viral protein trafficking, and viral assembly and budding (Nayak and Barman, 2002; Nayak, Hui, and Barman, 2004; Ono and Freed, 2005; Schmitt and Lamb, 2004). Non-raft-associated proteins may be brought to lipid rafts by protein-protein interaction. For example, M1, non-raft associated protein, could be brought to lipid rafts by interacting with HA and NA

(Ali et al., 2000). M2 was recently been shown to bind cholesterol, which is proposed to target M2 to the raft periphery where it may bridge raft domains (Schroeder et al., 2005).

For assembly and budding, both M1 and the vRNPs must be transported to the assembly site. The association of M1 with vRNP/NEP complex is well documented. It was believed that M1 is the major driving force in directing vRNPs to the plasma membrane. In the absence of M1, virus budding does not occur. M1 was shown to be a determinant for viral morphology and size, expression of M1 alone can form virus-like particles in transfected cells (Gomez-Puertas et al., 2000).

1.3.3.7 Virion budding

Influenza A virus release from the host cell is described as a "pinching-off", which requires the local curvature of the host plasma membrane to form a bud, followed by the formation of a lipid stalk and then fission. As described above, viral integral membrane proteins HA, NA and M2 are concentrated at the lipid raft domains and form the outer spikes, interaction of M1 with the cytoplasmic tails of HA and NA could facilitate the assembly of vRNPs into the assembly sites. Incorporation of all eight vRNP segments is required for the formation of infectious virus particles. However, the mechanisms of how these segments are incorporated into virus particles remain elusive. Many negative sense virus matrix proteins contain late (L) domains which contain highly conserved motifs that are involved in recruiting the host proteins required for bud formation and virus release [see review (Freed, 2002)]. L domains can be grouped into three general classes, namely, PT(S)AP, PPPY (or PPXY), and YPDL (or YXXL), which have been shown to interact

with a number of host proteins involved in endosomal sorting and endocytosis, such as TSG101 (Demirov et al., 2002; Garrus et al., 2001), Nedd4 (Demirov et al., 2002; Garrus et al., 2001; Kikonyogo et al., 2001) and ubiquitination machinery (Kikonyogo et al., 2001; Vogt, 2000). Influenza A virus M1 helix 6 (H6) domain was reported to possess a L domain-like motif and may be involved in interaction with host proteins (Hui et al., 2003b). However, the interaction of cellular protein with influenza late domain that is involved in budding has not been identified.

Virus release is facilitated by the enzymatic activity of NA that catalyze the removal of sialic acid from the cell surface, because HA anchors the virus to the cell by binding to sialic acid-containing receptors. It also removes sialic acids from viral glycoproteins HA and NA to prevent virus self-aggregation.

1.3.4 Influenza A virus NS1 and host immune response

1.3.4.1 Synthesis of the NS1 protein

Influenza A virus genome segment 8 is transcribed into two separate mRNAs. The first encodes the non-structural protein (NS1) that is collinear with the viral RNA segment, and the second is derived from a spliced version of the mRNA primary transcript, which is translated into the 121 amino acid nuclear export protein (NEP). The 26 kDa NS1 protein is 219-237 amino acids (aa) in length, which is species specific. All avian-origin influenza viruses have a putative NS1 sequence of 230aa (Suarez and Perdue, 1998), swine origin NS1 has 219aa. Human-origin NS1 showed some size variation. During the 1940s the 230 aa NS1 protein of human H1N1 viruses gained an additional 7 aa at its C-terminus. This extension was then retained in human viruses until the 1980s, when both co-circulating H1N1 and H3N2 viruses lost the extension and reverted to the original NS1 sequence. The significance of the extension and why it was subsequently lost is not entirely clear, although it has recently been implicated in the nuclear and nucleolar localisation of NS1 (Hale et al., 2008; Melen et al., 2007).

1.3.4.2 Sub-cellular localization of NS1 protein

The sub-cellular localization of NS1 protein appears to be dependent on many factors, including viral strain, cell type, time-post infection, and expression level. The differential localization of sub-cellular compartments of NS1 may contributes to its biological activity against host innate immune response. Generally, at early time point post infection (4-6 hours), NS1 is predominately found in cell nucleus, whilst, after 8-12 hours post-

infection, NS1 is found not only in the nucleus, but also in the cytoplasm (Melen et al., 2007). Nuclear localization of NS1 is mediated by its nuclear localization signals (NLSs) interacting with cellular importin-α families. Most influenza strains contain two NLSs (NLS1, amino acids 34-38; NLS2, amino acids 203-237), whilst certain strains appears to have only one NLS (Greenspan, Palese, and Krystal, 1988).

Recently, a nucleolar localization signal (NoLS) has been identified in NS1 (Melen et al., 2007). The nucleolus is a relatively large, highly organized non-membranous sub-compartment of the nucleus, and is the site for rRNA synthesis, processing, and ribosome assembly. Recently, it has become apparent that nucleoli are dynamic structures composed of more than 700 different proteins and involved in cell cycle regulation, tumor suppression, and stress response (Olson, 2004; Olson, Hingorani, and Szebeni, 2002), and nucleoli are often targeted by DNA viruses, retroviruses and RNA viruses [reviewed in (Hiscox, 2002; Hiscox, 2007)]. The nucleoli targeting of influenza virus NS1 appears to depend on viral strain and cell type infected. All NS1 proteins have an amino acid sequence forming a functional NoLS in avian cells, however, only a few viral strains NS1 showed nucleoli targeting in mammalian cells. A/Udorn NS1 contains the mammalian NoLS, in contrast, A/WSN/33 NS1 failed to accumulate in the nucleoli (Melen et al., 2007). It has been shown that many viruses, such as plant rhabdovirus maize fine streak virus (MFSV), Borna disease virus (Pyper, Clements, and Zink, 1998), and hepatitis delta virus (Li et al., 2006b), hijack the nucleolus for genome replication. However, the role of influenza A virus NS1 targeting the nucleolus is unknown. Mutant influenza A virus (A/Udorn/72) expressing a truncated NS1 protein (lacking of its C terminal 17 amino acids) that was unable to localize into the nucleolus was not attenuated

for replication (Melen et al., 2007). Therefore, nucleolus localization of NS1 may be only affecting host functions and thus viral pathogenesis.

1.3.4.3 NS1 and host immune response

Influenza virus NS1 protein is widely regarded as a multifunctional protein with regulatory activities that affect a variety of host cell functions. It plays a key role in the viral anti-host defense mechanisms (Hale et al., 2008; Li et al., 2006c; Noah and Krug, 2005). It is only found in cells infected with influenza viruses and not in the virion structure (Krug and Etkind, 1973). NS1 contains two distinct structural/functional domains: (i) an N-terminal RNA-binding domain (residues 1-73aa), which binds with high affinity to double-stranded RNA (dsRNA) for which both NMR and crystal structures have been published (Chien et al., 2004; Qian et al., 1995). Mutational analysis has further demonstrated that dimer formation is crucial for RNA-binding, mutation in R38 and K41 abolish the dsRNA binding activity (Wang et al., 1999). (ii) C-terminal effector domain with the binding sites for poly (A)-binding protein II (PABII) and the 30kDa subunit of cleavage and polyadenylation specificity factor (CPSF30) [reviewed in (Krug et al., 2003)]. NS1 has also been reported to interact with several other host factors, including the eukaryotic translation initiation factor 4GI (eIF4GI) (Aragon et al., 2000; Burgui et al., 2003; Wolff, O'Neill, and Palese, 1996; Wolff, O'Neill, and Palese, 1998), which mediates interactions with a number of host-cell proteins (Krug et al., 2003).

1.3.4.3.1 NS1 Inhibits type I IFN production

Evidence that the NS1 protein of influenza A virus inhibits the expression of IFN stimulated genes came from experiments showing that infection with delNS1 virus, the recombinant influenza virus lacking the NS1 gene, resulted in significant stimulation of an ISRE-containing promoter at conditions in which this activation was not observed in cells infected with wild-type influenza A virus (Garcia-Sastre et al., 1998). Further more, wild type influenza virus induces far less IFN than does mutant delNS1 virus and this difference lies at the transcriptional level, which suggests that NS1 protein prevents the synthesis of type I IFN during influenza A virus infection (Talon et al., 2000; Wang et al., 2000). A number of studies suggested that NS1 protein limits IFN induction by both pre-transcriptional (cytoplasmic) and /or post-transcriptional (nuclear) events.

(i) Pre-transcriptional limitation of IFN-β induction by NS1

The transcriptional activation of IFN-β in response to virus infection is regulated by IFN enhanceosome that includes the transcription factors IRF-3, NF-κB and AP-1 (see **Fig. 1.2**). Each one of these transcription factors has been shown to be inhibited in wild type (wt) influenza virus infected cells but not in mutant virus delNS1 infected cells which corresponds with the differential induction of IFN-β by these viruses (Ludwig et al., 2002; Talon et al., 2000; Wang et al., 2000).

The precise mechanism for how NS1 inhibits IFN synthesis is still elusive but its dsRNA-binding activity plays a key role. It is now evident that influenza virus ssRNA genome containing 5'-triphosphate is a potent agonist of RIG-I (Pichlmair et al., 2006; Rehwinkel et al., 2010). As such, recent publications indicate that NS1 may mediate its block on IFN-β induction by forming a complex with RIG-I (Guo et al., 2007; Mibayashi et al., 2007; Opitz et al., 2007), and NS1 was shown to sequester the stimulatory RNA to prevent recognition by RIG-I. It has been demonstrated in vRNP reconstitutions that wtNS1 forms a trimolecular complex with the stimulatory RNA and RIG-I (Rehwinkel et al., 2010), and the complex formation is largely dependent on R38 and K41 residues in NS1 (Pichlmair et al., 2006; Rehwinkel et al., 2010).

Not only does NS1 sequester stimulatory RNA, but also it inhibits tripartite motif (TRIM) proteins to prevent RIG-I activation. The ubiquitin ligase TRIM25 has recently been shown to induce Lys63-linked ubiquitination of the N-terminal CARD domain of RIG-I, resulting in a significant increase in RIG-I downstream signalling activity (Gack et al., 2007). In virus-infected cells, NS1 specifically inhibits TRIM25 activity by physical interaction, thus blocking TRIM25 oligomerization and RIG-I ubiquitination (Gack et al., 2009).

(ii) Post-transcriptional limitation of IFN-β induction by NS1

The nucleus is the site where the cellular mRNAs are synthesized, and their maturation involves the endonucleolytic cleavage of the primary transcripts, followed by polyadenylation of the cleaved products. Cellular mRNAs are targeted by FLUAV NS1

at various stages. It was firstly shown NS1 prevents the nucleo-cytoplasmic transport of poly(A)-containing mRNAs (Alonso-Caplen et al., 1992; Nemeroff et al., 1998; Qiu and Krug, 1994), and this inhibition results, at least partly, from the intervention of NS1 with the 30 kDa protein subunit of the cleavage and polyadenylation specificity factor (CPSF30) (Nemeroff et al., 1998) and poly(A)-binding protein II (PABPII) (Chen, Li, and Krug, 1999). As a consequence of the interaction, cellular pre-mRNAs that contain short (about 12 nucleotide) poly(A) tails are retained in the nuclei of influenza virus-infected cells, providing a pool of cap-donors for viral polymerases.

Besides targeting the 3' end processing of cellular pre-mRNAs, the 5' end cellular caps are also used by influenza virus to prime viral RNA transcription, and only those capped cellular RNAs synthesized after infection, and not those synthesized before infection, are used as primers (Herz et al., 1981a), thus these mechanisms provide an effective means to limit host antiviral responses.

1.3.4.3.2 Ability of NS1 to limit IFN production is not absolute

Several groups have demonstrated that type I IFN mRNA is induced and upregulated upon influenza virus infection in dendritic cells, macrophages and A549 cells (Huang et al., 2001; Matikainen et al., 2000; Ronni et al., 1997), implying that there is leakage in blocking IFN-β mRNA induction. Infection with wild-type influenza A virus resulted in both the activation of IRF-3 (Kim, Latham, and Krug, 2002) and the production of IFN-β mRNA albeit at low levels (Noah, Twu, and Krug, 2003). These results show that even though NS1 targets cellular pre-mRNA processing and transport by interacting with

CPSF30 and PABPII, some IFN-β pre-mRNA escapes the inhibition of post-transcriptional processing mediated by this binding site. Some post-transcriptional processing of several other virus-induced cellular pre-mRNAs also occurs in wild-type influenza A virus-infected cells, as detected by microarray analysis, e.g., the production of cellular mRNAs involved in the STAT and apoptotic pathways (Geiss et al., 2001; Geiss et al., 2002; Huang et al., 2001). These observations suggest that the function of the NS1-binding site for 30-kDa CPSF and PABPII is regulated during wild-type virus infection. One possibility is that the inhibition of posttranscriptional processing of cellular pre-mRNAs is relieved as a result of the activation of the nuclear export signal (NES) by the NS1 protein (Chen, Li, and Krug, 1998).

1.3.4.3.3 NS1 Inhibits OAS and PKR activity

OAS and PKR are both activated by dsRNA, a putative by-product of virus replication. NS1 can indirectly inhibits OAS and PKR activation by binding and sequestering dsRNA, thereby inhibiting host antiviral defense. PKR expression is induced by IFN and therefore the NS1-induced block in IFN synthesis will reduce the level of PKR in infected cells. NS1 can directly inhibit the function of PKR (Min and Krug, 2006). NS1 has also been observed to form a complex with PKR to block its activation (Tan and Katze, 1998). The other mode of PKR inhibition by influenza virus infection is to recruit the cellular p58(IPK), which interacts with the ATP-binding region of PKR in the C-terminal catalytic domain (amino acids 244 to 296) and involves in PKR dimerization (Gale et al., 1996). The interaction of P58IPK with this region of PKR prevents PKR

dimerization and autophosphorylation (Tan and Katze, 1998), thus interfering with PKR's ability to phosphorylate eIF2α and inhibiting translation initiation in response to viral infection.

1.3.4.3.4 NS1 activation of PI3K signalling

Influenza A virus infections induce a variety of pro- and antiviral-acting signalling processes (Ludwig et al., 2003; Ludwig et al., 2006). Very recently the phosphatidylinositol 3-kinase (PI3K) and its downstream effector Akt/protein kinase B have been added to the list of influenza A virus-induced signalling mediators (Hale et al., 2006; Shin et al., 2007). PI3K signalling contributes to many processes, including cell metabolism, cell cycle progression, cell proliferation, survival and migration, and intracellular vesicular transport [reviewed in (Vanhaesebroeck et al., 2010)]. Class IA PI3K is a heterodimeric protein/lipid kinase that consists of a regulatory subunit (usually p85α, p85β, or p55γ), and a p110 catalytic subunit (Talon et al., 2000). The p85β regulatory subunit is made up of five domains: an N-terminal Src homology 3 (SH3) domain, a GTPase activating protein domain, and two SH2 domains (nSH2 and cSH2), separated by an inter-SH2 (iSH2) domain (Okkenhaug and Vanhaesebroeck, 2001).

PI3K is activated upon influenza virus infection in a short and transient manner, followed by a second stage of prolonged activation later in the infection cycle (Ehrhardt et al., 2006). Furthermore, it was reported NS1 effector domain is responsible for activating PI3K by interacting with iSH2 domain of p85β subunit (Hale et al., 2006; Hale et al.,

2008; Shin et al., 2007). Recently, the crystal structure of the NS1 effector domain in complex with the inter-SH2 (coiled-coil) domain of p85β has been solved (Hale et al., 2010), and it was proposed that NS1 acts by physically blocking normal inhibitory contacts between the p85β nSH2 domain and p110.

Activation of PI3K causes the generation of the second messenger PIP3, which acts to recruit pleckstrin homology (PH) domain-containing effectors to the membrane, and is involved in a wide variety of cellular events, generating lipid signals downstream of receptors and influencing diverse cellular pathways. Activated PI3K is regulated by a negative feedback loop mediated by phosphatase PTEN (Carracedo and Pandolfi, 2008).

Activation of PI3K is involved in phosphorylation and activation of IRF-3 (Sarkar et al., 2004) but the role of PI3K in influenza virus infection was proposed to support effective virus uptake in early stages as well as preventing apoptosis late in infection (Ehrhardt et al., 2006; Hale et al., 2006). However, recent results from recombinant mutant viruses with loss of function of NS1 that is unable to activated PI3K was shown to promote apoptosis, but this is not due to an inability to activate PI3K (Jackson et al., 2010).

Aims of Thesis

The overall aim of this study is to compare influenza A viruses (FLUAV) and PIV5 in dealing with cells in a pre-existing interferon-induced antiviral state. Since the mechanisms of PIV5 in dealing with the IFN response is well characterized, the knowledge of PIV5 may shed lights on understanding how FLUAV deals with the IFN response. It may facilitate better understanding of viral pathogenesis and epidemiology. In particular, it is of interest to study which stages of influenza A virus life cycle is inhibited by IFN, and to identify the key antiviral molecules induced by IFN in establishing the antiviral state. To achieve this, it requires us to be able to look into each stage of virus life cycle in details and to be able monitor the fate of incoming genome. Since MxA was reported to be an important antiviral molecule induced by IFN and inhibit a number of viruses, it would also be of interest to characterize the antiviral mechanisms of MxA against influenza virus infection.

2.1 Mammalian cells and tissue culture

2.1.1 Cell types used in this study

A549 cells - Human lung epithelial cells.

Vero cells – fibroblast-like cell line originating from kidney cells of African Green monkey.

Vero-MxA – Vero cells constitutively expressing human MxA (kindly provided by Prof. Otto Haller, Freiburg).

MDCK cells – canine kidney cells.

MDCK/V cells – MDCK cells stably expressing the V protein of PIV5 (Precious et al., 2005b).

293T cells – Human embryonic kidney cells constitutively expressing SV40 large T antigen. Kindly provided by Prof. Richard Iggo, University of St Andrews.

A549/BVDV-Npro cells – A549 cells constitutively expressing Bovine Diarrhoea Virus (BVDV) Npro protein, which targets IRF-3 for degradation.

A549/shMxA cells – A549 cells transducted with lentivirus overexpressing shRNA against MxA.

A549/IFNβ-GFP cells – A549 cells overexpressing GFP under the control of IFNβ promoter, generated by Ms. Shu Chen (University of St Andrews).

A549/shUbcH8 cells – A549 cells transducted with lentivirus over-expressing shRNA against ISG15 conjugating enzyme E2 (UbcH8).

A549/MxA cells – A549 cells constitutively over-expressing MxA.

A549/pk-MxA cells – A549 cells constitutively overexpressing pk-tagged MxA.

A549/shMxA/wMxA cells – A549 cells with MxA knockdown constitutively over-expressing wobble MxA.

A549/NS1B cells – A549 cells constitutively overexpressing pk-tagged influenza B virus NS1 protein.

A549/FLAG-E3L cells – A549 cells constitutively overexpressing FLAG-tagged vaccinia virus E3L protein.

2.1.2 Cell maintenance

Cell monolayers were maintained in $25cm^2$ or $75cm^2$ tissue culture flasks (Greiner) in DMEM (Dulbecco's modified Eagle's medium; Invitrogen) supplemented with 10% (v/v) heat-inactivated fetal bovine serum (FBS; Biowest) and incubated at 37°C/5% CO_2 incubator. Cells were routinely passaged using trypsin/EDTA (Becton Dickinson Ltd.), at a frequency dictated by the rate of cell growth.

2.1.3 Cell stock preparation and resuscitation

Adherent cells were trypsinised, resuspended in DMEM/10% FBS, and centrifuged at ~500×*g* for 5mins. Pelleted cells were resuspended in DMEM supplemented with 20% FBS and 10% DMSO and aliquoted into cryovials. Cell stocks were frozen at 70°C before long-term storage in liquid nitrogen. For resuscitation of cells, cryovials were thawed at 37°C before centrifugation at ~500×*g*. Pelleted cells were then resuspended and grown in DMEM/10% FBS at 37°C/5% CO_2. Medium was replaced after 24 hours in order to remove traces of DMSO.

2.1.4 Treatment of cells

2.1.4.1 IFN treatment

Cells were treated overnight (at least 16 hours, unless where otherwise stated) with media

supplemented with Roferon recombinant human IFNα-2a (Roche Diagnostics) or IFN-γ (Invitrogen) at a concentration of 10^3 units/ml.

2.1.4.2 Transfections with plasmid DNA

Mutant viruses were generated by substituting the pHH-NS "rescue" plasmid for mutated versions in the rescue transfection (plasmids were kindly provided by Robert A. Lamb, Northwestern University). These plasmids (pHH-NS1-Y89F, pHH-NS1-P164/7A, pHH-NS1-P162/4/7A, pHH-NS1-R38A, pHH-NS1-123/4, pHH-NS1-184-8, pHH-NS1-103/106, pHH-NS1-Δ99) were generated by site-directed mutagenesis of pHH-NS using the QuikChange mutagenesis kit (Stratagene) and specific primers. The sequence of the mutated plasmids was confirmed by DNA sequencing. Transfection of cells with plasmid DNA was carried out using FuGENE 6 transfection reagent (Roche) according to the manufacturer's instructions.

2.2 Viruses and viruses infection

2.2.1 Influenza viruses

1. **rWSN**: Recombinant wild-type influenza A virus (A/WSN/33) generated and provided by Dr. Dave Jackson (University of St Andrews).
2. **rUd**: Recombinant wild-type influenza A virus (A/Udorn/72) generated and provided by Dr. Dave Jackson (University of St Andrews).
3. **rUd-Y89F**: recombinant Udorn virus expressing NS1 with the Y89F amino-acid substitution. Generated and provided by D. Jackson, Northwestern University, USA (Hale et al., 2006).
4. **rUd-P164/7A**: The NS1 protein carries two amino acid substitutions at positions 164 and 167 that are reported to abrogate PI3K binding (Shin et al., 2007).
5. **rUd-P162/4/7A**: The NS1 protein carries three amino acid substitutions at positions 162, 164 and 167. Virus isolation took much longer than for other mutants, and was initially a mixed population of virus, also containing rUd NS1-Δ99.
6. **rUd-Δ99**: This virus encodes an NS1 protein carrying only amino acids 1-98, and was isolated during rescue of the rUd-P162/164/167A virus.
7. **rUd-184-8(L)**: At positions 184-188, five amino acids, GLEWN have been substituted for RFLRY. This mutation eliminates the binding site for CPSF30 (Noah et al., 2003). This virus took much longer to rescue than other mutants, and isolated virus was initially a mixed population, also containing rUd -184-8(P).
8. **rWSN-Y89F**: recombinant WSN virus expressing NS1 with the Y89F amino acid substitution. Generated and provided by D. Jackson Northwestern University, USA (Hale et al., 2006).
9. **rUd-184-8(P)**: Isolated during the rescue of rUd-184-8(L). rUd-184-8(P) differs from rUd-184-8(L) at position 186, where it carries a proline instead of a leucine residue.

10. **rUd-103/106B**: The NS1 protein carries F103L and M106I amino acid substitutions. Residues 103 and 106 are important for stabilizing the NS1:CPSF30 interaction (Kochs, Garcia-Sastre, and Martinez-Sobrido, 2007; Twu et al., 2007).
11. **rUd-123/4**: The binding site for PKR (residues 123-127) is mutated in the NS1 protein. Mutation of this site abrogates the ability of NS1 of inhibit PKR during virus infection (Min et al., 2007).
12. **rUd-R38A**: The NS1 protein carries an amino acid substitution at position 38. This residue is absolutely required for the dsRNA binding activity of NS1, and replacement of arginine with alanine abrogates this activity (Wang et al., 1999).

2.2.2 Parainfluenza virus 5

1. **W3** Wild-type laboratory strain of PIV5 (Choppin, 1964)
2. **CPI-** canine isolate of PIV5 unable to block IFN signalling. (Baumgartner, Krakowka, and Blakeslee, 1987; Baumgartner et al., 1982; Baumgartner et al., 1981)

2.2.3 Virus infection

To infect with paramyxovirus isolates, monolayers were inoculated with virus diluted in DMEM supplemented with 2% fetal bovine serum (FBS) at an appropriate multiplicity of infection (MOI), or DMEM only (for mock infections). For virus infections in 6-well plates, cells were inoculated in a volume of 1ml per well and placed on a rocking platform at 37°C for an adsorption period of 1-2 hours. Virus inoculum was then removed and replaced with DMEM/2% FBS. Cells were incubated at 37°C/5% CO_2 until harvested.

FLUAV infections were carried out in serum-free DMEM. Monolayers were washed in DMEM prior to infection to remove all traces of serum. Cell monolayers were infected

with 400µl virus (per well of a 6-well plate) diluted in serum-free DMEM at an appropriate MOI (or DMEM only for mock infections). Cells were incubated for 1hr at 37°C, with gentle agitation at regular intervals. Virus inoculum was removed and replaced with serum-free DMEM. Cells were incubated at 37°C/5% CO_2 until harvested.

2.2.4 Preparation of virus stocks

Stocks of PIV5 isolates were kindly maintained and provided by Dan Young (University of St. Andrews).

To prepare stocks of influenza A virus, 100% confluent MDCK or MDCK/V monolayers in T75 cm^2 flask were washed twice in serum-free DMEM. Cells were inoculated at an MOI of ~ 0.001 plaque-forming units (PFU)/cell in 5ml serum-free DMEM. After a 1 hour adsorption period with constant rocking at 37°C, virus inoculum was removed and monolayers were washed with serum-free DMEM. 12 ml (per 75cm^2 flask) of serum-free DMEM supplemented with 2.5µg/ml N-acetyl trypsin (NAT) were added to the monolayers and cells were incubated in incubator at 37°C, 5% CO_2. Supernatants were harvested when 80-90% CPE (cytopathic effect) was observed (about 2 days). Cell debris was removed by centrifugation (~3000 ×*g* for 10mins) and aliquots were frozen at -70°C. Virus titers were determined by plaque assay as described below.

2.2.5 Titration of virus preparations

Titration of FLUAV was carried out on confluent MDCK monolayers in 6-well plates. The procedure was carried out essentially as described elsewhere (Takeda et al., 2002). Cells were washed twice in serum-free DMEM in order to remove all traces of serum.

Confluent monolayers of cells were inoculated with 0.5 ml 10-fold serial dilution of viruses and were incubated at 37°C/5% CO_2. Plates were agitated every 10 minutes to ensure even adsorption of the virus among the monolayers. During this period, 2× overlay medium (13.4g DMEM, 3.7g $NaHCO_3$, and 10mM HEPES pH 7.4 per 485ml water) supplemented with 2 μg/ml N-actetyl trypsin (NAT, Sigma) was incubated at 37°C. 2% agarose (NuSieve® GTG®) in water was melted in a microwave oven and placed in a 55°C water bath until required. After an adsorption period of 2 to 3 hours, virus inoculum was removed, monolayers were washed with FBS free DMEM, the 2× overlay medium and the 2% agarose were mixed in a 1:1 ratio, and 2ml of this molten agarose was added into each well. After the overlay had solidified (after about 30 minutes), plates were inverted and incubated at 37°C incubator with 5% CO_2 until distinct plaques had formed (2 to 3 days). Cells were fixed by adding 2ml 5% formaldehyde/1% sucrose in PBS on top of the agarose plugs, and the monolayers were left to fix for 2 hours. Agarose plugs were then removed and washed with PBS, plaques were stained with crystal violet (0.1% crystal violet, 3.6% formaldehyde, 1% methanol, 20% ethanol in H_2O) or visualized by immunostaining as described below.

2.2.6 Immunostaining of influenza virus plaques

To stain the plaques for immunological specificity, the monolayers were fixed with 5% formaldehyde (in PBS) for at least 2 hours and the agarose overlay were removed, cell monolayers were washed with PBS and blocked with 500µl of PBN (PBS, 1% BSA, 0.02% sodium azide) for one hour. Then the cells were incubated for 1 h with first antibody: goat anti-A/Udorn/72 virus serum in PBN (1:1000). The monolayers were then washed with PBS and incubated for 1 h with peroxidase-conjugated donkey anti-goat

immunoglobulin G (Santa Cruz Biotechnology, UK, sc-2022) in PBN. After the monolayers were washed with PBS, the peroxidase-conjugated antibody was reacted with fast red BCIP/NBT (Sigma) that is dissolved in water for 1-2 hours.

2.3 DNA subcloning

2.3.1 cDNA synthesis by reverse transcription

To clone IFN-induced genes, total cellular RNA were extracted using Trizol (Ambion) from A549 cells treated with IFNα (1000u/ml) and used as a template in a reverse transcription reaction for synthesis of cDNAs with M-MLV Reverse Transcriptase (Sigma). The procedures were carried out according to manufacture's instruction (Promega). 0.5μg Oligo$(dT)_{15}$ were mixed with 1μg of total RNA and heated at 70°C for 5min to melt any secondary structure with the template. Samples were then immediately immersed on ice. The annealed primer/template were added with 5× M-MLV RT reaction buffer, 10mM dNTP and M-MLV reverse transcriptase and incubated at 40°C for the initial 10 mins, then 42°C for the final 50 mins. After that, the reaction was inactivated by heating for 15min at 70°C, and the cDNA was used as a template for amplification by PCR with gene-specific primers.

2.3.2 Restriction enzyme digestion of DNA

DNA backbone vectors and purified PCR products (typically 2-3μg) were mixed with 5 units of the desired restriction enzymes, enzyme buffers, in a reaction mixture made up to 30μl with de-ionised water. Reactions were incubated for 2 to 4 hours at the appropriate temperature. All restriction reagents were purchased from Promega or New England Biolabs. Where a single enzyme was used for digestion of backbone vector to create cohesive ends, Calf intestinal Alkaline Phosphatase (CIAP, Promega) was added into reaction tube following digestion to prevent vector self-ligation.

2.3.3 Transformation of competent cells

0.1μg plasmid was added directly to 50μl of thawed, chemically competent cells (Invitrogen). After incubation on ice for 30min, cells were heat-shocked at 42°C in water bath for 30sec before being immediately immerged in ice for a further 2min. Cells were resuspended in 1ml LB broth and incubated at 37°C for 1h. The cell suspension was plated out onto LB-agar plates supplemented with ampicillin (90 mm-diameter Petri dishes; Scientific Laboratory Supplies Ltd., U.K.). Plates were inverted and incubated at 37°C overnight.

2.3.4 Preparation of plasmid DNA

For small-scale preparations, bacterial cell cultures of 10 ml (in LB broth containing 100μg/ml ampicillin) were grown overnight in a 37°C shaking incubator. DNA was extracted from cells and purified using the QIAGEN DNA mini-prep kit according to the manufacturer's instructions (QIAGEN). To produce larger-scale DNA preparations, 100 – 500 ml of bacterial culture was grown overnight in a 37°C shaking incubator. DNA was extracted from cells using the QIAfilter Plasmid Maxi Kit according to the manufacturer's instructions (QIAGEN).

2.3.5 Colony PCR screening

Colony PCR screening was used to quickly test for the presence of a target DNA fragment in E.coli without the need for growth and mini-prep of transformed bacterial colonies. Basically, a master mix of PCR reaction with primers for gene of interest was prepared and dispensed into PCR tubes. Bacterial colonies were picked up individually and resuspended into the PCR reaction tube, and PCR amplification was performed at

appropriate time and temperature for the primerts and PCR product expected for ~25 cycles according to the manufacturer's protocol (Promega). PCR products were analysed by agarose gel electrophoresis and bacterial colonies corresponding to positive PCR product were used for plasmids mini-prep.

2.3.6 Agarose gel electrophoresis

Plasmids preparations and PCR digestions were analysed by agarose gel electrophoresis in 1% (w/v) agarose (Sigma-Aldrich) gel in 1× TAE buffer added with ethidium bromide. DNA samples were mixed with appropriate volume of 6× DNA loading buffer (Promega) and electrophoresis was performed in 1× TAE buffer at 100V. Resolved DNA bands of interest were visualized on a UV transluminator.

2.4 Protein analysis

2.4.1 SDS polyacrylamide gel electrophoresis

Cell lysates were prepared by adding 2× disruption buffer (6M Urea, 2M β-mercaptoethanol, 4% (w/v) SDS, with bromophenol blue colouring) to cells in a 1:1 ratio. Viscosity of samples was reduced by sonication (2 × 20s) or digestion with Benzonase (Novagen). Polypeptides were separated through 4-12% NuPAGE polyacrylamide gradient gels (Invitrogen) by electrophoresis (200V for 50 min) in 1×MOPS buffer [3-(*N*-morpholino)propanesulphonic acid] (Invitrogen).

2.4.2 Immunoblotting

Protein lysates were subjected to SDS-PAGE as described above, and transferred to polyvinylidene difluoride (PVDF) membrane using the XCell II Blot Module according to the manufacturer's instructions (Invitrogen). After transfer, membranes were blocked for one hour in 5% (w/v) skimmed milk powder, 0.1% (v/v) Tween 20 in PBS (blocking buffer), and incubated for either 1h or overnight with primary antibody diluted (in blocking buffer) according to the manufacturer's instructions. After 3 washes with PBS/ 0.1% Tween 20, interactions between protein and antibody were detected by incubating the membrane for 1h with horseradish peroxidase (HRP)-conjugated antibody raised against the appropriate species, diluted appropriately in blocking buffer. Unbound antibody was washed away with PBS/ 0.1% Tween 20, and specific polypeptide bands were visualised using western blotting chemiluminescence luminal reagent according to the manufacturer's instructions (Santa Cruz).

2.4.3 Immunofluorescence

For immunofluorescence analysis, cells were grown on 10mm-diameter coverslips (General Scientific Co. Ltd., Redhill, United Kingdom) in individual wells of 6-well plates. Treatment with exogenous IFN was as mentioned above. Cells were infected with FLUAV or PIV5, and the inoculum was adsorbed for 1 h. At various times p.i., coverslips were removed and incubated in fixing solution (5% formaldehyde and 2% sucrose in PBS) for 30 min at room temperature, then permeabilized (0.5% Nonidet-P40, 0.5% Triton X-100, and 2% sucrose in PBS) for 15 min, and washed three times in PBS containing 1% fetal calf serum and 0.1% azide. After that, monolayers of cells were blocked with 5% FBS. To detect the proteins of interest, cell monolayers on coverslips were incubated with 20-30 µl of appropriately diluted primary antibody for 1 h. The antibodies used to detect PIV5 proteins were the MAbs NP and P. To detect influenza viral proteins, purified sheep anti-NS1 or monoclonal anti-NP antibody (Abcam) was used. Cells were subsequently washed (1% fetal calf serum, 0.1% azide in PBS) several times, and the antibody-antigen interactions were detected by indirect immunofluorescence (1 h incubation) with a secondary Texas Red-conjugated goat anti-mouse immunoglobulin, or FITC-conjugated goat anti-Rabbit IgG (Seralab, United Kingdom). In addition, cells were stained with the DNA-binding fluorochrome 4′,6-diamidino-2-phenylindole (DAPI, 0.5 µg/ml; Sigma-Aldrich) for nuclear staining. Following staining, monolayers were washed with PBS, mounted with Citifluor AF-1 mounting solution (Citifluor Ltd., United Kingdom), and examined under a Nikon

Microphot-FXA immunofluorescence microscope. For images that were meged with DAPI, SPOT software (version 4.6) was used.

2.4.4 Preparation of radio-labelled antigen extracts, immunoprecipitation, and SDS-PAGE

At various times after the addition or no addition of IFN, cells grown in T-25 flasks were metabolically labelled for 1 h with 2µl L-[35S]methionine (500 Ci/mmol; Amersham International Ltd., United Kingdom) in methionine-free tissue culture medium. At the end of the labelling interval, cells were washed with ice-cold phosphate-buffered saline (PBS) and lysed in immunoprecipitation buffer (10 mM Tris-HCl, pH 7.8, 5 mM EDTA, 0.3% Nonidet P-40, and 0.65 M NaCl) by sonication with an ultrasonic probe. 50µl of total cell antigen extract sample was kept as a control to examine total cellular protein synthesis. Soluble antigen extracts were prepared by subjecting the remainder of the cell lysates to centrifugation at 12, 000 ×g for 30min to remove insoluble material. To detect PIV5 viral proteins, immune complexes were formed by incubating 1ml samples of the soluble antigen extracts with a pool of monoclonal antibodies (MAbs) to the NP, P, M, and HN proteins of PIV5 and a polyclonal antiserum to the P/V proteins of PIV5 for 2 h at 4°C. To detect influenza viral proteins, immune complex were formed by incubating with 1ml of the soluble antigen extract with polyclonal anti-FLUAV antibody. The immune complexes were isolated by incubation with protein G-Sepharose 4B Fast Flow (Sigma) (1 h at 4°C). The proteins in the immune complexes were dissociated by heating in 2× disruption buffer (6M Urea, 2M β-mercaptoethanol, 4% sodium dodecyl sulphate) at 100°C for 5 min and analyzed by SDS-PAGE. After electrophoresis, gels were fixed, stained, and dried. The resolved labelled polypeptide bands were visualized by autoradiography and quantitated by phosphorimager analysis.

2.5 RNA analysis

2.5.1 RNA extraction

Monolayer of cells grown on 6-well plates were washed twice with ice-cold PBS and lysed directly in the culture dish by addingin 1ml Trizol (Ambion) and incubated for 5 minutes at room temperature. Cell suspension was then transferred into an eppendorf tube and mixed with 200µl chloroform. Samples were vortexed vigoriously for 15sec and incubated at room temperature for 5 minutes. The solution was then subject to centrifugation for 20min at 4°C, and total RNA was pelleted from supernatant by mixing with 0.5ml isopropanol. Finally the pellet was washed with 70% enthanol and dissolved in DEPC-treated water.

2.5.2 in situ hybridization

2.5.2.1 Generation of DIG-labelled probes

To generate Digoxigenin (DIG)-labelled ssRNA probes against FLUAV segment 8, we have to firstly generate a probe construct. Briefly, PCR amplicon was synthesized with primers sequence of A/Udorn Segment 8 (nucleotides169-610), and cloned into pGEM-T easy vector according to the manufacturer's instructions (Promega, see **A.3** for more details). The construct was then linearized by digestion with NdeI or NcoI and purified with QIAquick PCR purification kit (QIAGEN). T7 or SP6 polymerase was used to generate the digoxigenin (DIG)-labelled "run-off" transcript probes against the negative-sense vRNA or positive-sense RNA antigenome, respectively, using DIG RNA labelling kit (Roche). DIG-labelled RNA probes were subjected to LiCl/ethanol precipitation to

remove unincorporated DIG nucleotides, and the yield of DIG-labelled RNA probes was estimated using a spot test.

2.5.2.2 *in situ* hybridization

For *in situ* hybridization analysis of viral RNA localization, cells were grown on 10mm-diameter coverslips (General Scientific Co. Ltd., Redhill, United Kingdom) pre-coated with poly-L-lysine (sigma) in individual wells of 6-well plates. Cells were infected with FLUAV at 4ºC on ice for one hour to allow virus attachment to cells before the cells were incubated at 37ºC to synchronize the process of infection. At various times p.i., monolayers were fixed with 5% formaldehyde and 2% sucrose in PBS for 30 min at room temperature and washed three times in PBS.

Deproteinization was carried out with Proteinase K solution (2μg/ml Proteinase K, 20mM Tris pH7.5, 2mM $CaCl_2$) at 37 ºC for 15min. Cells were then rinsed with 0.1% Tween-20 in PBS and post-fixed in 5% paraformaldehyde in PBS for 20 minutes followed by permeabilization in 0.5% Triton X-100 in PBS with 2mM Vanadyl Ribonucloside Complex (VRC, Sigma) for 15 min at RT. After washing for 3 times with PBS, cells were allow to equilibrate for 5 minutes in 2× sodium chloride–sodium citrate (SSC) with 0.025% Tween-20 at 37ºC. DIG-labelled probes (1ng/μl) in hybridization buffer (50% deionised formamide; 1mg/ml sonicated salmon sperm; 1mg/ml tRNA; 0.1M Tris-HCl, pH8.0; 1.6mg/ml NaH_2PO_4; 1.4mg/ml Na_2HPO_4; 0.2mg/ml Ficol; 0.2mg/ml polyvinyl-Pirrdidone; 1mM DTT; 1×SSC and 20× RNasin) were heated for 5 minutes at 80°C on a heating block, and quenched on ice before being applied to the cells. Hybridization was allowed to proceed overnight at 55 ºC in a humidified chamber.

After overnight hybridization, sections were thoroughly washed once for 15 minutes with 2×SSC/10mM Tris pH7.5 at room temperature, twice in 0.1×SSC for 15 minutes (room temperature), and once with stringent buffer (30% Formamide/0.1%×SSC/10mM Tris, pH 7.5) for 30 minutes at 55 ºC. Cells were subsequently washed 3 times with TBST buffer (150mM NaCl; 10mM KCl; 50mM Tris-HCl pH7.5; 0.1% Tween-20) and blocked for one hour at room temperature with 2% blocking reagent (Roche) in TBST supplemented with 20% heat inactivated sheep serum.

For detection of hybridization, cells were incubated with alkaline phosphatase conjugated anti-DIG Fab fragments (Roche) diluted 1:1000 in TBST with 2% blocking reagent (Roche) and 1% heat-inactivated sheep serum for two hours at room temperature. After three washes with TBST buffer, viral RNAs and bound anti-DIG antibody complex was then detected with the highly sensitive Fast Red tablet (Roche) dissolved in 0.4M NaCl/Tris-HCl 0.1M, pH8.2 for two hours at room temperature. The reaction was stopped by washing of cells with 0.1% Tween-20 in PBS, followed by several washes with PBS. Cells nuclei were then stained with 4',6-Diamidino-2-phenylindole (DAPI) for 10 minutes at room temperature. Finally, cells were washed in PBS and coverslips were mounted onto microscope slides with Mowiol mounting medium (CALBIOCHEM).

2.6 Miscellaneous assays

2.6.1 Lentivirus-based shRNA cell line construction

2.6.1.1 Generation of shRNA constructs

The lentivirus-based small hairpin RNA (shRNA) transfer plasmids targeting human MxA at positions 1325 to 1349 (CCA GGA CUA CGA GAU UGA GAU UUC G) (pLKO/shMxA) was constructed, this sequence showed efficient MxA gene silencing (Oelschlegel, Kruger, and Rang, 2007). Briefly, the double strand hairpin oligos were synthesized by annealing two single strand DNA sequence, MxA Forward (GATCCCCAGGACTACGAGATTGAGATTTCGTTCAAGAGACGAAATCTCAATCTCGTAGTCCT GGTTTTTCCCGGGCTAGAG) and MxA reverse (AATTCTCTAGCCCGGGAAAAACCAGGACTA CGAGATTGAGATTTCGTCTCTTGAACGAAATCTCAATCTCGTAGTCCTGGG) and ligated into the backbone vector pLKO.1 (see **A.1** for more details), which was digested with EcoRI and BamHI. SmaI sequence was introduced into the hairpin oligos to allow screening of constructs by restriction digestion after transformation into E.coli. MxA sequence and its reverse complement sequence were marked in red color; SmaI (underlined) sequence was introduced into the hairpin oligos to allow screening of constructs by restriction digestion after transformation into E.coli.

2.6.1.2 Lentivirus production

Recombinant lentiviruses expressing MxA shRNA was prepared by transfecting 293FT

cells (Invitrogen) with the lentivirus vector plasmids (pLKO/shMxA) together with plasmids pCMVR8.91 (that expresses the gag/pol, tat, and rev genes of human immunodeficiency virus) and pMD_VSVG (that expresses the envelope gene of vesicular stomatitis virus) using FuGENE6 according to the manufactory's protocol (Roche). Two days after transfection, the culture media containing the lentiviruses were harvested, and new medium was added into the cells and harvested 24 hrs later. Medium from day 2 and day 3 after transfection was pooled and cell debris was removed by centrifugation at 3000×g for 10 minutes. The supernatant was filtered through a 0.45 μm Tuffryn membrane filters (Invitrogen) and stored at -70°C.

2.6.1.3 Transduction of target cells

30% confluent A549 cells were transduced with the lentivirus stock A549 cells in the presence of 8 μg/ml Polybrene (Sigma-Aldrich, UK). Selection with Blasticidin (InvivoGen; 10μg/ml) was carried out 2 days after infection. Stocks of stable cell line were frozen at -70°C. The cell line generated was verified by immunofluorescence and western blot assay.

2.6.2 Lentivirus-based overexpression cell line construction

The procedure of overexpressing lentivirus-based target gene is very similar to lentivirus-based shRNA knockdown system, except that the pdlNotI'IRESpuro vector is used (the map of the vector is attached at the **Appendix** section), and lentivirus transducted cells were selected in the presence of puromycin (2μg/ml) or Blasticidin (10μg/ml).

Antibodies

Antibodies were used in immunoblotting, immunostaining and immunofluorescence as described in the according section. The primary antibodies specific for viral proteins and cellular proteins used in this study are shown in table 2.1.

Table 2.1: Primary antibodies and target proteins:

Antibody	Target Protein	Source
PIV5-NP-a (mAb)	PIV5 NP	(Randall et al., 1987)
PIV5-P-e (mAb)	PIV5 P (unique C domain)	(Randall et al., 1987)
PIV5-P-k (mAb)	PIV5 V and P (common N domain)	(Randall et al., 1987)
Influenza A virus anti-NP (mAb)	Influenza NP	Abcam Ab20343
Polyclonal Anti X-31 (Sheep)	Influenza A virus	Diagnostics Scotland
Polyclonal anti -NS1 (Sheep)	NS1	Purified from sheep anti-NS1 serum
monoclonal Anti-actin (human)	Actin	Alexis Biochemicals ALX-210-935
Polyclonal anti-STAT1α (human) (RbAb)	Human STAT1 Alpha (AA 712-750)	Abcam Ab2071
Anti-Mx1/2/3 (H-285) (Rabbit)	Mx1/2/3	Santa Cruz Biotechnology sc-50509
Monoclonal anti-MxA 143	MxA	Kindly provided by Georg Kochs (University of Freiburg, Germany)

The following antibodies were used as secondary antibodies to detect primary antibodies:

- **Donkey anti-Goat IgG-AP alkaline phophatase conjugated** (Santa Cruz Biotechnology, UK, sc-2022)
- **Donkey anti-Goat IgG: Texas Red® conjugated** (Santa Cruz Biotechnology, UK, sc-2783)
- **Donkey anti-Sheep IgG-AP alkaline phospatase conjugated** (Abcam, UK, ab6898)

- **Goat anti-mouse IgG-AP alkaline phosphatase conjugated** (Santa Cruz Biotechnology, UK, sc-2008)
- **Goat anti-mouse IgG: FITC conjugated** (AbD Serotec, UK, 103002)
- **Goat anti-mouse IgG: Texas Red® conjugated** (AbD Serotec, UK, 103007)
- **Goat anti-rabbit IgG-AP alkaline phosphatase conjugated** (Santa Cruz Biotechnology, UK, sc-2007)
- **Goat anti rabbit IgG-HRP linked** (Santa Cruz Biotechnology, UK, sc-2004)
- **Goat anti-rabbit IgG+IgM: Texas Red® conjugated** (AbD Serotec, UK, 401007)
- **Goat anti-rabbit IgG**: Cyanine5 conjugated (kindly provided by Dan Young, University of St. Andrews

2.8 Primers

PRIMERS	DESCRIPTION	SEQUENCE
MxA5'(BamHI)	Primers used for cloning of human MxA gene into overexpression plasmid	5'-CGGGATCCATGGTTGTTTCCGAAGTGGAC-3'
MxAr(NotI)		5'-ATAAGAATGCGGCCGCTCAACCGGGGAACTGGGCA AGCC-3'
Ud-Seg8_169f	Primers used for generation of probes from constructs with segment 8 sequences (169-610)	5'-CGGAATTCGCACTCTCGGTCTAAACATC-3'
Ud-Seg8_610r		5'-CGGGATCCGAGACTCGAACTGTGTTATC-3'
pLKOfwd	Primer used for sequencing shRNA constructs	5'-CAAGGCTGTTAGAGAGATAATTGGA-3'
PK-fwd	Primer used for sequencing pk-tagged constructs	5'-ATGGGAAAGCCGATCCCAAAC-3'
UbcH8-fw	Used for verification of UbcH8 knockdown by RT-PCR	5'-CATGATGGCGAGCATGCGAGTG-3'
UbcH8-rev		5'-CTGGACAGGTTCCGCAGGTATGGG-3'
Herc5-fw	Used for verification of Herc5 knockdown by RT-PCR	5'-GGTCATAATTCAACACAGAATG-3'
Herc5-rev		5'-TCCCTTTTTGTGCTCTGCCACCG-3'
IFITM3-fwd	to amplify IFITM3 and clone into	5'-GGACTAGTATGAATCACACTGTCCAAACC-3'

IFITM3-rev	pdl'pk-Npro(Bla)	5'-GGAATTCCATATGCTATCCATAGGCCTGGAAGATC-3'
Herc5Forward2	shRNA oligos targeting Herc5 E3 ligase of ISGylation pathway	5'-GATCCGGACTAGACAATCAGAAAGTTTTCAAGAGAA ACTTTCTGATTGTCTAGTCCTTTTTCCCGGGCTAGAG-3'
Herc5Reverse2		5'-AATTCTCTAGCCCGGGAAAAAGGACTAGACAATCAG AAAGTTTCTCTTGAAAACTTTCTGATTGTCTAGTCCG-3'
UbcH8Forward2	shRNA oligos targeting UbcH8 E2 ligase of ISGylation pathway	5'-GATCCGCCTCCCATGATCAAATTCTTCAAGAGAGAA TTTGATCATGGGAGGCTTTTTCCCGGGCTAGAG-3'
UbcH8Reverse2		5'-AATTCTCTAGCCCGGGAAAAAGCCTCCCATGATCAA ATTCTCTCTTGAAGAATTTGATCATGGGAGGCG-3'
MxA_Fwd	shRNA oligos used for knocking down MxA gene	5'-GATCCCCAGGACTACGAGATTGAGATTTCGTTCAAGA GACGAAATCTCAATCTCGTAGTCCTGGTTTTTCCCGGGCT AGAG-3'
MxA_Rev		5'AATTCTCTAGCCCGGGAAAAACCAGGACTACGAGAT TGAGATTTCGTCTCTTGAACGAAATCTCAATCTCGTAGTC CTGGG-3'
MxA Wobble 5'	Primers used for cloning of synthetic wobble MxA gene into overexpression plasmid	5'-CGGGATCCATGGTGGTGTCTGAAGTCGATATTG
MxA Wobble 3'		5'-ATAAGAATGCGGCCGCTCAGCCAGGAAACTGAGCCAG-3'

2.9 Plasmids

PLASMIDS	DESCRIPTION
pMD-VSVG	Expressing the vesicular stomatitis virus glycoprotein (VSV-G), used in lentivirus production, provided by Y-H. Chen.
pCMVR 8.91	Expressing the gag/pol, tat and rev genes of HIV-1 (used in lentivirus production, provided by Y-H. Chen).
pdl'BVDV/NPro(N'V5)	Lentivirus vector expressing the N-terminally V5-tagged BVDV/NPro; provided by M. Galliano, University of St Andrews.
pLKO/shMxA(Bla)	Lentivirus vector expressing shRNA against MxA, cloned from backbone vector, pLKO-STAT1, which is kindly provided by Dr. Lena Andrejeva, University of St Andrews**.**
pLKO/shUbcH8(Bla)	Lentivirus vector expressing shRNA against ISGylation E2 conjugation enzyme UbcH8, cloned from backbone pLKO/shMxA(Blasticidin resistance).
pLKO/shHerc5	Lentivirus vector expressing shRNA against ISGylation E3 ligase gene Herc5.
pLKO/shIFITM3(puro)	Lentivirus vector expressing shRNA against IFITM3, cloned from backbone vector pLKO-TLR3(puro), provided by Dr. Andrejeva.
pdl'MxA(puro)	MxA gene was cloned into pdlNotI-IRESpuro plasmid (generated by Y.H. Chen) from pTM/MxA (kindly provided by Dr. Gjon Blakqori, University of St Andrews).
pdl'pk-MxA(puro)	Lentivirus vector expressing pk-tagged MxA. MxA gene were amplified from pTM-MxA, and cloned into pdl'SeV-V N-pk digested with SpeI/BamHI.
pHH/NS1	Influenza A virus segment 8 (strain A/Udorn) was cloned into pHH plasmid in reverse orientation (kindly provided by Dr. D., Jackson).
pdl'pk-Viperin(Bla)	Viperin gene were amplified from puro-viperin vector (provided by Dr. Andrejeva), digested with SpeI/NdeI, and cloned into pdl'pk-Npro(Bla) (provided by Dr. M., Killip).
pdl'FLUBV/NS1 (N'term-V5)	Lentivirus vector expressing influenza B virus NS1 gene (strain Yamagata/73), provided by Dr. Benjamin Hale, University of St Andrews.
pdl'FLAG-E3L	Lentivirus vector expressing vaccinia virus E3L gene, N-ter FLAG tagged, provided by Mara Galliano, University of St Andrews.

3.1 Comparing the mechanisms of influenza A virus and PIV5 in overcoming the IFN response

Type I interferon (IFN) constitutes the first line of defence against virus infection. IFN exert its antiviral function by induction of IFN-stimulated genes, which inhibit virus replication through a range of mechanisms. However, viruses have evolved different mechanisms to overcome the IFN response. Previous work has shown the paramyoxyvirus PIV5 can dismantle the IFN-induced antiviral state by targeting STAT1 for degradation, thus blocking IFN-signalling (Young et al., 2000b). Influenza A virus encodes the NS1 protein which antagonizes various steps of interferon induction and signalling pathways. It was of interest to compare the strategies employed by influenza A viruses (FLUAV) and PIV5, which may allow better understanding viral pathogenesis and epidemiology, and may also facilitate improved vaccine design and drug discovery.

3.1.1 Viral ability to overcome the IFN response is not absolute

Even though PIV5 and FLUAV encode powerful IFN antagonists, V and NS1 respectively; their ability to overcome the IFN response is not absolute (Carlos, Fearns, and Randall, 2005; Carlos et al., 2009). This is demonstrated by comparison of plaque size of both influenza A virus and PIV5 in A549 cells which could produce and respond to IFN is smaller compared with that in A549/BVDV-Npro cells (**Fig. 3.1A**), which can only respond to IFN but not produce IFN, because they were designed to constitutively overexpress BVDV N-terminal protease fragment (Npro), which targets IRF-3 for degradation (Hilton et al., 2006). A possible explanation for smaller plaques in naive

A549 cells is that, IFN released from cells in the developing plaque induce an antiviral state in neighboring cells, thus limiting virus spread.

Further evidence to support that influenza A virus is unable to completely block IFN production is demonstrated by double staining with anti-NS1 and anti-MxA antibody using immunofluorescence in A549 cells infected at low multiplicity with A/Udorn (MOI = 0.1pfu/cell) (**Fig. 3.1B**). MxA expression is only seen in uninfected cells surrounding those infected cells, indicating that uninfected cells responded to IFN released from infected cells and induced the expression of MxA, even though influenza A virus NS1 protein is a multifunctional protein involved in many aspects of antagonizing the IFN response.

Nevertheless, the effects on virus replication, when IFN induces an antiviral state within a cell, are not known. To address this question, PIV5 and FLUAV plaque formation was examined in cells in a pre-existing antiviral state induced by IFN. Plaque assays were performed in A549/BVDV-Npro cells that were or were not pretreated with IFN. IFN pre-treatment reduces not only FLUAV plaque sizes, but also its plaque numbers by a factor of about 100. In contrast, IFN pre-treatment significantly reduced PIV5 plaque size but not plaque numbers (**Fig. 3.1A)**. These preliminary results suggest that IFN treatment does not completely inhibit the replication of both PIV5 and FLUAV, and these viruses still manage to form plaques even through the cells are in an IFN-induced antiviral state. However, the viruses respond to IFN very differently, IFN does not prevent PIV5 from initiating plaque development, instead it slows down the spread of virus to neighboring

cells. In contrast, IFN not only significantly slows down FLUAV spread to neighboring cells, but also prevents FLUAV development from an initial focus of infection.

3.1.2 Comparison of FLUAV and PIV5 protein synthesis

To understand how PIV5 and FLUAV deal with the IFN response in detail, viral protein synthesis was examined by radioactive labelling at various time post-infection. A549 cells were, or were not, pretreated with IFN, then mock infected or infected with PIV5 or FLUAV at MOI of 5pfu/cell, and at various time post-infection, the cells were metabolically labeled with [^{35}S] methionine for one hour and the total cell lysate were prepared and protein synthesis were visualized by phospho-imaging (**Fig. 3.2**). The results from this analysis revealed that PIV5 viral proteins were efficiently expressed in cells untreated with IFN, and there was a significant overexpression of PIV5 NP protein (**Fig. 3.2A**). In cells pretreated with IFN (**Fig. 3.2B)**, the effect of IFN on different viral proteins was selective, the relative expression levels of NP, P and V proteins were similar in IFN-treated compared to non-treated cells. However, there was an obvious reduction in the relative protein levels of HN, M, and L.

In case of influenza virus, the synthesis of viral proteins in cells untreated with IFN is very rapid, and is accompanied by a dramatic inhibition of host cell protein synthesis (**Fig. 3.2C**), termed host cell shut-off. However, the synthesis of all detectable viral proteins is impaired in IFN-treated cells, and it is also evident that host cell shut-off is prevented by IFN-treatment.

3.1.3 Study of the mechanism of host-cell shut-off by Influenza A virus

Unlike PIV5, influenza virus infection of host cells leads to a progressive decline in the synthesis of cellular proteins, known as host-cell shut-off. The rapid synthesis of viral RNA and proteins requires the diversion of cellular resources away from cellular events. Several stages of host-cell mRNA translation stages are altered by influenza virus, which may lead to host-cell shut-off. It has been shown that an essential component of host cell transcriptional machinery, RNA polymerase II (pol II), was degraded in influenza virus-infected cells (Vreede et al., 2010). Previous studies have shown that the NS1 protein selectively promotes translation of viral mRNAs rather than cellular mRNAs (de la Luna et al., 1995), and it was also shown it targets cellular pre-mRNA processing and transport by interacting with CPSF30 and PABPII (Chen, Li, and Krug, 1999; Fortes, Beloso, and Ortin, 1994; Nemeroff et al., 1998), I therefore asked whether the interaction and inhibition of these factors by NS1 could lead to host-cell shut-off.

To address the question whether influenza NS1 protein is involved in shutting-off host protein synthesis, a panel of recombinant influenza A viruses generated by Dr. D. Jackson were used. These viruses contained mutations in the NS1 protein that have previously been shown to prevent interactions with dsRNA, PI3K, CPSF30 and PKR (Hale et al., 2006; Jackson et al., 2010; Nemeroff et al., 1998; Shin et al., 2007). Amino acid mutations were introduced into the NS1 protein of A/Udorn/72 by a plasmid-based reverse genetics system. The mutations introduced into NS1 and the functions inhibited by each mutant are summarized in **Table. 3.1**, and the relative position of each mutation

within NS1 is illustrated in **Fig. 3.3**. Two mutant viruses rUd-NS1-184-8(L) and rUd-NS1-103/106 were created in which the interaction of NS1 with CPSF30 is inhibited. Interestingly, a third mutant [rUd-184-8(P)] was generated during the recovery of rUd-NS1-184-8(L) virus, and these two viruses were separated by plaque purification. The rUd-NS1-123/4 mutant was created to prevent the binding of NS1 to PKR. The rUd-NS1-R38A mutant was designed to prevent NS1 binding to dsRNA. The mutant rUd-Δ99 was created that the effector domain of NS1 is deleted. Three mutant viruses were created to prevent the interaction with the p85β subunit of PI3K: (i) rUd-Y89F, (ii) rUd-NS1-P164/7A, and (iii) rUd-NS1-P162/4/7A.

The expression of cellular and viral genes in cells infected with these mutant viruses was studied. A549 cells were infected with mutant viruses, wt A/Udorn or WSN strain at a MOI of 5 pfu/cell and labelled by incorporation of [^{35}S] Met for 1 hour at 12 h post-infection. Total cell lysates were separated by SDS-PAGE, and the proteins were visualized by phosphoimaging. The synthesis of cellular proteins was significantly inhibited in cells infected by both wild type and mutant viruses. Despite the loss of NS1 functions for interactions with dsRNA, PI3K, CPSF30 and PKR, mutant viruses induced a shut-off similar to that observed after wild-type influenza virus infection, as seen by the diminished labelling and disappearance of prominent cellular bands (**Fig. 3.4**). Therefore, it was concluded that influenza virus induced host shut-off is independent of NS1 function.

3.1.4 PIV5 can dismantle the IFN-induced antiviral state

Even though the IFN response could limit virus spread to neighboring cells as demonstrated in above figures, PIV5 was still able to overcome the IFN-induced antiviral state. This was demonstrated by examining the effect of IFN pretreatment on the distribution of virus proteins in infected cells. A549 cells were, or were not pretreated with IFN for 16 h and then infected at a high MOI with PIV5. Cell monolayers were fixed at one, and two days p.i. and examined by immunofluorescence using a mixture of monoclonal antibodies against NP and P proteins **(Fig. 3.5)**. In cells untreated with IFN, NP and P proteins were distributed diffusely throughout the cytoplasm during the time course of virus infection (Panels A and B). However, virus replication is severely impaired in cells pretreated IFN at one day post infection, as NP and P proteins are seen as inclusion bodies in the majority of infected cells, although in a few cells, NP and P were distributed throughout the cytoplasm (Panel C). However, by two days p.i., the size of the inclusion bodies increased slightly, and the proportion of the cells strongly positive for virus antigen was much higher (Panel D) and the distribution of NP and P became closer to that observed in untreated cells, i.e. localized throughout the cytoplasm. Therefore, even in the continuous presence of IFN, PIV5 could still gradually overcome the inhibitory effects of IFN treatment.

3.1.5 PIV5 could overcome the IFN-induced antiviral state in the presence of neutralizing antibodies

In the above experiments, it was demonstrated that, in cells in a pre-existing IFN-induced antiviral state, PIV5 could gradually overcome the inhibitory effects of IFN. There are two explanations for this. PIV5 could initiate multiple cycles of replication with the functional attachment and fusion proteins and thus progeny viruses released from cells that were strongly positive for viral antigens could infect cells that were not strongly positive for viral antigens, or in cells that NP were seen as cytoplasmic bodies the viral genome was gradually recovered, and expressed high level of viral antigens. To address this issue, neutralizing antibody was used to prevent PIV5 virus from initiating multiple cycles of replication.

Firstly, the ability of neutralizing antibodies against F and HN proteins to prevent multiple cycles of replication of PIV5 was tested. A549 cells were inoculated at low MOI (0.1pfu/cell) of PIV5, 3 hours after virus adsoption, the inoculum was removed, cells were washed twice with PBS and then serum free DMEM was added in the presence (+antibody) or absence (-antibody) of neutralizing antibody. Cells were fixed at different times p.i. and stained with anti-NP/P antibodies (**Fig. 3.6**). In the absence of neutralizing antibody, only a few cells were positive for viral antigens at early times post infection (16 h; p.i; Panel A), but the virus managed to spread to neighboring cells, and by 48 hrs p.i, all the cells were positive for viral antigens (Panel C). In contrast, in the presence of neutralizing antibody, the virus was unable to undergo multiple cycles of replication, and

the majority of cells remained negative for viral antigens at 48 hrs p.i (Panel D). Therefore, this demonstrated that neutralizing antibody was able to block PIV5 multiple cycles of replication.

Given that neutralizing antibody could neutralize PIV5, it was of interest to then ascertain whether the ability of PIV5 to undergo multiple cycle growth accounts for the breakthrough of IFN-induced antiviral effects. A549 cells pretreated or untreated with IFN were infected with PIV5 at high MOI to ensure all cells were infected, at 3 hours p.i., neutralizing antibody was added, or not added into the medium. Cells were fixed and stained with anti-NP/P antibodies at different times p.i (**Fig. 3.7**). As a control, in the absence of neutralizing antibody, all untreated cells were positive for NP and P proteins, while in cells pretreated with IFN, NP and P were strongly positive in a minority of cells, and localized in cytoplasmic bodies in the majority of cells at one day post infection. As discussed above, PIV5 was able to overcome the IFN-induced antiviral state at late times post infection. By 3 days, all the cells were strongly positive for NP and P (Panel A). Surprisingly, even in the presence of neutralizing antibody, PIV5 was still able to overcome the IFN-induced antiviral state, at 3 days p.i., NP and P proteins were diffusely distributed throughout the cytoplasm in all cells (Panel B), indicating that the ability of PIV5 to undergo multiple cycle of replication is not responsible for dismantling the pre-existing IFN-induced antiviral state. Since PIV5 can eventually degrade STAT1 in cells that have entered an antiviral state (Didcock et al., 1999), those cells cannot maintain an antiviral state in the absence of continuous signalling. Consequently, although the PIV5 viral genome was initially relatively quiescent in cells in an IFN-induced antiviral state,

activity gradually recovered when the cells left the antiviral state, and subsequently achieved high level of antigen expression at late times post-infection.

3.1.6 Influenza virus and the IFN-induced antiviral state

Knowing that PIV5 can dismantle the pre-existing IFN-induced antiviral state, it was of interest to study whether influenza virus can overcome the antiviral state. A549 cells, untreated or treated with IFN, were infected at an MOI of 5pfu/cell to ensure all cells were infected. Cells were fixed at different times post-infection and analyzed for expression of NS1 by indirect immunofluorescence (**Fig. 3.8A**). In untreated cells, virtually all were positive for NS1 expression. By contrast, only a small fraction of IFN-treated A549 cells expressed detectable levels of NS1 protein in the cytoplasm (**Fig. 3.8A**). Even at two days post-infection, the majority of infected cells remain negative for NS1 expression, suggesting that in these cells, the influenza virus genome could not recover from the inhibitory effects of IFN treatment.

It is worthwhile to note that the inhibition, by IFN treatment, of virus replication is not 100%, and a small fraction of cells were positive for NS1 expression. It seemed very likely that this minority of cells had lost their antiviral state, and could account for the positive expression for viral antigens. To address this question, A549 cells, untreated or treated with IFN were infected with FLUAV and double stained with sheep anti-NS1 antibody and MxA monoclonal antibody (**Fig. 3.8B**). MxA is an IFN-induced antiviral molecule with a broad spectrum of antiviral activities, thus it is often used as a marker for the IFN-induced antiviral state. As seen in panel B, NS1 was strongly positive even in cells where MxA was also strongly positive. These findings clearly argue that FLUAV could overcome the antiviral state in a small percentage of cells.

3.2 Characterization of influenza A virus replication in cells in an IFN-induced antiviral state

Although the antiviral function of interferon was discovered using UV-inactivated influenza virus, the mechanism of IFN-inhibition of influenza virus replication is still elusive. The IFN-induced antiviral state is considered to result from the concerted action of a number of IFN-induced proteins. It is of particular interest to identify the stage(s) of virus replication cycle that are/is inhibited by the action of IFN and to identify the key antiviral molecules induced by IFN that are responsible for the inhibition of influenza replication.

3.2.1 IFN treatment after virus infection failed to inhibit virus replication

It has been demonstrated above that influenza A virus replication is severely impaired in IFN-pretreated cells, but it is not known at which stage of influenza virus life cycle inhibition occurs. To address this issue, A549 cells were infected at high MOI (5 pfu/cell) with influenza A virus, IFNα was added into the culture medium of infected cells one hours after infection and the cells were fixed and immunostained with anti-NS1 antibody at 8 and 24 hours post-infection (**Fig. 3.9**). In contrast to IFN-pretreatment, treatment after virus infection has no inhibitory effects on virus replication, all the infected cells showed positive staining for NS1 antigen. This result suggests that the late event of virus life cycle, such as viral protein synthesis, virion assembly and budding may not be affected. Therefore there may be a block before the expression of any viral functions, such as virus binding, endocytosis, uncoating, or transport to the nucleus. Alternatively, some early viral function, such as transcription and replication might be inhibited. Since

these events occur only at the beginning of an infection, delayed IFN treatment would be ineffective against virus infection.

3.2.2 Multiplicity-dependent leakiness of the IFN-induced inhibition of virus replication

If the early events of influenza virus life cycle are inhibited, this raised the question as to whether the IFN-induced antiviral state could be swamped by high multiplicity of infection (MOI). To answer this, monolayers of A549 cells were, or were not pretreated with IFN then infected with FLUAV at different MOI, the cells were fixed at 12h p.i. and immunostained with anti-NS1 antibody. The percentage of cells positive for NS1 antigen was calculated by counting cells positive for NS1 versus the total number of cells per field of view under the microscope. As shown in **Fig. 3.10**, the degree of inhibition does depend on the virus MOI. The number of IFN-pretreated cells which were positive for viral antigens is only about 5% at MOI of 0.5pfu/cell, the degree of protection by IFN gradually decreases with increasing MOI and the percentage of cells positive for viral antigens increased to about 35% at MOI of 50 pfu/cell (**Fig. 3.10A**). These results indicate that the inhibition of all the known early events of FLUAV can be overcome by infecting the cells at a higher multiplicity.

3.2.3 Effects of IFN treatment upon vRNP import

Previous results on the effects of IFN treatment on influenza virus replication have shown that I, IFN-mediated inhibition only occurs when treatment with IFN precedes influenza A virus infection and treatment after virus infection has no inhibitory effect and II, that the degree of protection decreases with increasing MOI. The conclusion from these results is that inhibition by IFN can be narrowed down to early event(s) in the virus life cycle. If these early events are blocked, this can explain both the requirement for pretreatment with IFN and the decreased inhibition at higher multiplicity of infection. For instance, if the cell has only a few molecules induced by IFN which can block the early events, they could be overwhelmed by high amount of input virus.

Given that FLUAV is one of the few RNA viruses that rely on the host cell nucleus for viral transcription and replication, it was of interest to study whether viral genome import into the nucleus is blocked in IFN-treated cells. To undertake this study, it was necessary to be able to monitor the fate of incoming (parental) nucleocapsids. This was accomplished in two ways: by using immunofluorescence with antibody specific for viral NP protein, and by *in situ* hybridization using Digoxigenin (DIG)-labelled probe to FLUAV genome. The accumulation of parental vRNP in infected cell nuclei was evaluated at a time before the onset of viral protein synthesis (2h after inoculation). A549 cells that were, or were not treated with IFN were inoculated with very high MOI of influenza virus (500 pfu/cell) in the presence or absence of cycloheximide (CHX) at 4ºC to allow the viruses to bind to the cells. 45 mins after inoculation, pre-warmed (37ºC) serum-free medium was added to synchronize the process of virus infection. The infected cells were fixed and immunostained at 2h p.i. with anti-NP antibody. To ensure that we

were detecting only the incoming vRNP, a control group of cells were added with protein synthesis inhibitor cycloheximide (CHX) at the time of infection. No detectable synthesis of NP protein occurred under these conditions as judged by radioactive labelling of proteins (data not shown), so that the only NP protein detected in the infected cells was that associated with parental viral nucleocapsids. In cells not treated with IFN, viral NP proteins were readily detected in the nuclei. However, only a few cells showed nuclear staining of NP protein in cells pretreated with IFN (**Fig.3.11a**).

It was not clear where the viral nucleocapsids were localized in those cells pretreated with IFN but without nuclear staining of NP. To address this issue, *in situ* hybridization, using ssRNA probes specific for sequences within the FLUAV segment 8 gene, was used to detect negative-sense viral RNA genome. The procedure for infection was basically the same as carried out above. In the absence of IFN treatment, virus genomes were readily seen inside the nuclei (Panel A). As expected, in cells pretreated with IFN, very few cells showed nuclear staining of FLUAV vRNA. In the majority of cells, vRNA was localized outside the nucleus at the perinuclear region (Panel B).

Taken together, by immunofluorescence it was shown that parental NP protein was prevented from entering the cell nucleus in IFN-pretreated cells, and by *in situ* hybridization it was also shown that negative-sense viral genomes were localized at the perinuclear region and prevented from entering the nucleus in IFN-pretreated cells, thus IFN-pretreatment must inhibits at the stage of nuclear import of viral genome.

3.2.4 Effect of MxA knockdown upon virus genome import

Previous reports have shown that MxA is an important antiviral molecule induced by IFN which inhibits replication of a number of DNA and RNA viruses. In particular, MxA has been shown to block the replication of La Crosse virus, hantaviruses, Dugbe virus, and Crimean-Congo hemorrhagic fever virus (CCHFV) by interacting with the viral nucleocapsid proteins. It has also been shown that MxA targets influenza virus NP protein in minireplicon systems, and that over-expression of NP effectively rescues viral transcriptional activity from the MxA inhibition (Turan et al., 2004). In light of the observations above, that IFN-treatment inhibits FLUAV viral genome import, it was of interest to study whether MxA plays a major role in blocking influenza viral genome import within cells in an IFN-induced antiviral state. To address this question, an MxA-knockdown cell line was generated using a lentivirus-based shRNA system. The procedures for generation of MxA-knockdown cells are described in detail in **Materials and Methods**.

Western blot analysis and immonofluorescence were used to determine whether MxA was efficiently depleted in cells transduced with lentivirus expressing shRNA against MxA (**Fig. 3.12**). Naïve A549 cells and MxA-knockdown cells were treated with 1000 u/ml IFNα. 16 h later, cells were fixed and immunostained with monoclonal anti-MxA antibody or lysed and subjected to SDS-PAGE and western blot analysis. As shown in Panel A, by western blot analysis MxA expression was seen only in IFNα-treated naïve A549 cells but was not detectable in cells not treated with IFNα. In contrast, MxA was not detectable in lentivirus-transduced MxA-knockdown cells (shMxA) even if the cells were treated with IFNα. As a control, the expression of STAT1 was upregulated in both

naïve A549 cells and MxA-knockdown cells following IFN treatment. By immunofluorescence analysis MxA was strongly expressed in the cytoplasm in naïve A549 cells treated with IFN, but it was undetectable in cells not treated with IFN (Panel B). In contrast, in MxA-knockdown cells, MxA was down regulated in almost 100% of the cell population even after treatment with IFN, although a few cells showed weak staining of MxA.

To follow the fate of influenza viral nucleocapsids in cells in a pre-existing IFN-induced antiviral state in the absence of MxA expression, control cells and shMxA cells that were, or were not treated with IFN were infected at very high multiplicity with influenza virus (500pfu/cell). To selectively stain incoming nucleocapsids, cells were incubated in the presence of CHX to block *de novo* viral protein synthesis. **Fig. 3.13** showed that in control cells in the absence of IFN treatment, NP almost completely moved into the nucleus of control cells at 2 hour post-infection, whilst in the presence of IFN treatment, there was no obvious nuclear staining of NP. In contrast, irrespective the presence or absence of IFN-treatment in shMxA cells, the viral nucleocapsids were efficiently imported into the nucleus, suggesting that MxA plays an essential role in blocking viral genome import in IFN-treated A549 cells.

3.2.5 Effects of MxA over-expression upon virus genome import

Previous studies have shown that avian strain of influenza virus (FPV-B) replication was severely impaired in murine 3T3 cells over-expressing MxA, and it was suggested that MxA inhibits a poorly defined step after primary transcription (Pavlovic, Haller, and Staeheli, 1992). To demonstrate the role of MxA, MxA-over-expression cell line was generated by transducing A549 cells with lentivirus encoding human MxA and subsequently selected in the presence of puromycin. The procedure for generation of MxA-over-expression cell line is described in details in **Materials and Methods**. Over-expression of MxA in A549 cells was tested by western blot and immunofluorescence (**Fig. 3.14**). By western blot analysis, in control A549 cells, MxA is only expressed in cells treated with IFN, whilst in A549 cells over-expressing MxA (A549-MxA), MxA was shown to be strongly positive even in the absence of IFNα treatment (Panel A). By immunofluorescence analysis, MxA accumulated in the cytoplasm of A549-MxA cells and showed a granular staining pattern similar to MxA pattern observed in that of A549 cells treated with human IFNα (Panel B). It was clear that MxA was expressed in more than 95% of the total cell population as judged by immunofluorescence.

The fate of incoming nucleocapsids in MxA-over-expressing cells was also investigated (**Fig. 3.15**). The procedure was basically the same as previously described. Nucleocapsids moved into the nucleus of naive A549 cells, leaving the cytoplasm virtually clear at 2 hours post-infection. In A549-MxA cells, the transport of parental nucleocapsids into the nucleus was similar to control cells, suggesting that MxA over-expression alone is insufficient to block incoming genome import.

3.2.6 Effects of introducing MxA back into MxA-knockdown cells upon virus genome import

It has been demonstrated above that without IFN pretreatment, over-expression of MxA in naïve A549 cells does not block FLUAV genome import. Two possible explainations for the observations in the above experiment are, firstly, that exogenously expressed MxA (by lentivirus transduction) is nonfunctional, or secondly, that whilst MxA is required to block the nuclear import of FLUAV genome. To do so, it requires additional auxiliary molecules induced by IFN. In IFN-pretreated A549-MxA cells, the MxA population constitutes the endogenously expressed IFN-induced MxA and exogenously expressed lentivirus-transduced MxA. To determine whether exogenously expressed MxA was nonfunctional, "wobble" MxA (kindly provided by Prof. Peter Staeheli) was introduced into the MxA-knockdown cells (shMxA). The amino acid sequence of the wobble MxA gene is identical to endogenous MxA. However, no shRNA-complementary sequences are present due to the introduction of synonymous substitutions. As a consequence, even after IFN-treatment of these cells, only the "wobble" MxA would be expressed. If the expression of the wobble MxA restored the ability to block the nuclear import of FLUAV genome in the shMxA cells, it would demonstrate that whilst MxA is essential in blocking viral genome nuclear entry, additional IFN-induced factors are required for its functionality.

Briefly, wobble MxA was cloned into lentivirus backbone vector, then MxA-knockdown cells were transduced with lentivirus encoding wobble MxA and subsequently selected in

the presence of puromycin. Immunofluorescence analysis showed that MxA was strongly expressed in the cytoplasm in naïve A549 cells treated with IFN, but was not detected in A549 cells that had not been treated with IFN. As previously shown, MxA was not detected in shMxA cells regardless of whether the cells had or had not treated with IFN. In contrast, in shMxA/wMxA cells, MxA was detected in the cytoplasm of a proportion of shMxA cells and showed a granular staining pattern similar to the pattern observed in A549 cells treated with IFN (**Fig. 3.16**). Therefore, wobble MxA was successfully expressed in shMxA cells even though the cells constituively express shRNA against endogenous MxA.

The fate of incoming nucleocapsids in shMxA/wMxA cells was then investigated. The procedure was basically the same as previously described. As seen in **Fig. 3.17B**, in naïve A549 cells, in the absence of IFN treatment, NP almost completely moved into the nucleus at 2 hour p.i., whilst in the presence of IFN treatment, there was no obvious nuclear staining of NP, and NP was seen as cytoplasmic bodies inside the cytoplasm. In MxA-knockdown cells (shMxA), irrespective of the presence or absence of IFN-treatment, NP was efficiently imported into the nucleus (Panel C). In contrast, in shMxA/wMxA cells, in the absence of IFN treatment, NP was imported into the nucleus even if the cells are positive for MxA expression. However, in the presence of IFN treatment, NP nuclear import was blocked (Panel D). This result showed that the ability of IFN to block the nuclear import of influenza virus genome could be restored by introducing MxA back into MxA knockdown cells.

Taken together, these experiments showed that influenza viral genomes were efficiently imported into the nucleus in cells in a pre-existing antiviral state in the absence of MxA

expression. However, over-expression of MxA alone is insufficient to account for the observed blockage of genome import, implying that there must be other molecules induced by IFN which function together with MxA to restrict genome import, or that MxA function requires modification by IFN.

3.2.7 Effects of MxA in plaque reduction assay

It has been demonstrated above that MxA is an essential molecule in blocking viral genome import in cells in a pre-existing IFN-induced anti-viral state, but failed to confer resistance of viral genome import in stable cell lines by following the fate of incoming parental nucleocapsids using immunofluorescence and *in situ* hybridization. These studies also suggested that there are other ISGs which act in concert with MxA to block viral genome import. Plaque reduction assays were performed to confirm the role of MxA in blocking FLUAV genome import, in addition, and to identify potential ISGs in modulating MxA activities.

Naïve A549 cells, MxA-knockdown cells and MxA-over-expressing cells, pre-treated or left untreated with IFN, were infected with 10-fold serial dilutions of influenza A viruses, after virus attachment to cells, unbound virus was removed and monolayers of cells were overlaid with agarose. Virus plaques were allowed to develop for 4 days, and plaque numbers were counted (**Fig. 3.18**). The number of plaques was reduced by 100-fold in A549 cells pretreated with IFN compared with untreated cells. Reduction in plaque numbers were partially relieved in MxA-knockdown cells pretreated with IFN, suggesting that MxA is an important antiviral molecule in the IFN-induced antiviral state. However, there is still about a 10-fold reduction in plaque numbers, suggesting the existence of other molecules induced by IFN that contribute to the IFN-induced antiviral state. Immunofluorescence studies above have shown that there is no restriction in blocking viral genome import in MxA-expressing A549 cells, this is also supported by plaque reduction assays. There was only a slight reduction in plaque numbers in MxA-expressing A549 cells compared to that in naïve A549 cells, suggesting that over-

expression of MxA alone is insufficient to confer resistance to influenza virus in stable cell lines. However, surprisingly, IFN treatment proved to be more effective in MxA-expressing A549 cells than naïve A549 cells, reduction in plaque numbers following IFN-treatment were 1000-fold and 100-fold respectively, suggesting that stable expression of exogenous MxA in A549 cells enhances IFN-mediated resistance against influenza virus replication. Furthermore, this also points out a role of IFN treatment in enhancing MxA antiviral activity either by the modification of MxA itself, or modulation by other molecules induced by IFN.

Overall, from the observations presented above, it is clear that MxA is essential but is not the only molecule in IFN-mediated antiviral resistance, and its activity strongly depends on IFN treatment.

3.3 Identification of potential ISGs involved in the establishment of IFN-induced antiviral state

Over-expression and small interfering RNA (siRNA)-mediated knockdown of ISG expression are two common approaches to identify ISGs involved in the establishment of an antiviral state by IFNs. It has been shown that the activity of MxA against FLUAV depends on IFN treatment, suggesting that there are other auxiliary ISGs induced by IFN which act in concert with MxA to bring about its full functionality. It will be important to identify those molecules essential for MxA function. I aimed to clone ISGs and over-express them either alone or in combination with MxA, but because of the time limit, I did not have enough time to screen all the ISGs.

3.3.1 Effects of viperin in plaque reduction assay

Viperin was recently reported to be an important antiviral molecule and showed a broad spectrum against viruses. In particular, viperin has been reported to inhibit influenza virus release by perturbing lipid-rafts (Wang, Hinson, and Cresswell, 2007). The lentivirus system was used to generate A549 cell lines that stably express N-terminal Pk-tagged viperin by selection with blasticidin. The viperin gene was cloned with specific primers by RT-PCR using total RNA extracted from A549 cells treated with IFNα. As seen in **Fig. 3.19**, over-expression of viperin was able to reduce plaque numbers by 10-fold compared to naïve A549 cells. However, IFN treatment of viperin-expressing A549 cells and naïve A549 cells, compared with untreated cells, resulted in reduction in plaque

numbers both around 100-fold, suggesting that IFN treatment does not enhance the antiviral function of viperin. These results clearly suggest that viperin is an important molecule in establishing the antiviral state induced by IFN.

3.3.2 Role of ISG56 in plaque reduction assay

In search of the ISGs that are able to inhibit FLUAV infection and potentially modulate MxA function, my colleague Dr. Lena Andrejeva was also looking for ISGs that are able to inhibit PIV5. She identified ISG56 was an important antiviral molecule against PIV5 infection. Thus her cell line was used to investigate whether ISG56 also has antiviral activity against FLUAV. The role of ISG56 upon PIV5 was demonstrated in **Fig. 3.20**, PIV5 only formed pinpoint plaques in Hep2 cells, which could respond and produce IFN, whilst the plaque size was significantly bigger in Hep2/Npro cells, that cannot produce IFN because they constitutively express BVDV-Npro, which targets IRF-3 for degradation. In Hep2 cells with ISG56 knockdown mediated by lentivirus expressing shRNA, the plaque size was in between that in Hep2 and Hep2/Npro cells (Panel A).

To examine whether ISG56 has antiviral activity against influenza A virus, plaque development was observed in naïve A549 cells and A549 cells with ISG56 knockdown. As seen in Panel B, FLUAV plaque size and numbers were comparable in naïve A549 cells and ISG56 either pretreated with IFN or left untreated, suggesting that ISG56 is not involved in inhibiting FLUAV replication in IFN-induced antiviral state.

3.3.3 Effects of ISGylation on IFN-mediated plaque reduction

ISG15 is an IFN-induced antiviral molecule and mediates the ISGylation of hundreds of proteins in IFN-treated cells. It has also been reported that a few IFN-induced molecules, RIG-I, PKR and MxA are targeted for ISGylation (Zhao et al., 2005). To understand the role of ISGylation in the IFN-induced antiviral state, the knockdown approach was used to generate cell lines expressing shRNA against the E3 ligase, Herc5, a component of the ISGylation pathway. An NS1B-over-expressing cell line was also genenated, as it has been reported influenza B virus NS1 protein (NS1B) inhibits ISGylation. These cell lines, treated or left untreated with IFNα, were infected with FLUAV, and virus plaques were allowed to develop for 4 days. As seen in **Fig. 3.21**, in NS1B-over-expressing cells, the size of viral plaques were significantly larger, although there was no increase in plaque numbers compared to naïve A549 cells. This suggests NS1B over-expression could facilitate FLUAV spread to neighbouring cells. However, it was not clear whether the inhibition of ISGylation by NS1B accounts for the increase in plaque size. In IFN-treated Herc5 knock down cells (shHerc5), there was a slight reduction in plaque numbers compared to IFN-treated naïve A549 cells. This result is perplexing, and it is in conflict with previous publications that ISGylation negatively regulates FLUAV replication (Tang et al., 2010). Thus further studies need to be carried out to understand the role of ISGylation in cells in a pre-existing IFN-induced antiviral state.

3.3.4 IFNγ does not have a significant effect on viral plaque reduction

Both type I and types II IFNs activate antiviral defence mechanisms by inducing the expression of antiviral genes. Although the signalling pathways and genes activated by IFN-α/β and IFN-γ partially overlap, there are genes selectively regulated by distinct IFNs (Der et al., 1998). It has been shown that IFN-α and IFN-γ reduce Hantaan virus replication with same efficiency (Oelschlegel, Kruger, and Rang, 2007), thus it was of interest to compare the consequences of addition of each of the two types of IFN on influenza virus plaque formation to see whether IFN-γ reduces plaque numbers to same extent as IFN-α.

Monolayers of A549 cells were treated with 1000 u/ml IFN-α or IFN-γ for different times before inoculation with influenza virus, after adsoption, cell monolayers were washed with PBS and agarose overlay medium were added onto the cells to allow the plaques to grow. Cells were fixed and immunostaining with polyclonal antibody against influenza virus, and plaque numbers were counted. As shown in **Fig. 3.21**, IFN-γ, in contrast to IFN-α, did not significantly reduce plaque numbers. Rather, the plaque numbers in IFN-γ treated cells was very similar to that observed in untreated cells. It has been reported that no MxA protein and only minimal amount of MxA mRNA were detected in IFN-γ-treated cells (Andersson et al., 2006), thus the difference seen with IFN-α and IFN-γ-treated cells suggests that only those ISGs induced by type I IFN are responsible for the observed inhibition, and this correlation might be useful to identify potential effectors responsible for the IFN-induced block of influenza virus.

Discussions

Experimental data presented in this thesis revealed important observations that have contributed to understanding how PIV5 and FLUAV interact with the IFN system. Moreover, these findings have contributed to elucidating the mechanism by which IFN inhibits influenza virus replication.

4.1 Effect of interferon on the replication of PIV5

Studies on PIV5 replication in cells in a pre-exisiting IFN-induced antiviral state showed that IFN treatment had at least two effects on viral proteins. First, experimental data from the analysis of virus proteins isolated by immunoprecipitation from infected cells showed that the relative expression levels of NP, P, and V proteins were not significantly different between IFN-treated and untreated cells. However, IFN-treatment did cause an obvious reduction in the relative levels of M, HN, and L proteins (Figure 3.2, Panel B). These results suggest that IFN causes a specific down-regulation in the expression of genes that are downstream of the V/P gene (Carlos, Fearns, and Randall, 2005). Second, results presented in this thesis also showed that IFN alters the intracellular distribution of the viral proteins synthesized. In cells pretreated with IFN, the NP and P proteins rapidly became localized in cytoplasmic inclusion bodies, whilst in the absence of IFN treatment, the majority of NP and P proteins were more evenly distributed throughout the cytoplasm (**Fig. 3.5**). Therefore, PIV5 virus replication is severely impaired within cells in a pre-existing IFN-induced antiviral state.

4.2 Comparison of the mechanisms of PIV5 and FLUAV in dealing with the IFN response

The co-evolution of viruses with their hosts has resulted in diverse mechanisms, employed by viruses, to evade the host anti-viral immune system. Viruses infection of *in vivo* system results in the production of large amounts of IFN to mount an effective defence against viral replication. As a result, viruses will ultimately encounter cells that are already in an IFN-induced antiviral state at some stage during an ongoing infection. In this thesis, it has been shown that PIV5 and FLUAV have evolved strikingly different strategies in dealing with cells in a pre-existing IFN-induced antiviral state. Although the IFN response significantly slows down PIV5 spread to neighbouring cells [**Fig. 3.1A**, see also (Young et al., 2003)], PIV5 manages to limit the amount of IFN induction, as well as to block IFN signalling by targeting STAT1 for proteasome-mediated degradation (Andrejeva et al., 2004; Didcock et al., 1999). However, to survive, PIV5 must maintain its genome in a functional state within cells in an IFN-induced antiviral state until those cells exit the antiviral state. This is corroborated by plaque reduction assays, which showed that IFN pretreatment did not significantly reduce PIV5 plaque numbers, but rather reduce plaque size (**Fig. 3.1**). Our working model is that upon infection of cells in an IFN-induced antiviral state, although virus replication is initially severely impaired, PIV5 targets STAT1 for proteasome-mediated degradation. Without continuous IFN signalling, the cells cannot maintain their antiviral state indefinitely. When the cell exits the antiviral state, normal virus replication is restored and a diffuse cytoplasmic distribution of the nucleocapsid and P proteins is observed [**Fig. 3.5**, see also (Carlos et al., 2009)]. However, the virus has to wait for 24-48 hours to dismantle the pre-existing

antiviral state, thus the plaque size in IFN-pretreated cells is significantly smaller compared to untreated cells.

FLUAV also employs mechanisms to antagonize the IFN response. The expression of IFN-induced antiviral molecules is blocked in virus-infected cells (**Fig. 3.1B**). However, FLUAV replication is severely impaired within cells, which are in a pre-existing IFN-induced anti-viral state. Thus by plaque reduction assays, it was shown that there was a significant reduction in plaque numbers in IFN-pretreated cells, however, the plaque size was comparable regardless whether the cells were or were not pretreated with IFN (**Fig. 3.1A**). Thus it is reasonable to postulate that IFN pretreatment of naïve A549 cells inhibits FLUAV by preventing that one infectious virus particle from developing into a plaque at the initial foci of infection, so that only around 1% of the virus population is able to initiate productive infection in cells in a pre-existing IFN-induced antiviral state. Once virus replication is established in infected cells, a large number of progeny viruses can be produced in a short time and they are able to infect neighbouring uninfected cells by swamping the pre-existing IFN-induced antiviral state (**Fig. 3.10**). As a result, the size of plaques developed is compable regardless whether the cells were, or were not, pretreated with IFN (**Fig. 3.1A**). By immunofluorescence, it was shown that in the majority of infected cells in a pre-existing antiviral state, FLUAV failed to establish a productive infection (**Fig. 3.8**), and in those cells that are negative for viral antigens, the virus was unable to resume normal replication even if IFN was washed away and the infection was allowed to proceed. This led onto an investigation into the fate of FLUAV genomes within cells in an IFN-induced antiviral state.

4.3 Influenza A virus shut-off of host protein synthesis

Currently, there is no consensus on the strategies utilized by influenza viruses to shut-off host protein synthesis. Beloso et al reported that a role of cellular mRNA degradation during infection maybe responsible for protein synthesis shut-off (Beloso et al., 1992). NS1 is a multifunctional protein has been implicated in a number of functions related to regulation of gene expression in infected cells. Some of these functions, such as inhibition of cellular pre-mRNA processing and transport, by NS1 interacting with CPSF30 and PABPII, would result in inhibition of gene expression and possibily in shut-off host protein synthesis (Alonso-Caplen et al., 1992; Fortes, Beloso, and Ortin, 1994; Lu, Qian, and Krug, 1994; Nemeroff et al., 1998; Qian, Alonso-Caplen, and Krug, 1994; Qiu and Krug, 1994).

Various mutant influenza A viruses with corresponding loss of function in their NS1 genes have been used to study the effects of the NS1 protein on host protein expression during influenza A virus infection. Therefore, the overall levels of cellular protein synthesis was examined in cells infected with wild-type virus and mutant viruses loss the ability to interact with CPSF30 or PKR, to bind to dsRNA or to activate PI3K. Results from radioactive host cell labelling of proteins showed no major differences in cellular protein synthesis were found among these viruses (**Fig. 3.4**), the conclusion being that the NS1 protein does not play a major role in the shutoff of host protein synthesis. Clearly, some other viral protein plays a more crucial role than NS1 in the shutoff of cell protein synthesis in influenza virus-infected cells. Recently it has been reported host RNA polymerase II (Pol II), a multiprotein complex composed of 12 subunits responsible for the transcription of mRNAs, is targeted for degradation by influenza viral polymerase

(Rodriguez, Perez-Gonzalez, and Nieto, 2007; Vreede et al., 2010). Therefore it has been proposed that the viral RNA polymerase-mediated inhibition of Pol II plays an important role in inhibiting host gene expression and may contribute to the shut-off of host protein synthesis.

4.4 Stages of influenza A virus replication blocked by IFN

Influenza virus replication is highly sensitive to the antiviral action of IFN. The precise step of influenza virus life cycle that is inhibited in IFN-treated cells has been difficult to establish and to date, there is no consensus regarding which stage of influenza virus replication is inhibited. It has been reported that inhibition occurs at the level of primary transcription (Bean and Simpson, 1973; Ransohoff et al., 1985), however, Repick et al suggested that inhibition of viral replication occurs at a stage between primary transcription and secondary transcription (Repik, Flamand, and Bishop, 1974). Data presented here demonstrate that type I IFN triggers efficient inhibition of influenza virus replication. This inhibition appears to be at the stage of nuclear import of viral RNPs, since the nuclear translocation viral RNPs and subsequent synthesis of primary transcripts as well as viral proteins translation are absolutely essential virus replication. Subsequently, studies of FLUAV replication in cells in which MxA expression was knocked down clearly indicate that MxA is an essential component induced by IFN in blocking viral genome import, as the IFN-mediated inhibition is aborted in MxA-knockdown cells.

4.5 MxA recognition of NP protein

Exactly how MxA prevents nuclear import of viral RNPs is not clear, but it is conceivable that MxA recognizes influenza virus nucleocapsids and prevents their transport through the cells. Previous studies have shown that the human MxA protein blocks the genome import of Thogoto virus, an influenza virus-like orthomyxovirus, by recognizing the viral nucleocapsids (Kochs and Haller, 1999b). It was also reported that MxA targets influenza virus NP protein in minireplicon systems. Over-expression of NP effectively rescues viral transcriptional activity from the MxA inhibition (Turan et al., 2004).

Not only was MxA reported to target nucleocapsids of orthomyxovirus, it also targets bunyavirus N protein. MxA was found to recognize newly synthesized viral N protein and forms a complex in the perinuclear region, leading to a depletion of N protein, thus blocking virus genome amplification.

Influenza virus NP is associated with the viral genome, and together with viral polymerases, forms the nucleocapsid. However, there is no solid evidence that MxA directly associates with influenza virus NP protein, the interaction was only demonstrated in cross-linking conditions (Turan et al., 2004). Nevertheless, how exactly MxA recognizes viral RNPs and prevents their nuclear translocation is not clear. It is thought that MxA can self-assemble into ring-structures and recognizes the multimeric lattice of NP in the vRNPs and wraps around them to cover the nuclear import signals that are

normally exposed. Alternatively, MxA may direct vRNPs to other places in the cytoplasm where they are subsequently degraded.

4.6 Cell type and virus strain specificity of the antiviral function of MxA

The antiviral function of MxA appears to be rather complicated. Previous studies have shown that MxA inhibits a poorly defined step after primary transcription, this was demonstrated by infecting murine 3T3 cells stably over-expressing MxA with fowl plague virus (FPV-B) (Pavlovic, Haller, and Staeheli, 1992). Results presented here, from *in situ* hybridization and immunofluorescence in A549 cells infected with H3N2 viruses, suggest that MxA is an essential molecule induced by IFN in blocking influenza virus genome import. The difference is difficult to reconcile, but it could have been due to the different cell types used. Over-expression of MxA in Vero cells appears to be more effective in reducing influenza virus plaque numbers than in A549 cells (data not shown). Circumstantial evidence indicates that the cell type-specific antiviral function of MxA is not unique to influenza viruses. Previous studies which showed that Measles virus transcription is inhibited only in certain human cell lines (Schneider-Schaulies et al., 1994; Schnorr et al., 1993), strongly support the idea of the existence of auxiliary cellular factors that modulate the antiviral activities of MxA. Results presented here, from studies using MxA over-expressing cells, are in agreement with previous reports. As shown in the results section, constitutive expression of MxA in naïve A549 cells or introducing wobble MxA back into MxA knockdown cells is insufficient to block nuclear import of influenza virus RNPs (**Fig. 3.15** and **Fig. 3.17D**), but wobble MxA was able to restore the

ability of IFN to block the nuclear import of FLUAV genome in the shMxA cells, suggesting that other molecules induced by IFN are required for MxA function. Thus, it may be that MxA recognition of NP is facilitated by one or more auxiliary proteins. It is very likely that IFN-induced molecules bridge the interaction of MxA with NP, or rather, IFN-induced modification of MxA may modulate its antiviral activity. Previous studies have shown that MxA is ISGylated (Dastur et al., 2006; Zhao et al., 2005), and it has also been showed that ISG15 and/or its conjugates play an important role in protecting cells from infection by several viruses. However, the mechanism by which ISGylation exerts antiviral activity has not been established. It is plausible that by targeting MxA, ISG15 increases its stability or induces conformational change to form lattice strucrture for interaction with other proteins.

The difference of antiviral functions of MxA seen may be a consequence of differences in sensitivity to MxA between strains of influenza A viruses. Previous studies have shown that avian FPV-B virus replication in MxA over-expressing 3T3 cells was significantly reduced (Pavlovic et al., 1990b). It was also reported that human H1N1 (1918) virus-based mini-replicon system was almost insensitive to inhibition by MxA, whilst the avian H5N1 virus was significantly inhibited (Dittmann et al., 2008).

4.7 Future prospects

MxA belongs to the dymamin superfamily of GTPases that is involved in endocytosis and vesicle trafficking between intracellular membrane compartments. Mx GTPases have

a relatively low affinity for GTP and a intrinsic high rate of GTP hydrolysis. They consists of an N-terminal GTPase domain, the central interactive domain (CID), and a C-terminal GTPase effector domain (GED) (Haller, Stertz, and Kochs, 2007). The CID and GED were proposed to form a stalk which mediates oligomerization and self-assembly. Recently, the crystal structure of the stalk region has been resolved (Gao et al., 2010), and it was shown MxA assembles in a criss-cross pattern to form higher-order structures. This provides new insights into the structural basis for MxA antiviral functions, thus it will be of great interest to unravel the precise mechanism of MxA antiviral action by mapping the domain(s) that mediate(s) the interaction of MxA with viral nucleocapsid protein. Currently, I am in the process of generating the Pk-tagged MxA aimed at finding the cellular factors that are associated with MxA by immunoprecipitations.

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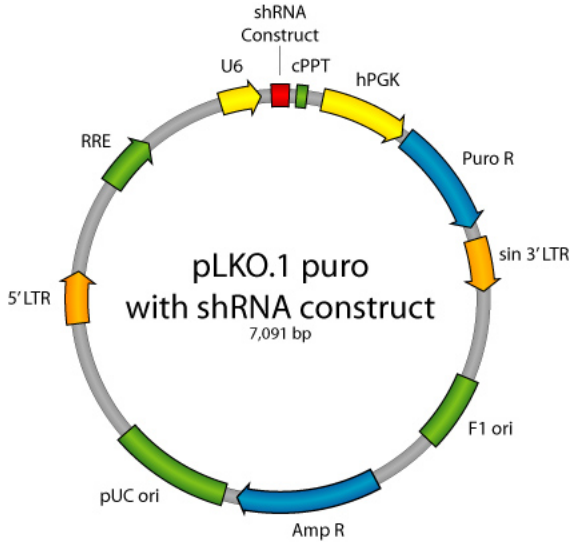
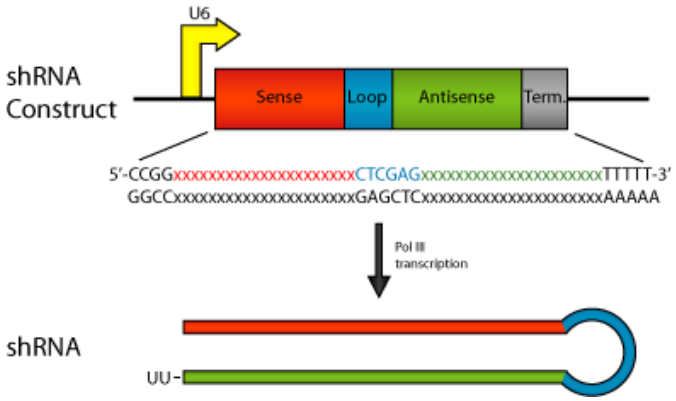
A.1 Plasmid map of the pLKO.1 with shRNA construct



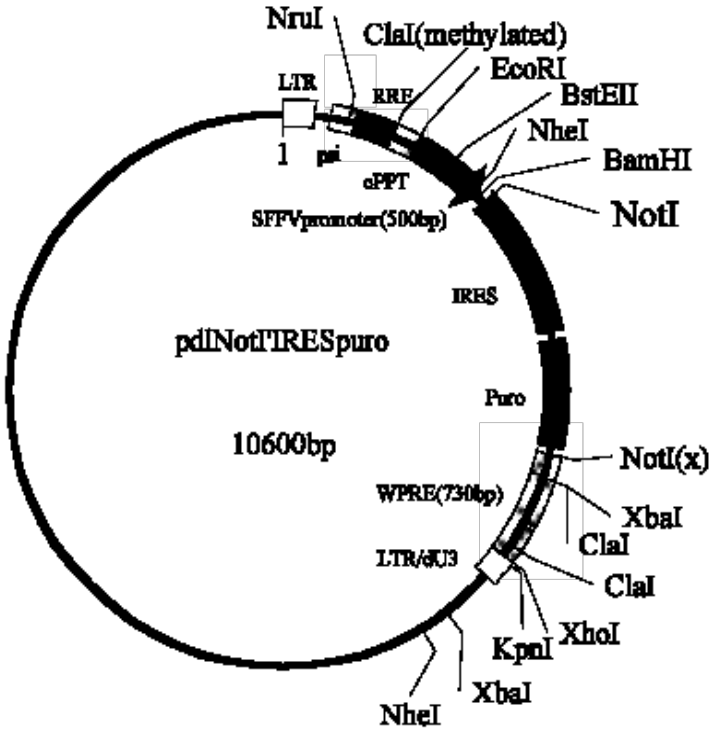
pLKO.1 is a cloning vector of shRNA. It contains the necessary *cis* elements for packaging, reverse transcription, and integration for subsequent production of the lentivirus particles.

Description	Vector Element
U6	Human U6 promoter drives RNA Polymerase III transcription for generation of shRNA transcripts.
cPPT	Central polypurine tract, cPPT, improves transduction efficiency by facilitating nuclear import of the vector's preintegration complex in the transduced cells.
hPGK	Human phosphoglycerate kinase promoter drives expression of puromycin.
Puro R	Puromycin resistance gene for selection of pLKO.1 plasmid in mammalian cells.
sin 3'LTR	3' Self-inactivating long terminal repeat.
f1 ori	f1 bacterial origin of replication.
Amp R	Ampicillin resistance gene for selection of pLKO.1 plasmid in bacterial cells
pUC ori	pUC bacterial origin of replication.
5'LTR	5' long terminal repeat.
RRE	Rev response element.



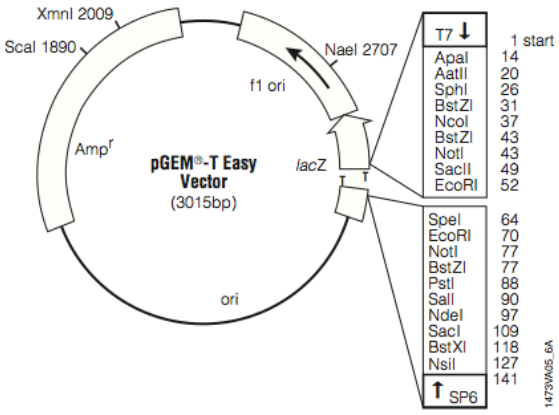
Detail of shRNA insert. The U6 promoter directs RNA Polymerase III transcription of the shRNA. The shRNA contains 21 "sense" bases that are identical to the target gene, a loop containing an XhoI restriction site, and 21 "antisense" bases that are complementary to the "sense" bases. The shRNA is followed by a polyT termination sequence for RNA Polymerase III.

A.2 Plasmid map of the pdlNotI'IRESpuro vector



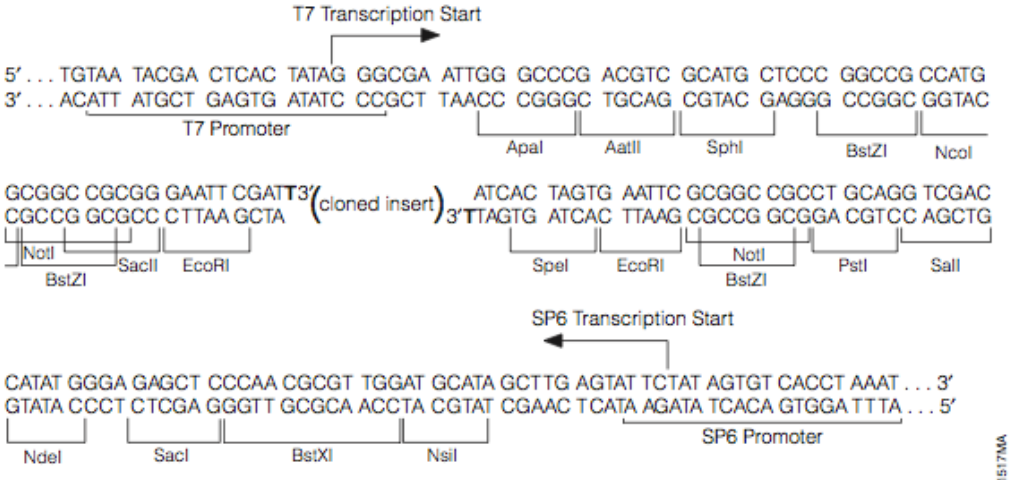
Plasmid generated by Dr. Yun-Hsiang Chen, University of St Andrews, UK.

A.3 pGEM-T Easy Vector Map and sequence reference points



pGEM®-T Easy Vector sequence reference points:

T7 RNA polymerase transcription initiation site	1
multiple cloning region	10–128
SP6 RNA polymerase promoter (–17 to +3)	139–158
SP6 RNA polymerase transcription initiation site	141
pUC/M13 Reverse Sequencing Primer binding site	176–197
lacZ start codon	180
lac operator	200–216
β-lactamase coding region	1337–2197
phage f1 region	2380–2835
lac operon sequences	2836–2996, 166–395
pUC/M13 Forward Sequencing Primer binding site	2949–2972
T7 RNA polymerase promoter (–17 to +3)	2999–3



The promoter and multiple cloning sequence of the pGEM-T easy vector. The top strand corresponds to the RNA synthesized by T7 RNA polymerase. The bottom strand corresponds to the RNA synthesized by SP6 RNA polymerase.

Virus	Mutation	Comments
rUd-NS1-R38A	R38A	Prevents binding of dsRNA to NS1 thereby preventing activation of 2'-5'OAS. Also known to be important in the co-precipitation of NS1 and RIG-I (Min and Krug, 2006; Wang et al., 1999).
rUd-NS1-123/4	I123A / M124A	Prevents binding of NS1 to PKR (Min et al., 2007).
rUd-NS1-103/106	F103L / M106I	Destabilisation of the NS1-CPSF30 interaction.
rUd-NS1-184-8(L)	Residues 184-188 GLEWN → RF**L**RY (L28F/N29S in NS2)	Prevents interaction of NS1 and CPSF30 (Noah, Twu, and Krug, 2003).
rUd-NS1-184-8(P)	Residues 184-188 GLEWN → RF**P**RY (L28F/N29Q in NS2)	Recovered with rUd-NS1-ΔCPSF-RFLRY as a mixed virus population.
rUd-NS1-Y89F	Y89F in Udorn background	Prevents interaction of NS1 with PI3-K (Hale et al., 2006).
rUd-NS1-P164/7A	P162A, P164A	Double proline mutant Shin *et al*. (2007). Reported to prevent the interaction of NS1 with PI3-K.
rUd-NS1-P162/4/7A	P162A, P164A, P167A	Triple proline mutant Shin *et al*. (2007) (although they reported it as a double mutant their sequence is a triple mutation). Causes increased apoptosis.
rUd-NS1-Δ99	138 amino acid C-terminal truncation of NS1 due to stop codon at residue 99	Recovered with rUd-NS1-P162/4/7A as a mixed virus population.

Table 3.1. Amino acid mutations introduced into the A/Udorn NS1 protein of mutant viruses.

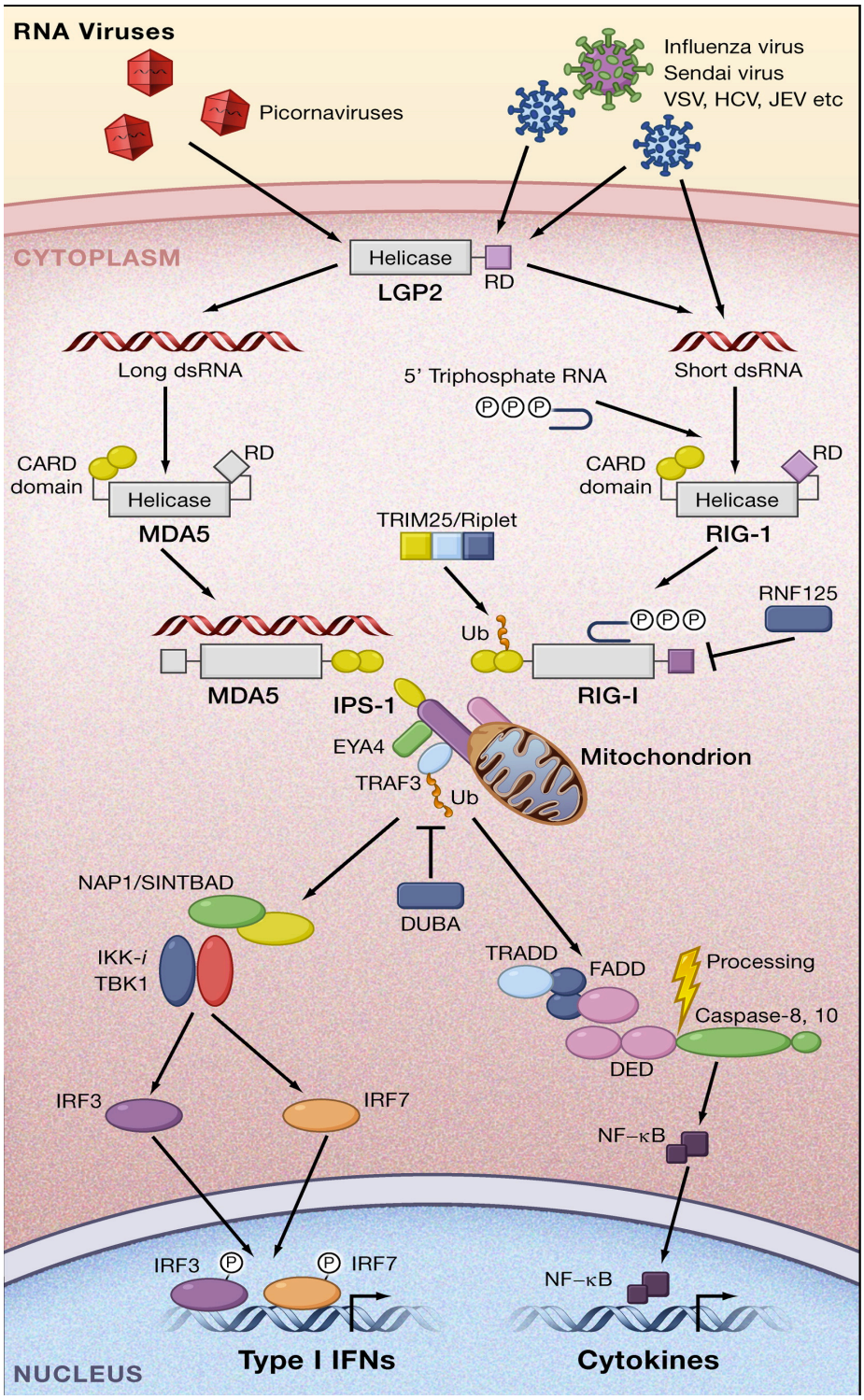


Fig. 1.1. Recognition of viral PAMPs by cellular sensors. RIG-I recognize different RNA viruses by detecting short dsRNAs and ssRNAs with 5'-triphosphate ends, whilst MDA-5 recoginizes long dsRNAs. LGP2 functions as a positive regulator in RIG-I and MDA5-mediated virus recognition. Activation of RIG-I is positively and negatively regulated by ubiquitin ligases TRIM25 and RNF125, respectively. RIG-I and MDA5 interact with a common downstream signalling molecule, MAVS through homophilic interactions between CARD domains. MAVS then activates signaling cascades leading to the expression of type I IFN genes (*diagram adapted from Takeuchi & Akira, 2010*).

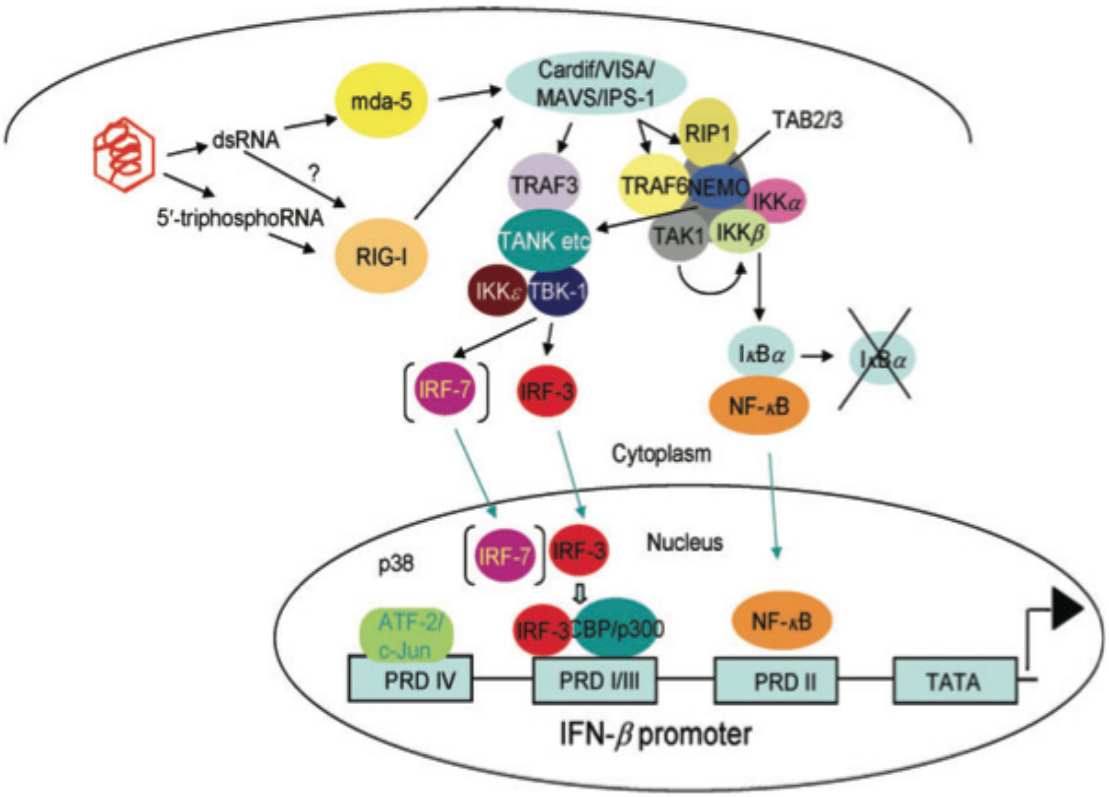


Fig. 1.2. Schematic representation of activation of IFN-β activation from RLRs signalling. Viral RNA, generated during virus replication inside the cells, activates the RNA helicases MDA-5 and RIG-I, and they subsequently recruit the adaptor Cardif/VISA/ MAVS/IPS-1. This adaptor, in turn, recruits signalling components essential for the activation and nuclear translocation of IRF- 3 or NF-kB. These transcription activators are essential in the assembly of transcription machinery for the activation of IFN-β promoter (*Diagram adapted from Randall and Goodbourn, 2008*)**.**

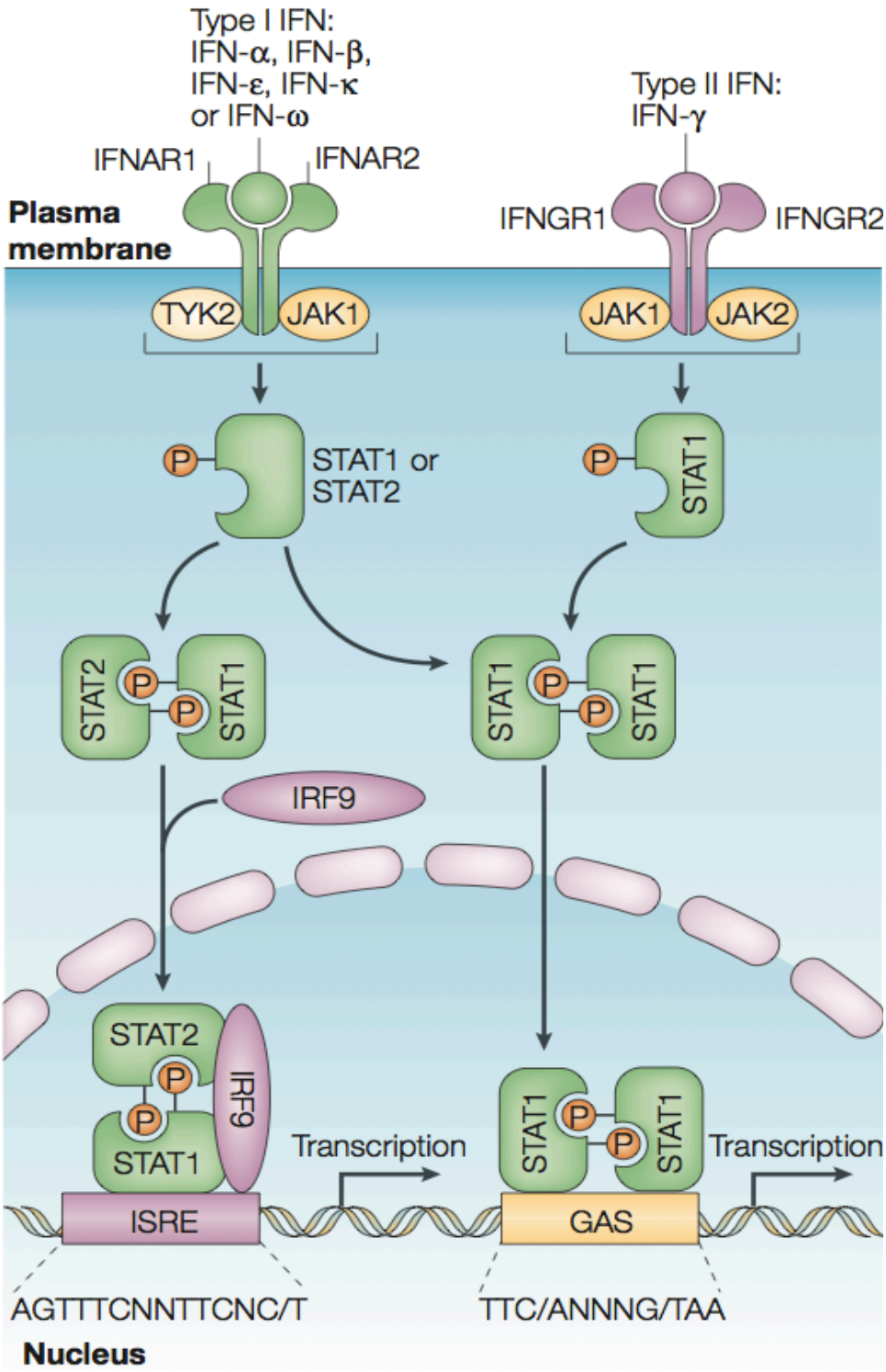


Fig. 1.3. Type I and type II IFN signalling pathways. Type I signalling is initiated by type I IFNs binding to a common receptor at the cell surface. This leads to the activation of the receptor-associated tyrosine kinases JAK1 and Tyk2, which phosphorylate STATs. Phosphorylated STAT1 and STAT2 form a heterodimer with each other by recognizing SH2 domains, and then translocated into the nucleus, where it interacts with the DNA-binding protein IRF-9, forming ISGF3. ISGF3 binds to a *cis* element, ISRE, located on in the promoter region of most ISGs. Type II IFN binds a distinct receptor, composed of IFNGR1 and IFNGR2, which are associated with JAK1 and JAK2, respectively. Activated JAKs phosphorylate STAT1, inducing the formation of STAT1 homodimers that translocate to the nucleus and bind to GAS elements of certain ISGs, thereby initiating their transcription (*Diagram adapted from Platanias, 2005*).

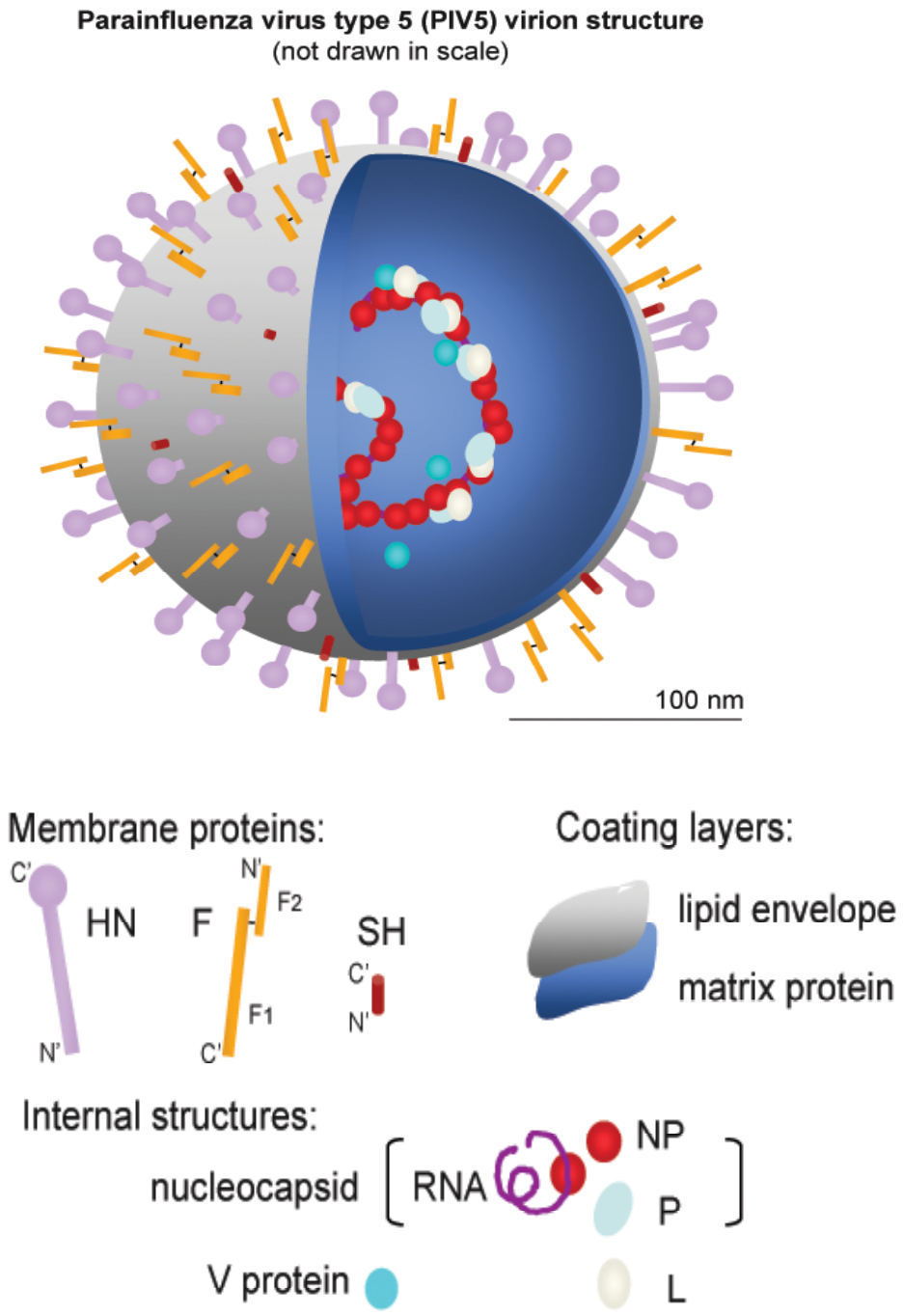


Fig. 1.4. Schematic representation of the Parainfluenza virus type 5 (PIV5) virion structure. The spherical particle of PIV5 consists of an outer lipid membrane with membrane protein hemagglutinin-neuraminidase (HN) attachment glycoprotein, and the fusion (F) glycoprotein. The small integral membrane protein, SH, is unique for PIV5. Underneath the lipid bilayer is matrix protein, essential for maintaining virus integrity. Inside the virion is the single-stranded, negative-sense RNA genome encapsidated by nucleocapsids protein (NP). Associated with NP are the L and P proteins, which have the RNA-dependent RNA polymerase activity. V protein of PIV5 is found as a virion component, whilst V proteins of other members of paramyxoviruses are found only in virus-infected cells.

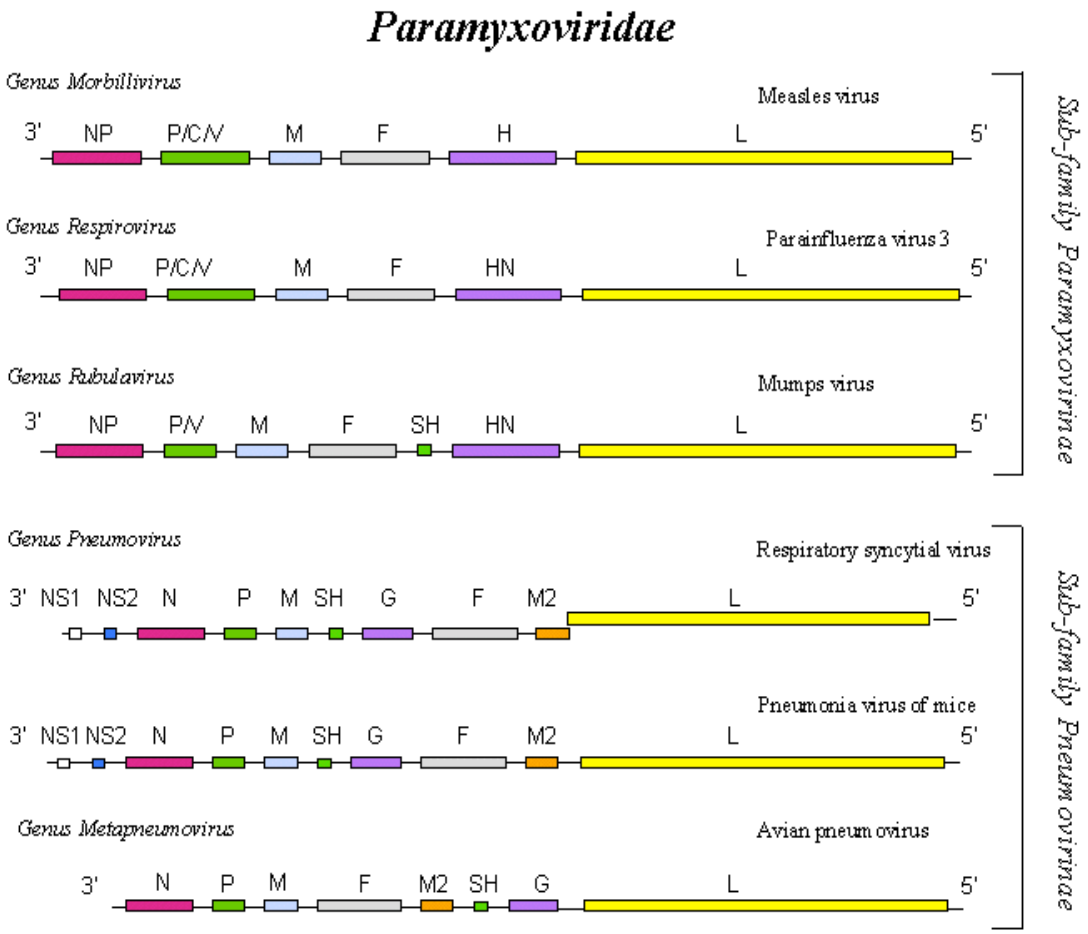


Fig. 1.5. Genetic map of a typical member of each genus of the Paramyxoviridae. The single-stranded, non-segmented, negative-sense RNA genome of paramyxoviruses consists of 6 to 10 tandem linked genes, depending on the species. The genes are shown as boxes that are drawn to approximate scale. These genes are separated by intergenic regions. Flanking the genome is a 3' extracistronic region of ~50 nucleotides known as the leader and 5' extracistronic region of 50 to 161 nucleotides known as the trailer.

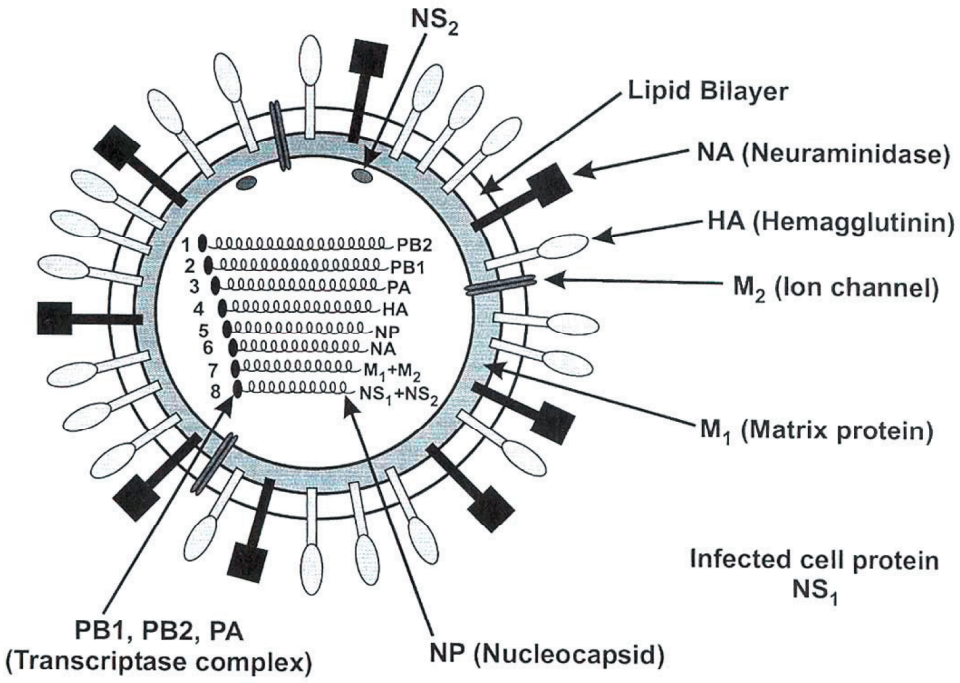


Fig. 1.6. Schematic representation of the influenza A virus particle. Virions are decorated with two surface glycoproteins, HA and NA. M2 ion channel protein is also inserted into the lipid bilayer. Underneath the lipid envelope is M1 protein. The genome is composed of eight segments of single-stranded negative-sense RNA genome encapsidated by the nucleoprotein. These ribonucleoproteins (RNPs) are also associated with components of polymerase complex (PB2, PB1, PA). NS1 and PB1-F2 are only found in infected cells, therefore they are not displayed in the diagram. The proteins encoded by each genomic segment are indicated. *Diagram adapted from Lamb & Krug, 2001.*

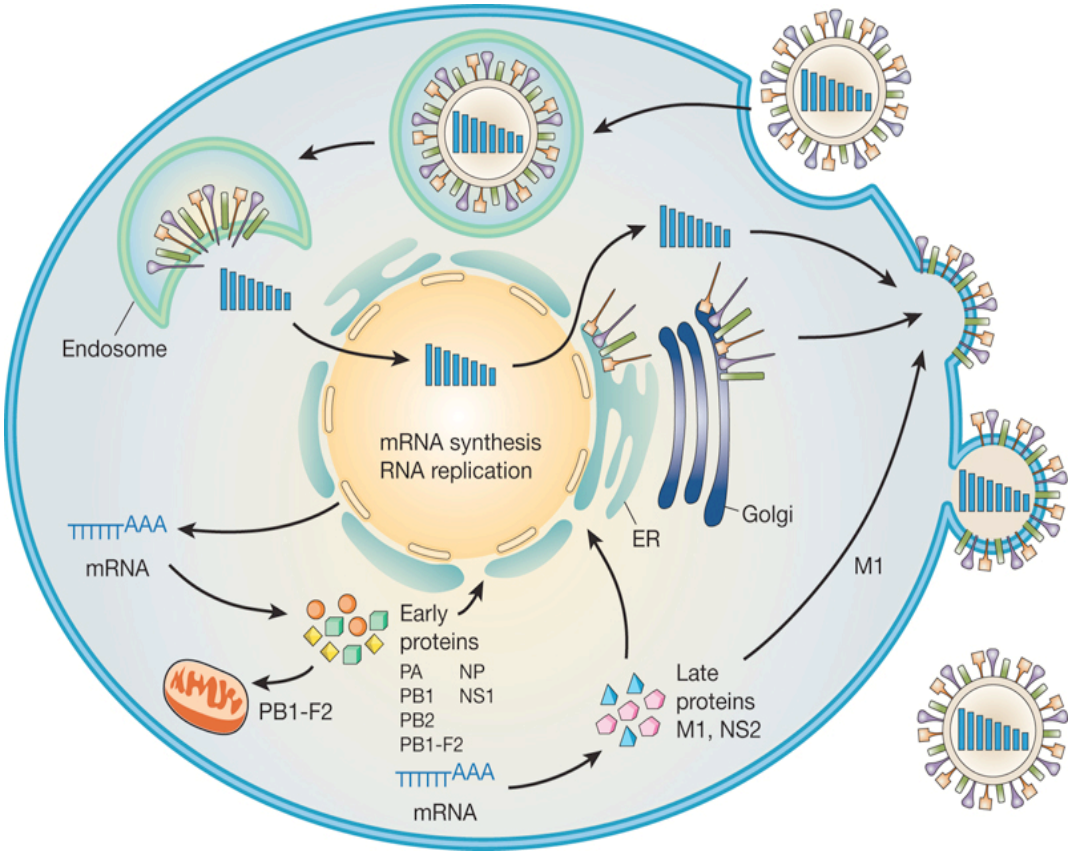


Fig. 1.7. Schematic diagram of the influenza viral life cycle. Following receptor-mediated endocytosis and fusion/uncoating process inside the endosome, the viral ribonucleoprotein (vRNP) complexes are released into the cytoplasm and subsequently transported to the nucleus, where replication and transcription occur. See text for more details. *Diagram adapted from Neumann and Kawaoka, 2010.*

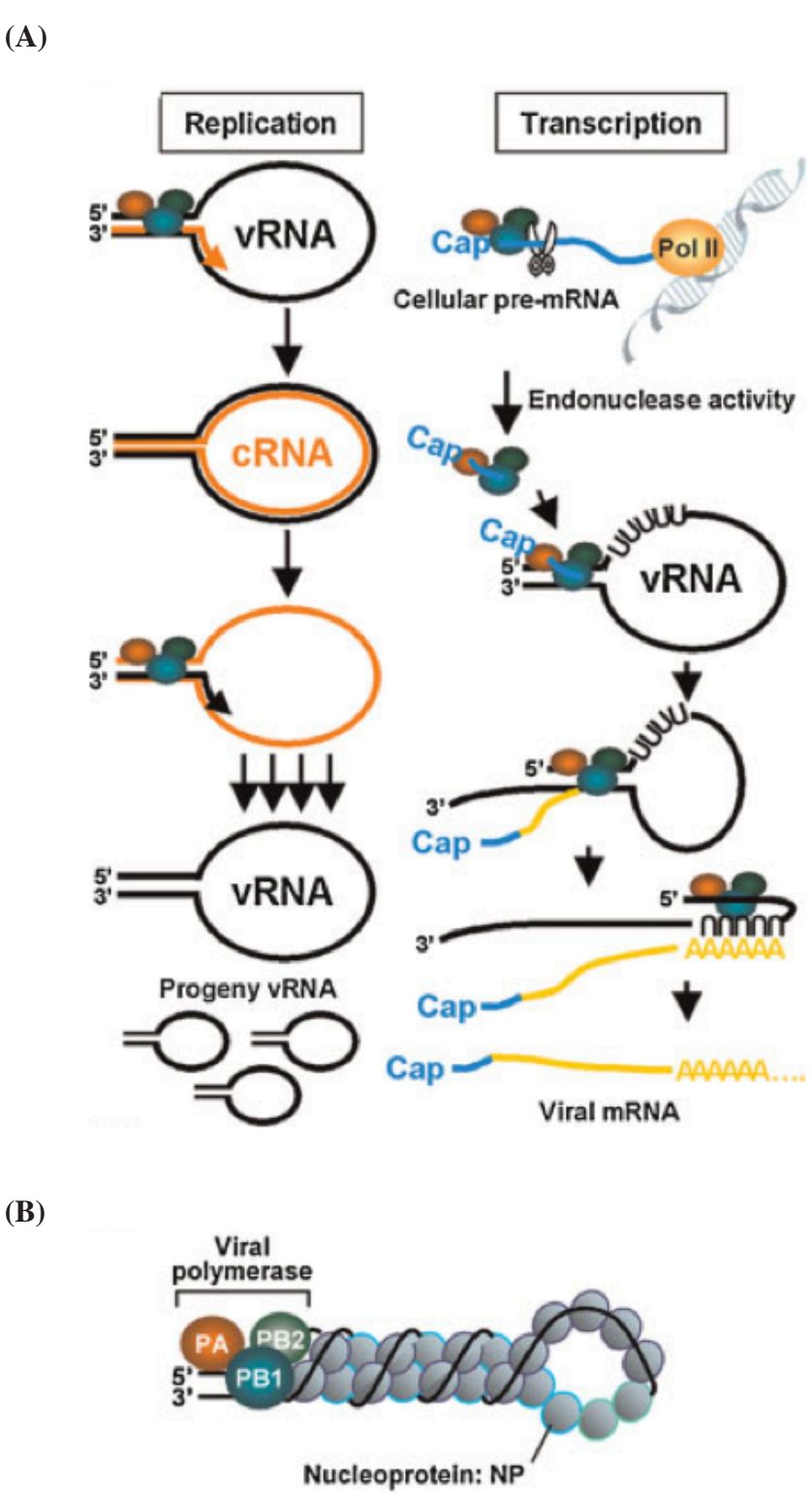
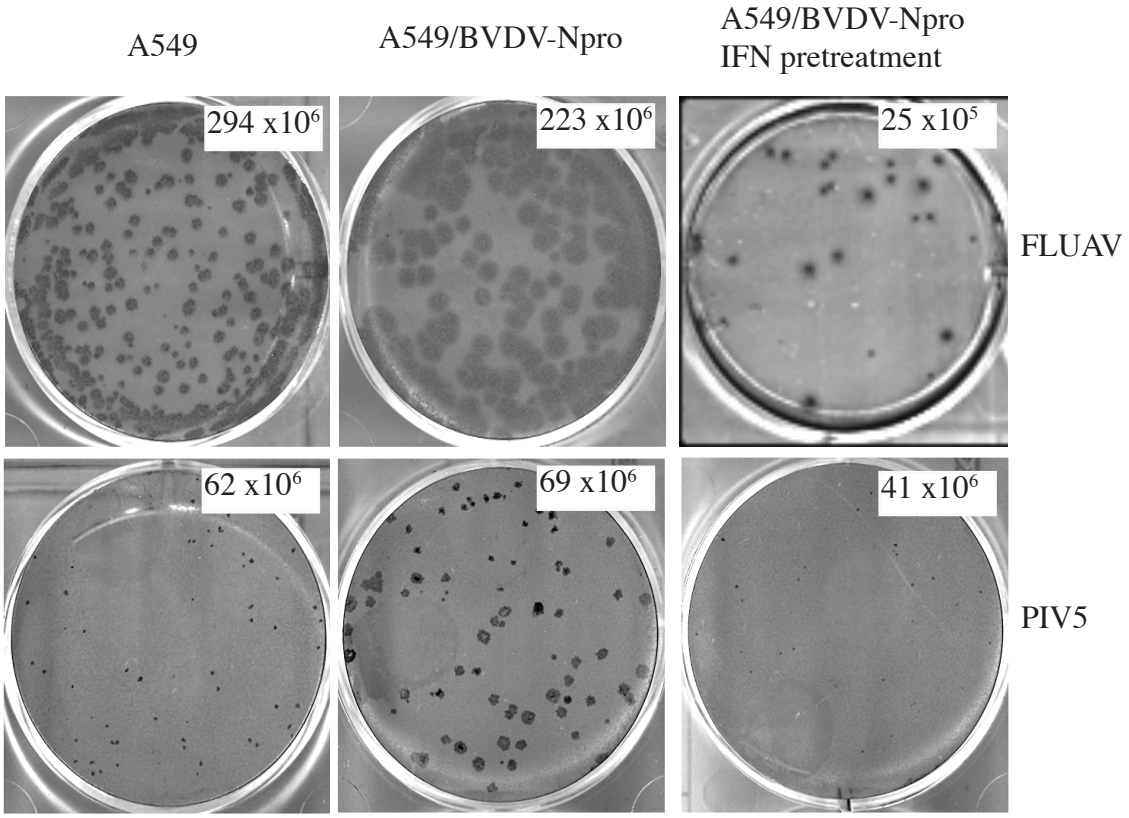


Fig. 1.8. Replication and transcription of the influenza virus genome. (A) The major steps of replication and transcription of the influenza virus genome. See main text for more details. (B) The "panhandle" model of influenza virus ribonucleoprotein complex. *Diagram adapted from Nagata and Naito, 2008.*

(A)



(B)

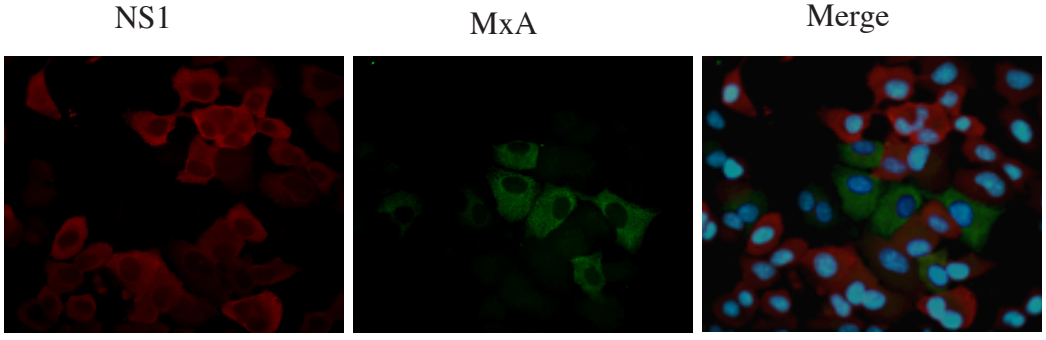


Fig. 3.1. **(A)**. Plaques of PIV5 or influenza A virus formed on monolayers of A549, A549/BVDV-Npro, or A549/BVDV-Npro pretreated with 1000u/ml IFN for 16h. note A549/BVDV-Npro cells cannot produced IFN in response to virus infection as BVDV-Npro targets IRF3 for degradation. influenza A virus infected cells were fixed at 5 days p.i. and immunostained with polyclonal anti-influenza A virus antibody, whilst PIV5 infected cells were fixed at 10 days p.i. and immunostained with an antibody to PIV5 NP. The plaque numbers formed on monolayer of cells were shown on the top right corner of each well. **(B)**. A549 cells were infected with influenza A virus at low MOI of 0.1 pfu/cell, cells were fixed at 16 hours post-infection and double-stained with antibodies against influenza A virus NS1 protein and cellular protein MxA. The cells were also counter-stained with DAPI to reveal the nuclei. NS1, MxA and DAPI imaged were merged using SPOT software (v4.6).

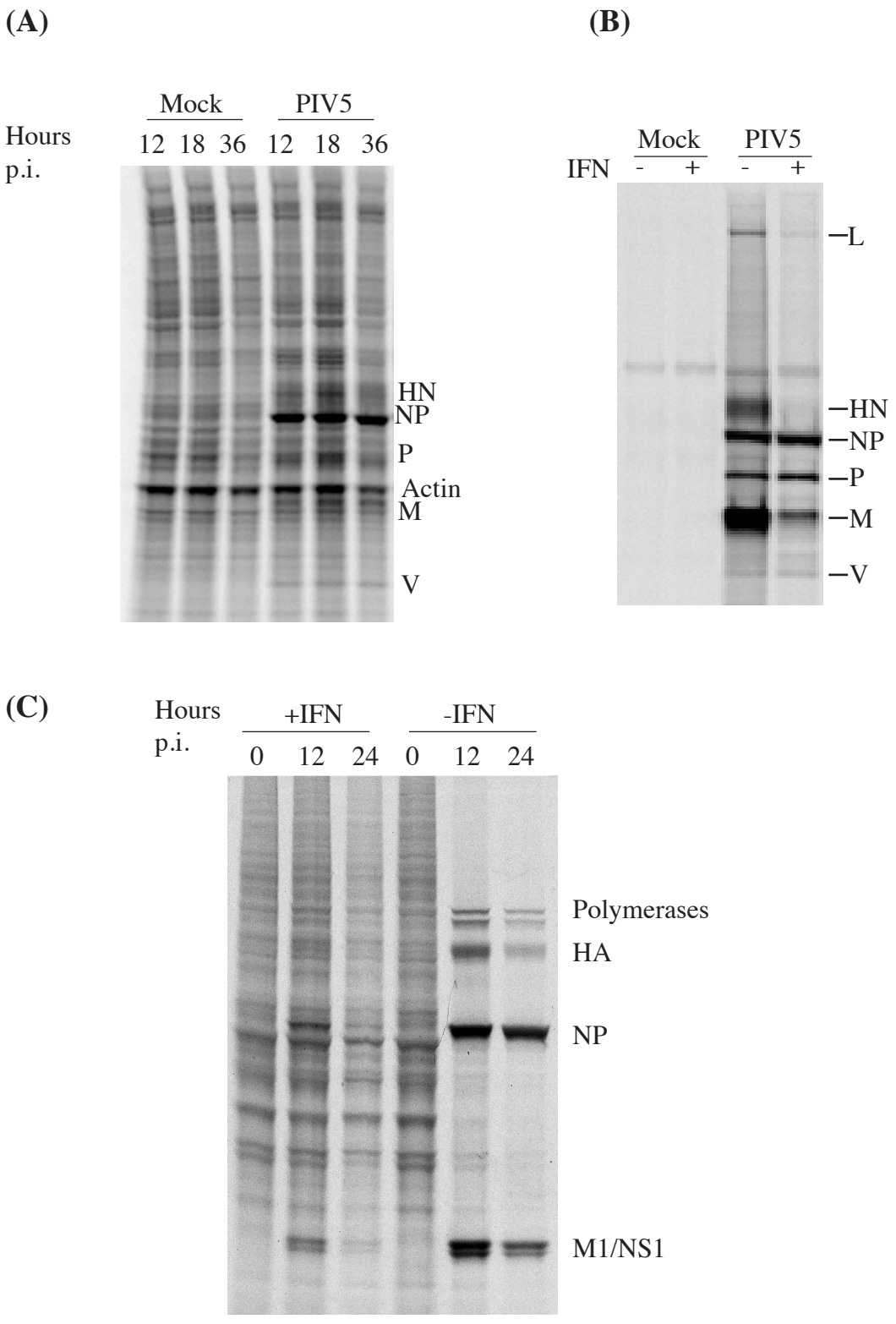


Fig. 3.2. Metabolic labelling of Influenza A virus and PIV5 infection of A549 cells. **(A).** A549 cells were infected with PIV5 or mock-infected at an MOI of 5pfu/cell, and at different time point post-infection, cells were labelled with S35-Methionine for one hour and cell lysate were prepared at the end of labelling period. Viral proteins were analysed with SDS-PAGE and phosphoimaging. A549 cells were, or were not pretreated with IFN were either mock-infected or infected with PIV5 **(B)** or FLUAV **(C)**, and at different time post-infection, cells lysate were prepared. PIV5 proteins were precipitated with a pool of monoclonal antibodies against PIV5 viral proteins and analysed by phosphoimaging.

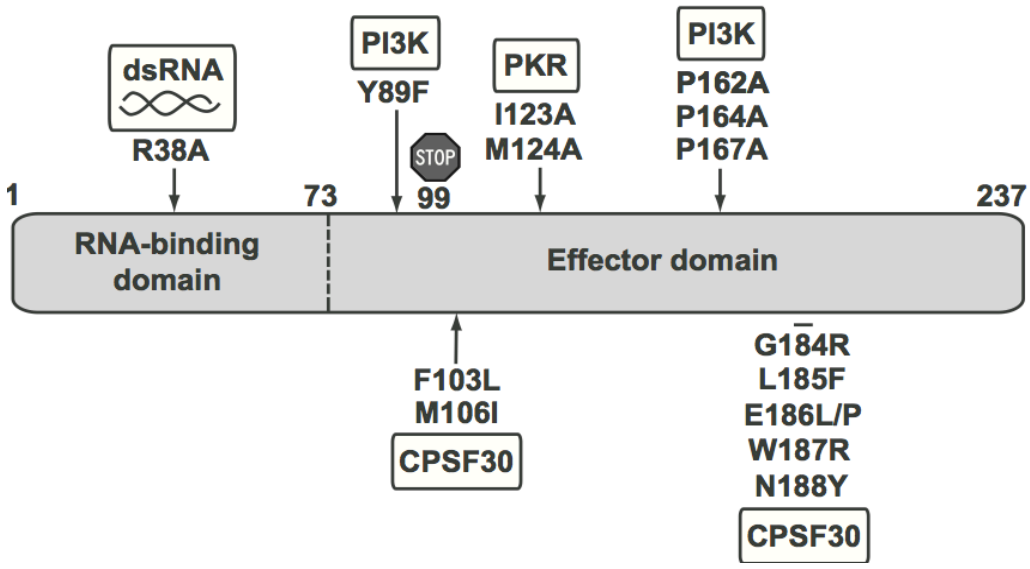


Fig. 3.3. Schematic diagram of the NS1 protein of recombinant mutant viruses. The NS1 protein of A/Udorn/72 virus is 237 amino acids (aa) in length. It is notionally divided into a 73 aa N-terminal RNA binding domain and a 164 aa C-terminal effector domain. The mutations introduced into the recombinant A/Udorn/72 (rUd) viruses described in **Table. 3.1** are shown. The NS1 and cellular protein/RNA interactions affected by each mutation is highlighted in the open boxes. The "stop sign" indicates the position of the termination codon introduced into the NS1 proteinof the rUd-Δ99 virus (Jackson D., et al. 2010).

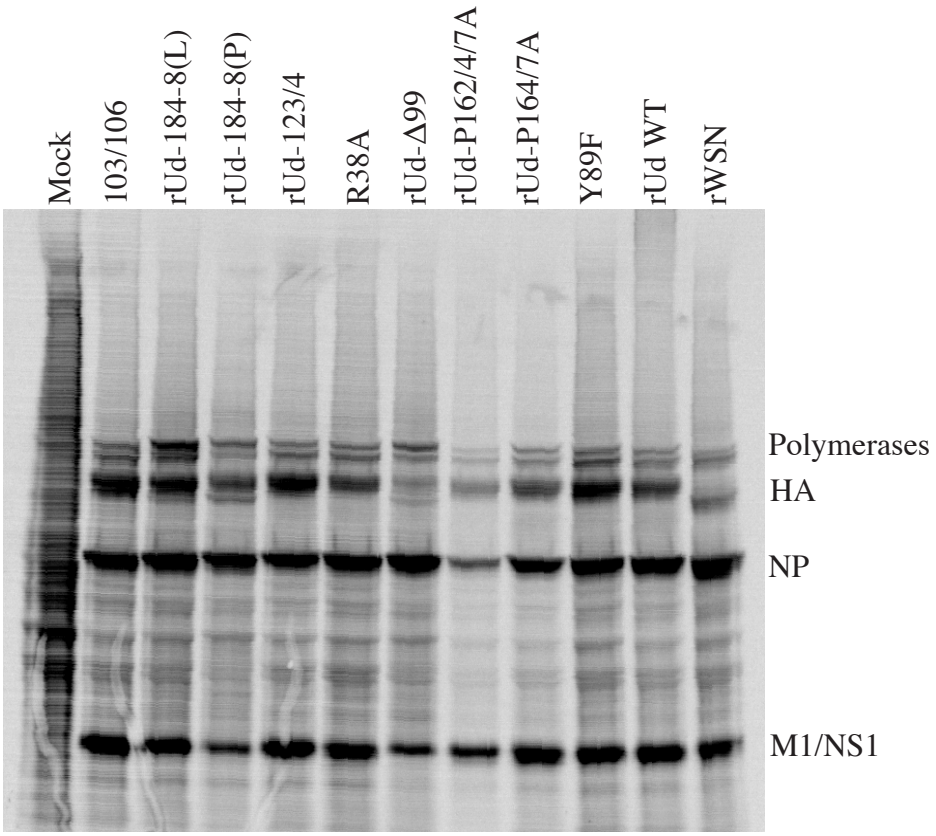


Fig. 3.4. Cell shut-off is not affected by mutations in NS1 protein. Cultures of A549 cells were infected with the recombinant wild type rWSN virus, A/Udorn virus or mutant rUd viruses in which the interactions with dsRNA, PI3K, CPSF30 and PKR were inhibited. The mutations introduced into NS1 and the functions inhibited by each mutant are summarized in **Table. 3.1**. Cells were infected at a MOI of 5 PFU/cell. The cultures were labelled at 12 h p.i. with [35S]Met-Cys (200 Ci/ml) for 1 h and the cells were lysed with immunoprecipitations buffer. The cell lysates were subjected to polyacrylamide gel electrophoresis and autoradiography.

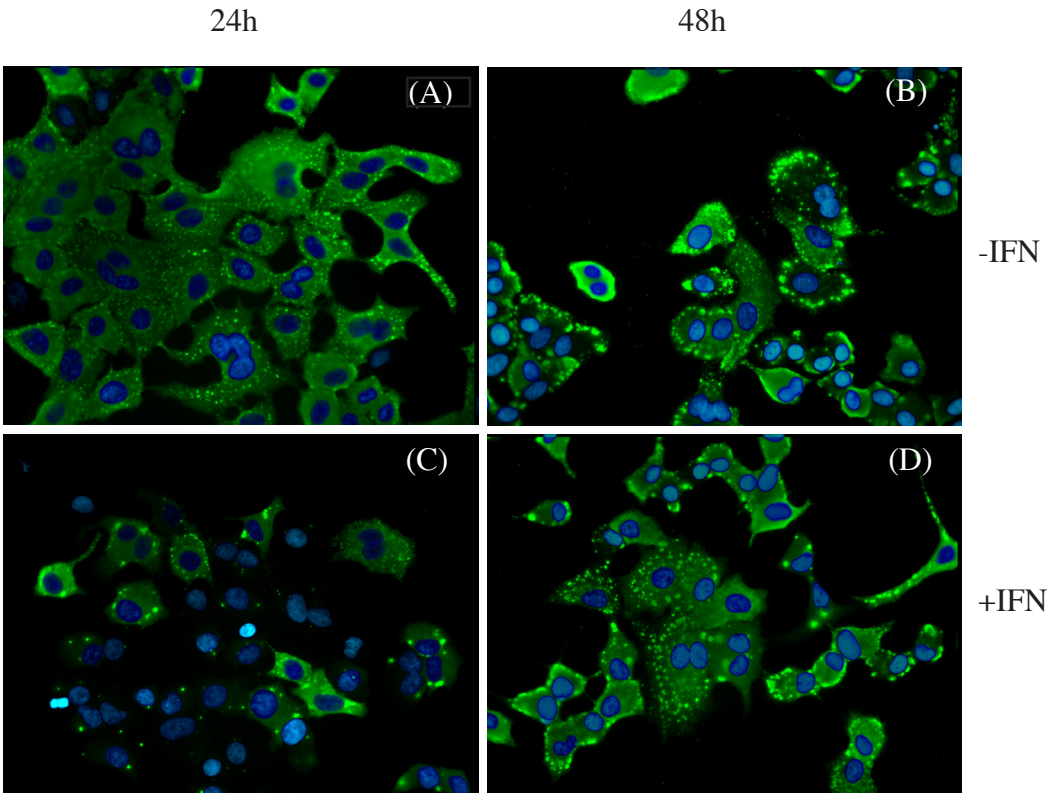


Fig. 3.5. Immunofluorescence of PIV5 virus in cell pretreated or left untreated with IFN. A549 cells were either pretreated (+IFN) or left untreated (-IFN) with 1000u/ml IFN for 16h and infected with PIV5 at an MOI of 5pfu/cell, at 24h (A and C) and 48 h (B and D) post-infection, cells were fixed and immunostained with monoclonal PIV5 NP and P antibodies. The cells were also counter-stained with DAPI to show the localization of the nuclei.

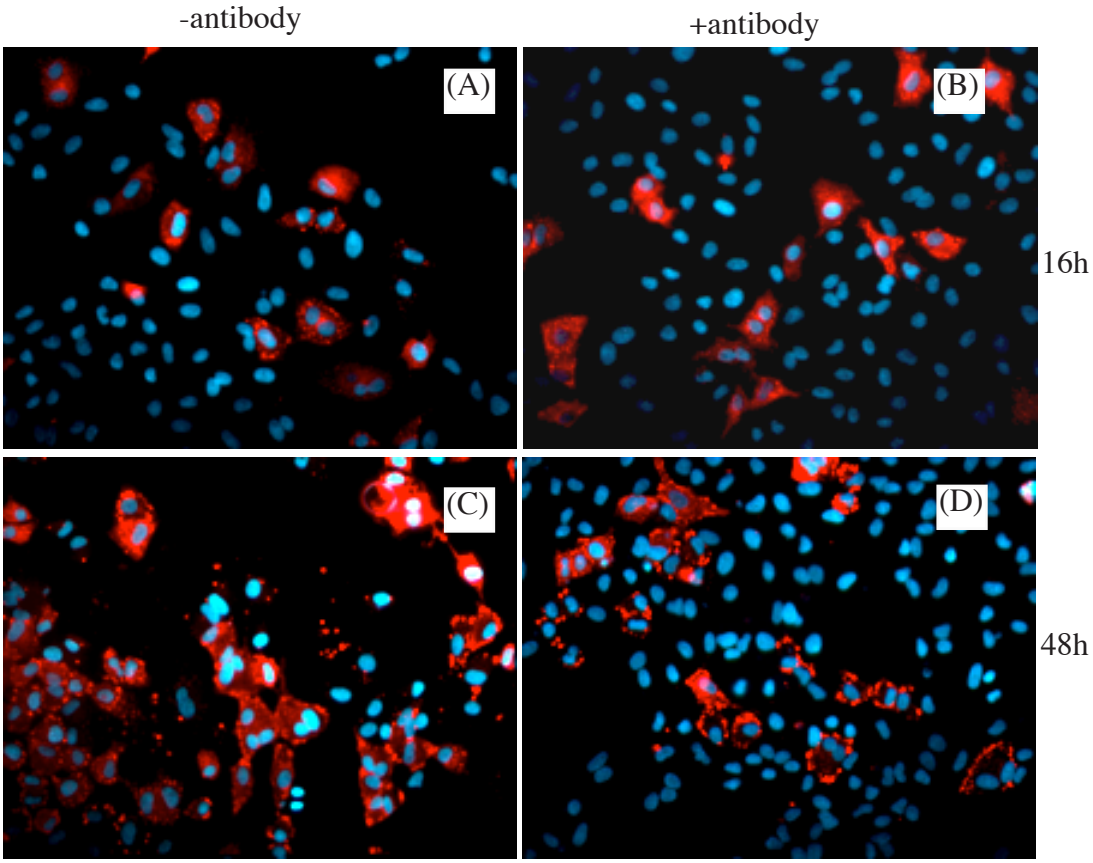


Fig. 3.6 Neutralization antibody was able to prevent PIV5 virus spread. A549 cells were infected with PIV5 at low MOI of 0.1 pfu/cell. After virus binding to cells, the inoculum were washed away and cells were added with FBS free DMEM in the presence (B and D) or absence (A and C) of neutralizing antibody. Cells were fixed and immunostained with NP and P antibodies at different time post-infection. The cells were also counter-stained with DAPI to show the localization of the nuclei. Images of NP/P and DAPI were merged using SPOT software.

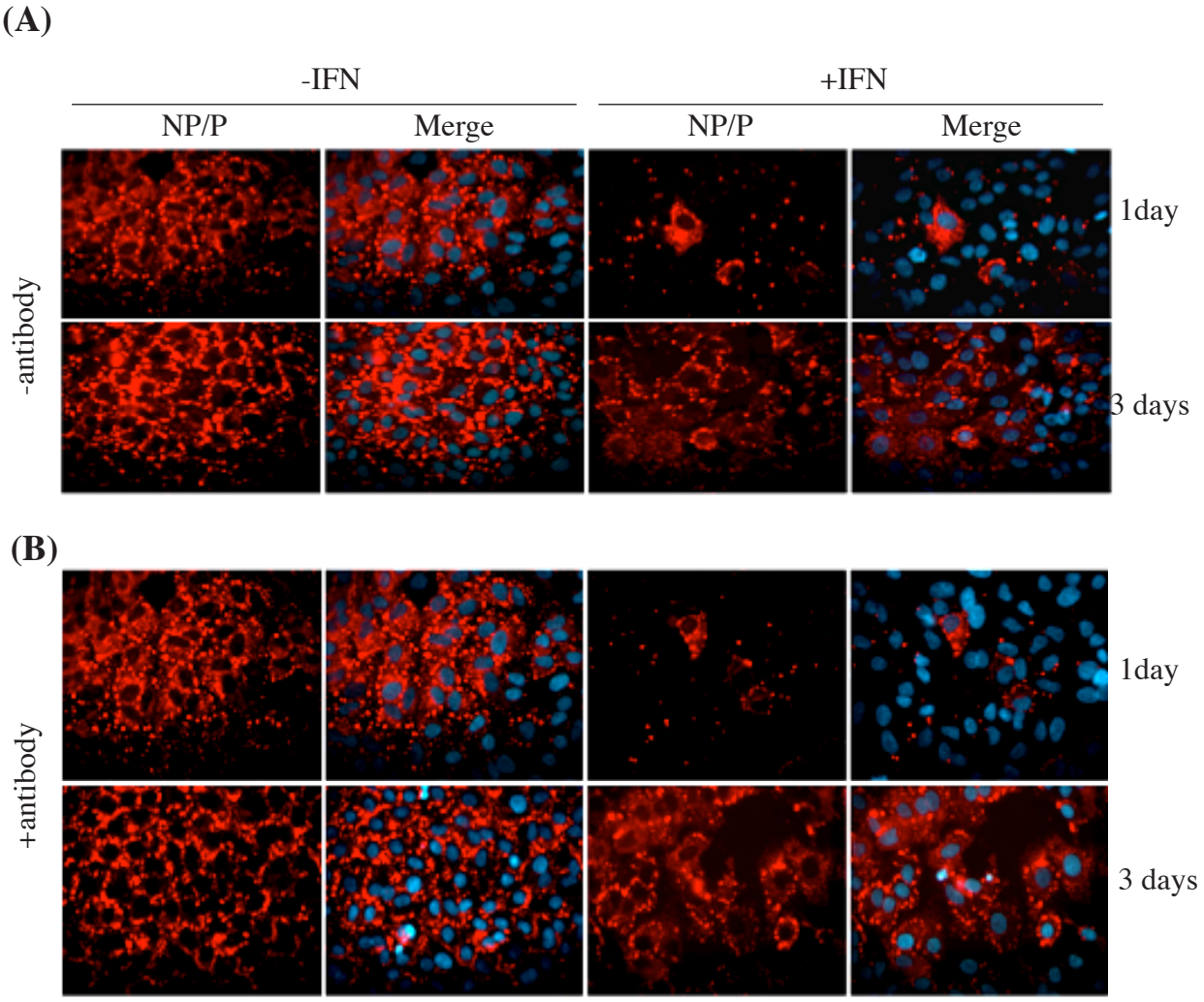


Fig. 3.7. PIV5 was able to overcome the antiviral state in the presence of neutralizing antibody. A549 cells pretreated (+IFN) or left untreated (-IFN) with IFN O/N were infected with PIV5 at a high MOI of 10 pfu/cell. After virus binding to cells, the inoculum were washed away and cells were added with FBS free DMEM in the presence **(A)** or absence **(B)** of neutralizing antibody. Cells were fixed and immunostained with monoclonal NP/P antibodies at different time post-infection. The cells were also counter-stained with DAPI to reveal the localization of nuclei, and the images of NP/P were merged with DAPI using SPOT software.

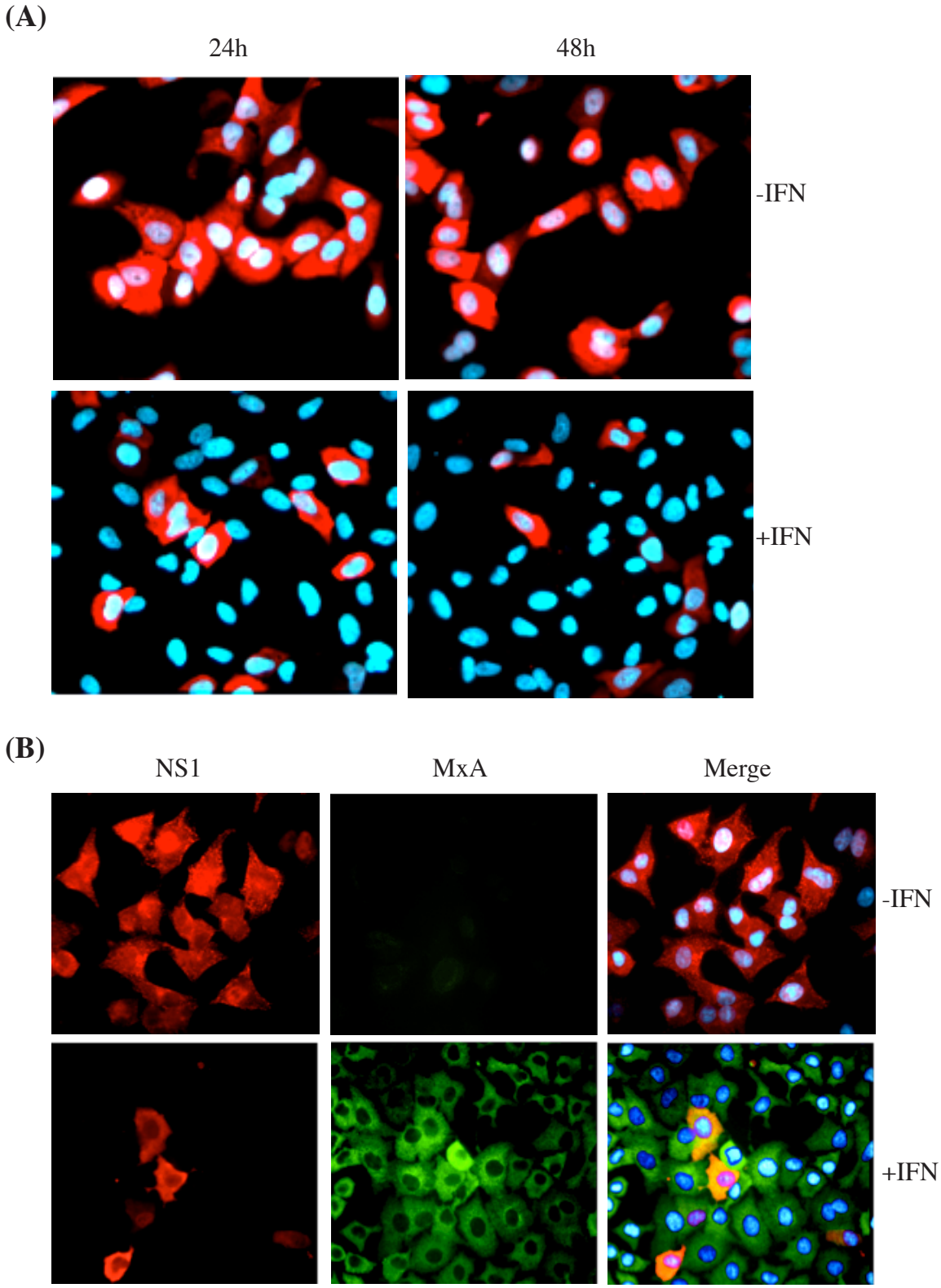


Fig. 3.8. (**A**). A549 cells were pretreated (+IFN) or left untreated (-IFN) with 1000u/ml IFN for 16h and infected with A/Udorn at an MOI of 5 pfu/cell. cells were fixed at 12p.i. and stained with anti-NS1 antibody and DAPI. Images were captured and merged using Nikon SPOT software.
(**B**). A549 cells were pretreated (+IFN) or left untreated (-IFN) with 1000u/ml IFN for 16h and infected with A/Udorn at an MOI of 5 pfu/cell. cells were fixed at 12p.i. and double-stained with sheep anti-NS1 antibody and monoclonal anti-MxA antibody. The cells were also counter-stained with DAPI and images of NS1, MxA and DAPI were merged using SPOT software.

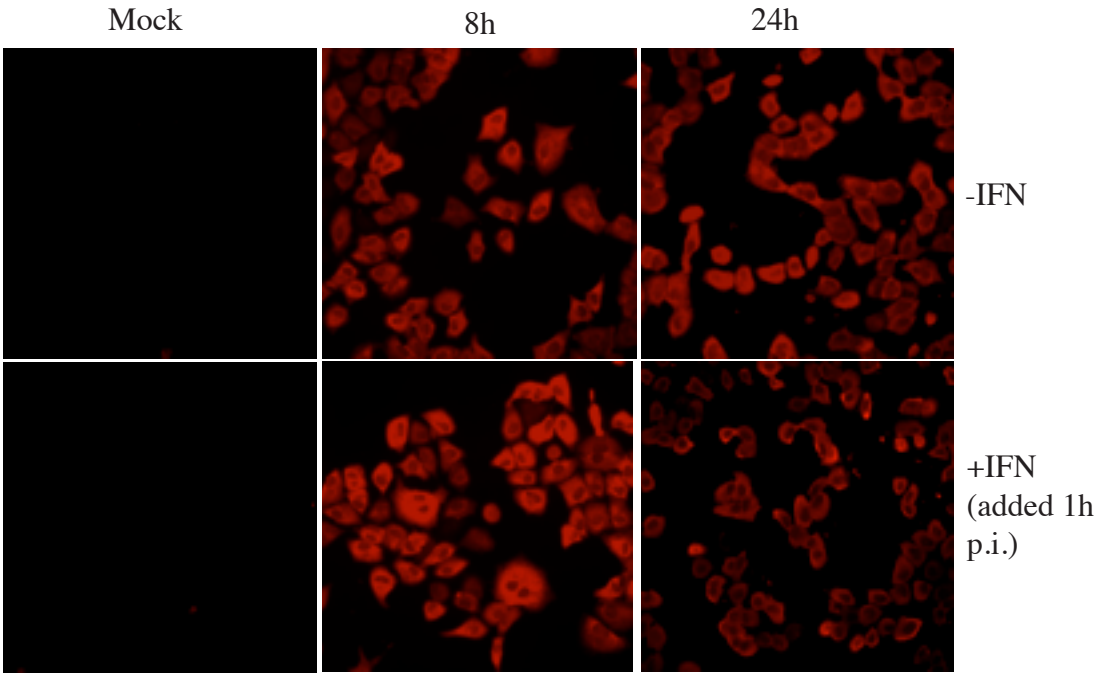
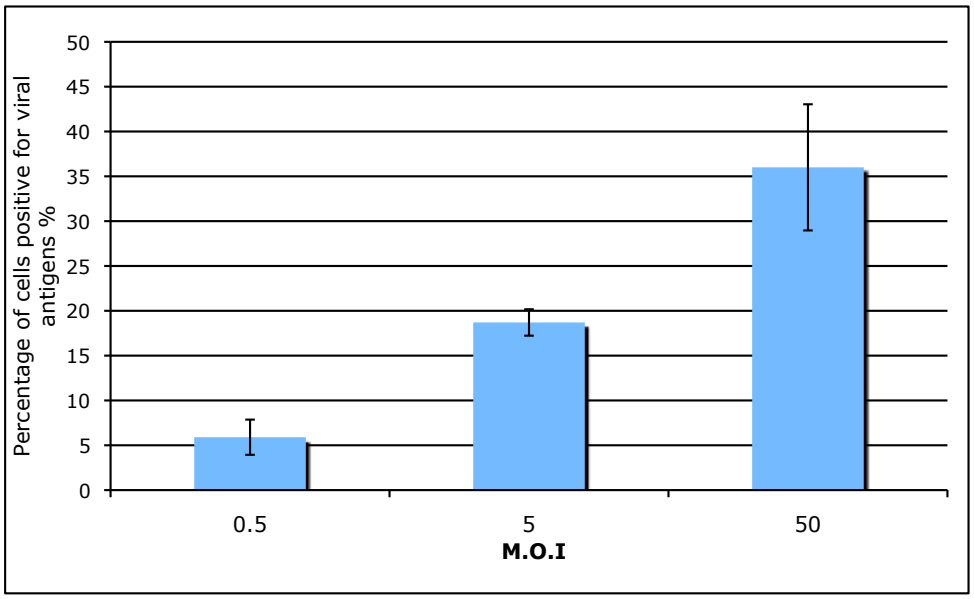


Fig. 3.9. IFN treatment after virus infection has no inhibitory effect on virus replication. A549 cells were infected with influenza A virus at MOI of 5 pfu/cell. one hour after infection, inoculum was removed, and cells were treated with 1000u/ml IFN (+IFN) or left untreated (-IFN). At different time point post-infection cells were fixed and immunostained with sheep anti-NS1 antibody.

(A)



(B)

Mock NS1

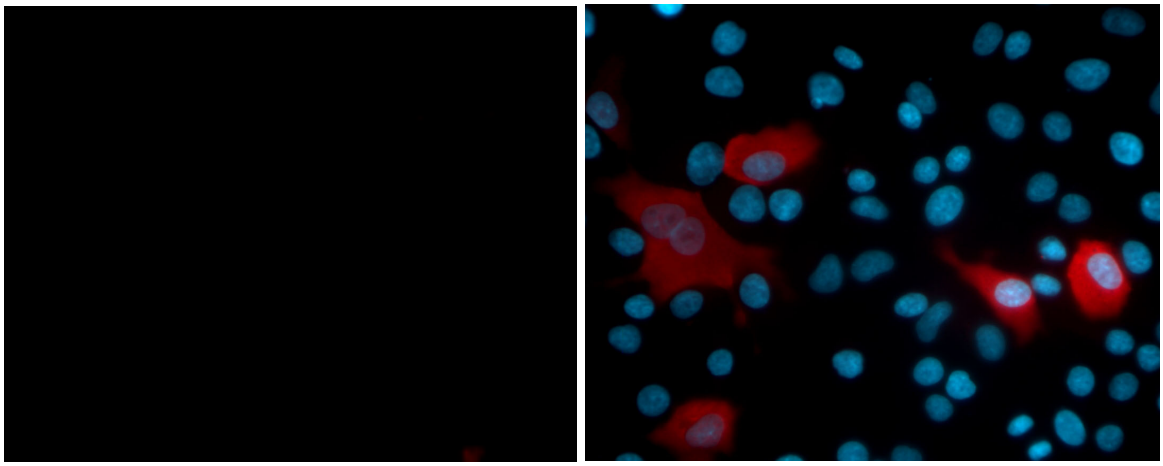


Fig. 3.10. The ability of influenza A virus to overcome the antiviral state depends on MOI. (**A**). Confluent A549 cells were pretreated with 1000u/ml IFNα and infected with influenza A virus at different MOI, cells were fixed and immunostained with anti-NS1 antibody 12 h p.i. and the percentage of NS1 positive cells was calculated by counting cells positive for NS1 versus the total number of cells per field of view under the microscope. (**B**). As a control, A549 cells untreated with IFN were infected with influenza A virus at MOI of 0.5 pfu/cell, at 12h p.i. cells were fixed and stained with sheep NS1 antibody and DAPI to reveal the nuclei. Images of NS1 and DAPI were merged using SPOT software.

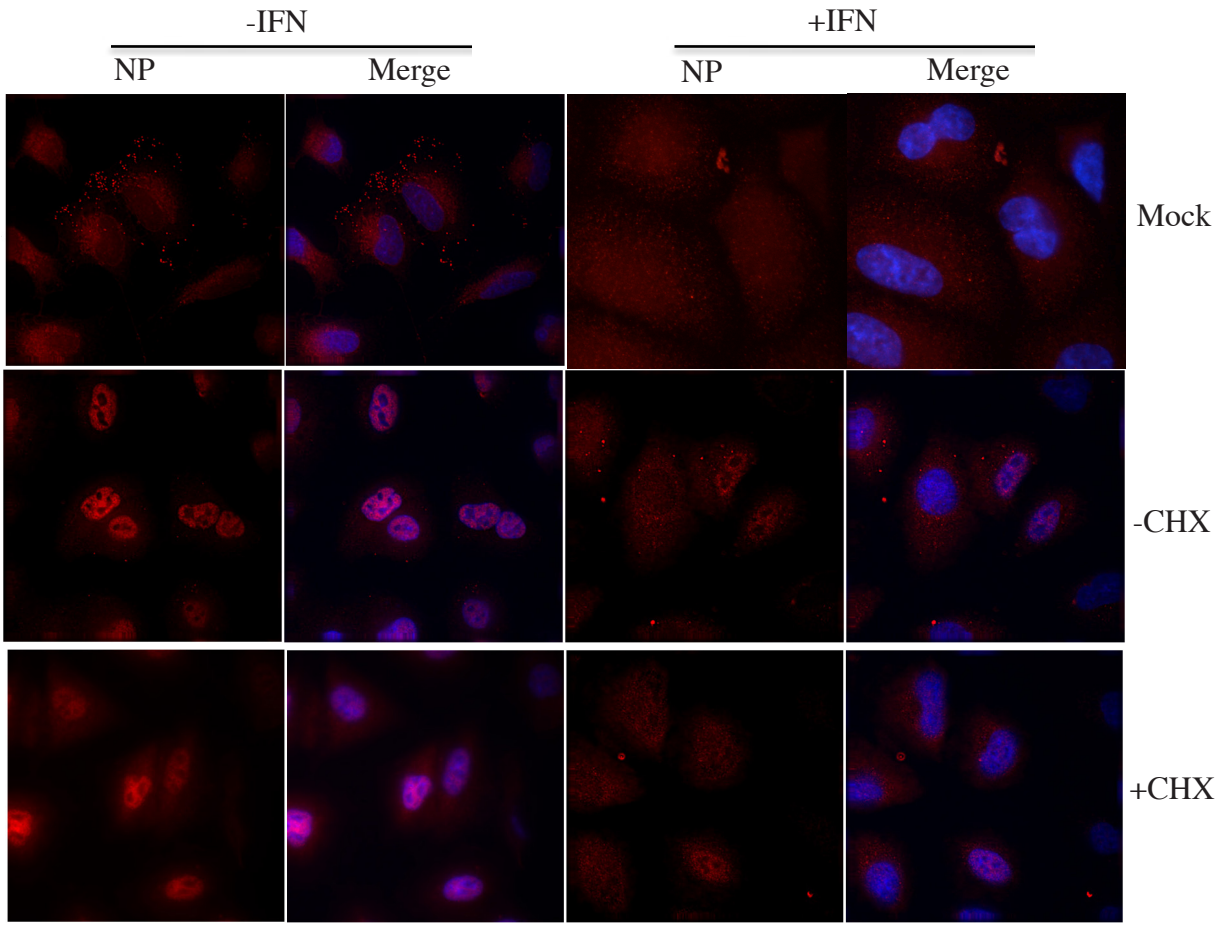


Fig. 3.11a. IFN pretreatment blocks the nuclear import of viral nucleocapsids. A549 cells pre-treated with IFN (+IFN) or left untreated (-IFN) were either mock-infected or infected with influenza A virus at a high MOI of 500 pfu/cell in the presence (+CHX) or absence (-CHX) of 100μg/ml cycloheximide (CHX) on ice for 45min to allow virus binding to cells. After that, virus inoculum was removed and unbound viruses were washed away with ice-cold PBS and virus infection was synchronized by adding pre-warmed FBS-free medium onto the cells. CHX was kept the whole time during infection. At 2 hours p.i., the cells were fixed and stained with monoclonal anti-NP antibody. The cells were also counter-stained with DAPI to reveal the location of the nuclei.The subcellular localization of NP was visualized using Nikon Microphot FXA, 20×.

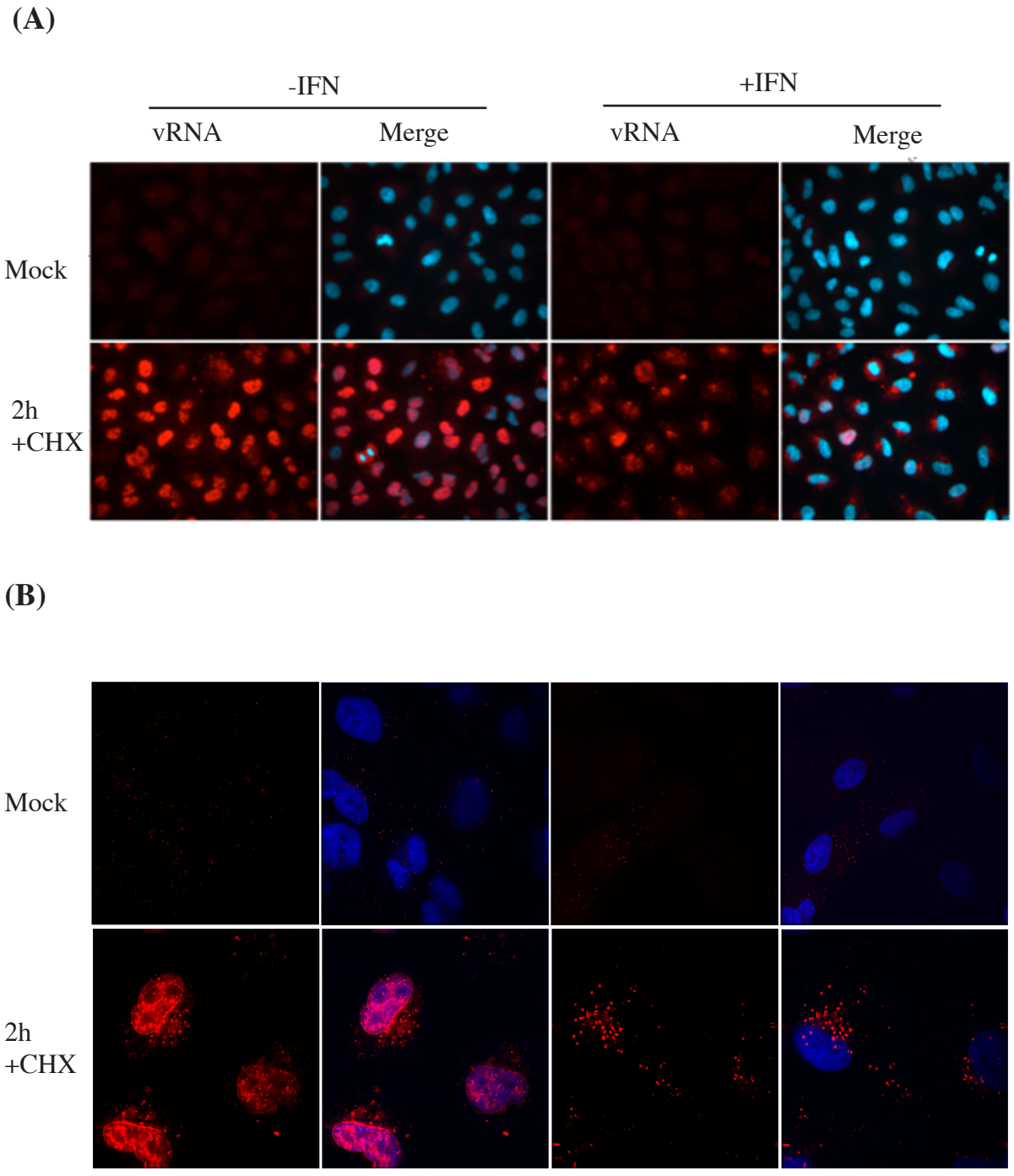


Fig. 3.11b. IFN pretreatment blocks the nuclear import of viral genome. A549 cells pre-treated with IFN (+IFN) or left untreated (-IFN) were either mock-infected or infected with influenza A virus at a high MOI of 500 pfu/cell in the presence of 100µg/ml cycloheximide (+CHX) on ice for 45 min to allow virus binding to cells. After that, virus inoculum was removed and unbound viruses were washed away with ice-cold PBS and virus infection was synchronized by adding pre-warmed serum-free medium onto the cells. CHX were kept the whole time during the infection. At 2 hours p.i., the cells were fixed and viral genome localization was revealed by *in situ* hybridization using DIG-labeled probes specific for genomic NS1. The cells were also counter-stained with DAPI to reveal the location of the nuclei.The subcellular localization of NS1 genome was visulized using Nikon Microphot FXA 20× (**A**) or with DeltaVision 60× (**B**).

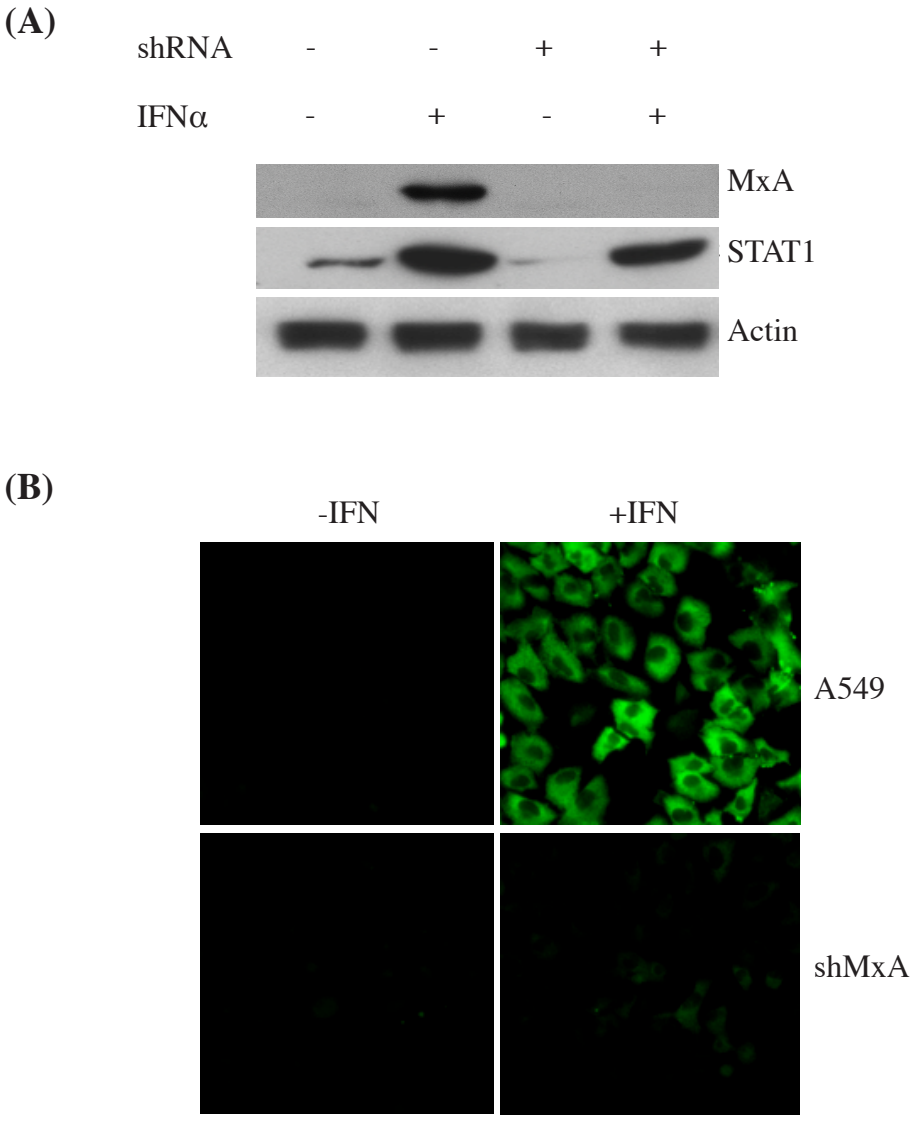


Fig. 3.12. Knock down of MxA expression in A549 cells with lentivirus expressing shRNA against MxA. Naive A549 cells or A549 cells transduced with lentivirus expressing shRNA against MxA were treated or left untreated with 1000u/ml IFN. The level of MxA expressed was determined by western blot **(A)** or immunofluorescence **(B)** 16 hours after IFN treatment. For western blot, cells were lysed using immunoprecipitation buffer and sonicated to reduce sample viscosity. MxA, STAT1 and actin expression was examined using rabbit anti-MxA, monoclonal anti-STAT1 and monoclonal anti-actin, respectively. For immunofluorescence, MxA expression was revealed by staining cells with monoclonal anti-MxA antibody.

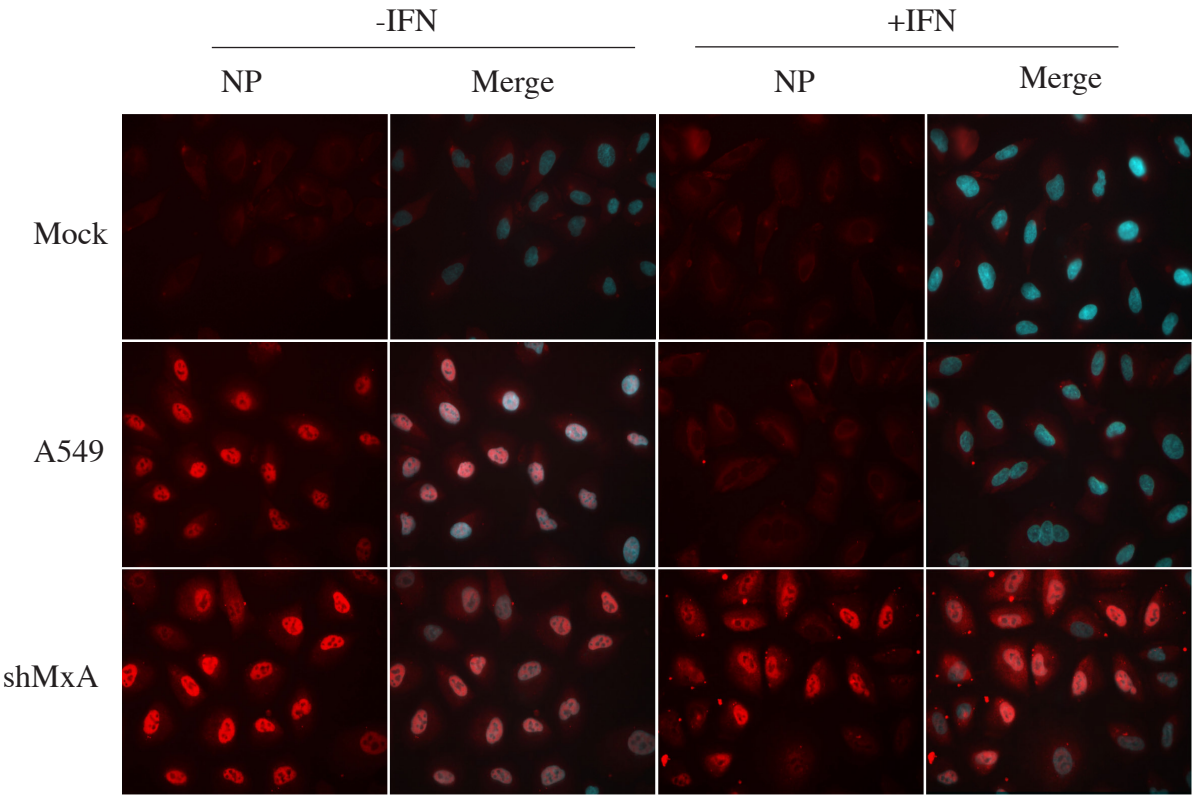


Fig. 3.13. MxA is essential in blocking FLUAV genome import in cells in a pre-existing IFN-induced antiviral state. A549 cells (A549) and MxA knockdown cellss (shMxA), pre-treated (+IFN) or left untreated (-IFN) with 1000u/ml IFN, were either mock-infected or infected with FLUAV at a very high MOI of 500 pfu/cell in the presence of 50µg/ml cycloheximide on ice for 45min to allow virus binding to cells. Subsequently, virus inoculum was removed and unbound viruses were washed away with ice-cold PBS and virus infection was synchronized by adding pre-warmed FBS-free medium onto the cells. CHX were kept the whole time during infection. At 2 h p.i., cells were fixed and stained with monoclonal anti-NP antibody. The cells were also counter-stained with DAPI to reveal the location of the nuclei.

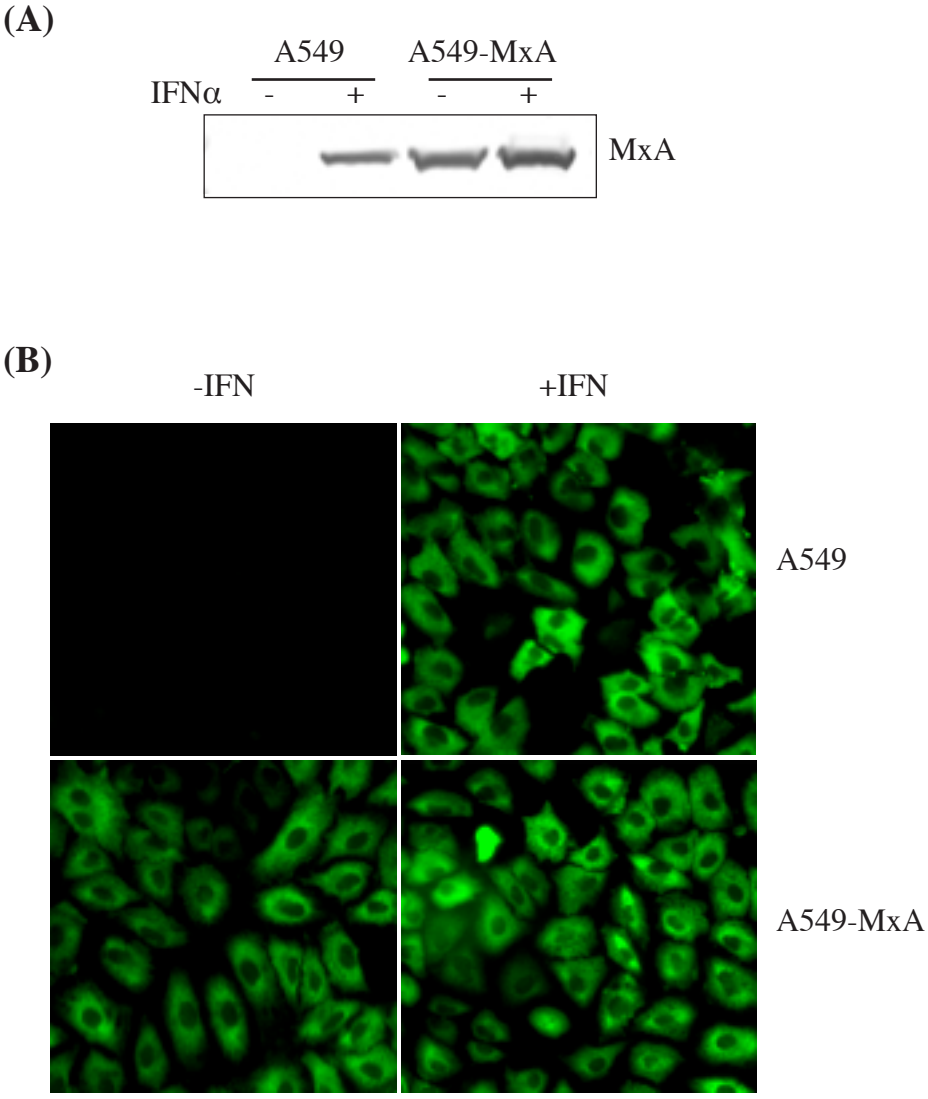


Fig. 3.14. Generation of MxA over-expression cell line in A549 cells. A549 cells were infected with lentivirus expressing MxA, or were left uninfected. Stable cell line were selected with puromycin 2ug/ml. Cells were treated with 1000u/ml IFNα as indicated. The amount of MxA expressed was determined by western blot **(A)** or immunofluorescence **(B)** analysis using monoclonal anti-MxA 16 hours after IFN treatment.

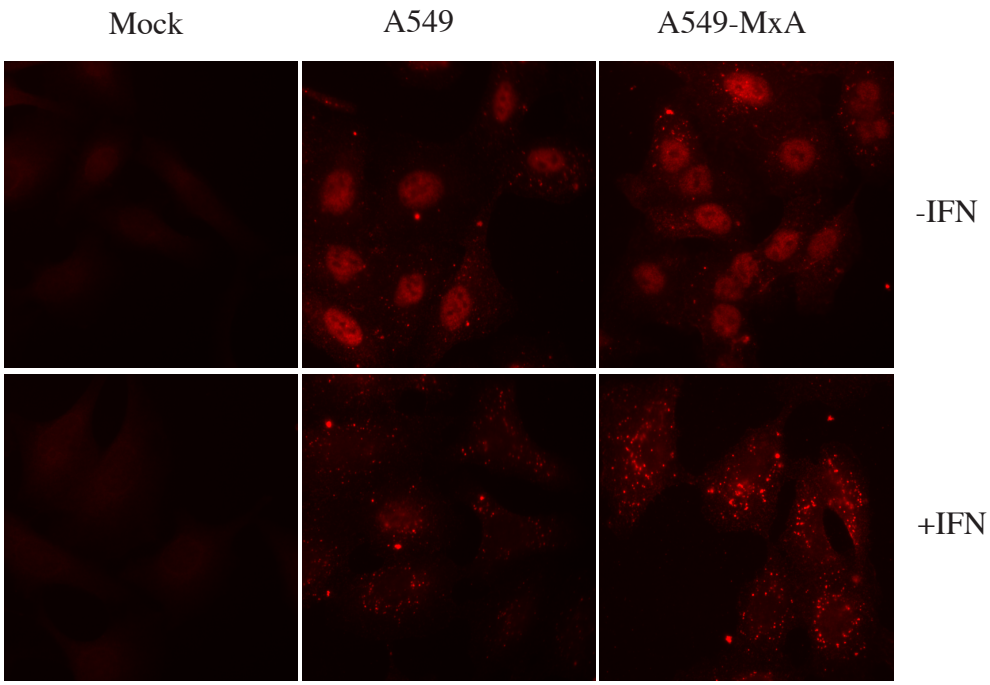


Fig.3.15. **Over-expression of MxA is insufficient to block FLUAV genome import**. A549 cells (A549) and MxA over-expression cells (A549-MxA), treated with 1000u/ml IFN (+IFN) or left untreated (-IFN), were either mock-infected or infected with FLUAV at a high MOI of 500 pfu/cell in the presence of 50μg/ml cycloheximide on ice for 45min to allow virus binding to cells. Subsequently, virus inoculum was removed and unbound viruses were washed away with ice-cold PBS and virus infection was synchronized by adding pre-warmed FBS-free medium onto the cells. CHX were kept the whole time during infection. At 2 h p.i., cells were fixed and stained with monoclonal anti-NP antibody.

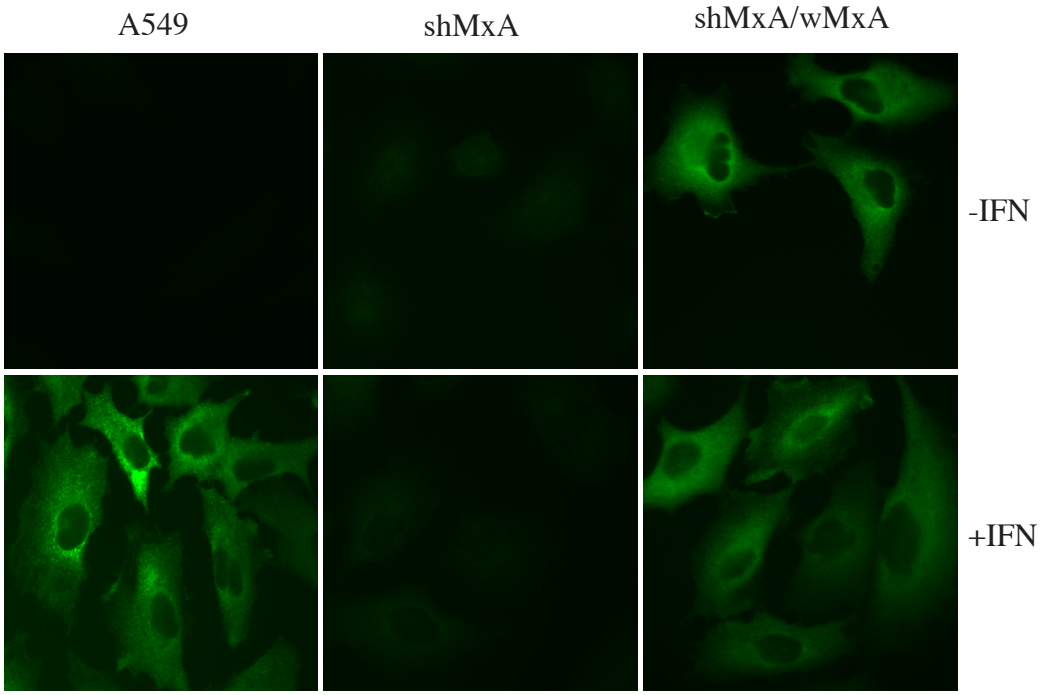


Fig. 3.16. Generation of wobble MxA over-expression cell line in shMxA cells. Lentivirus transduced, blasticidin-resistant shMxA cells were infected with lentivirus construct expressing wobble MxA, or were left uninfected. The amino acid sequence of wobble MxA is identical to endogenous MxA, but there is no complementary sequence to shRNA against MxA due to the introduction of synonymous substitutions. Stable cell line were selected with puromycin (2μg/ml). The level of MxA expression in naive A549 cells (A549), MxA knockdown cells (shMxA) or wobble MxA cells (shMxA/wMxA) was determined by immunofluorescence.

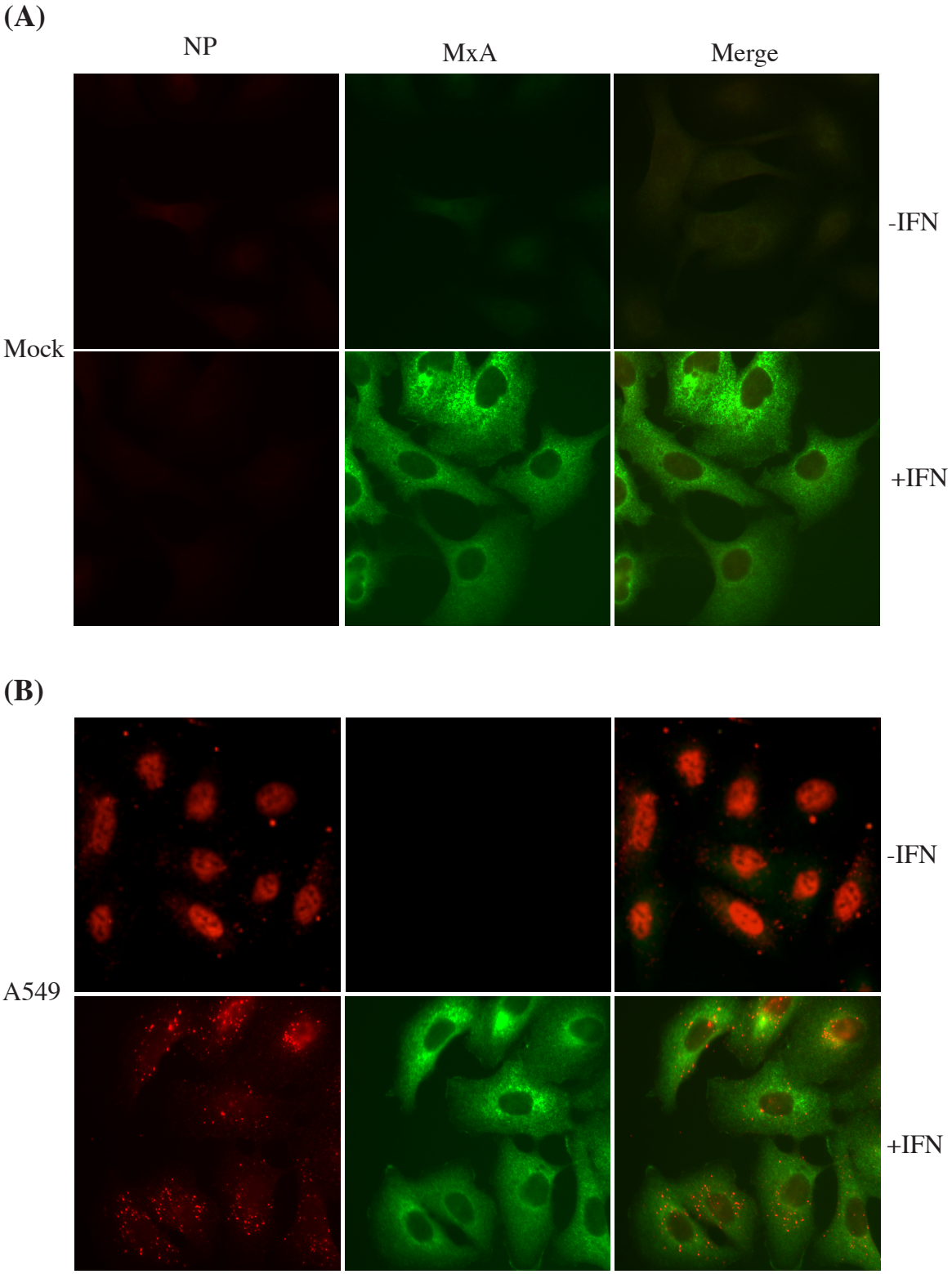


Fig. 3.17. Effects of introducing MxA back into MxA-knockdown cells upon virus genome import.

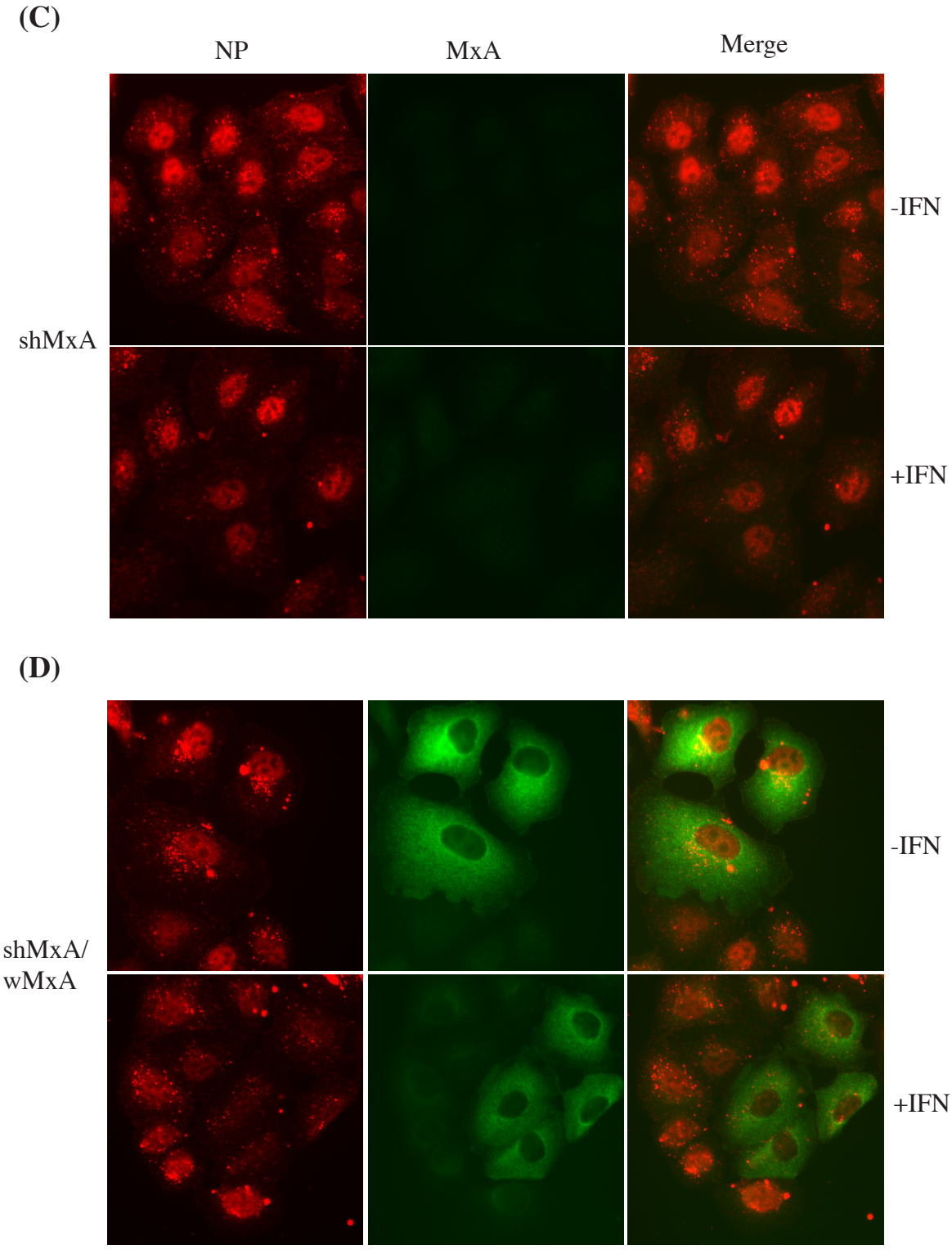
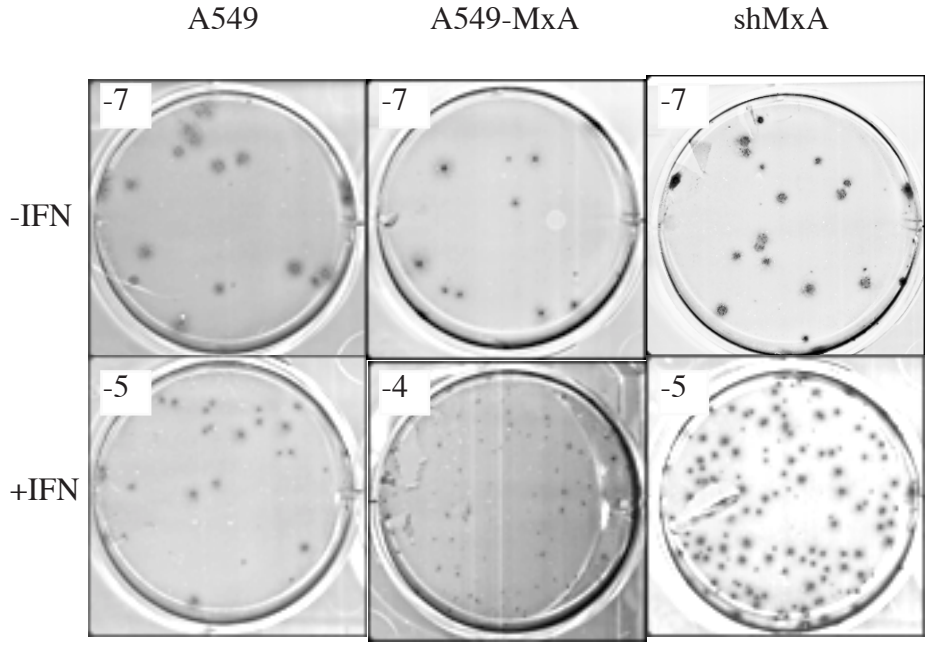


Fig. 3.17. Effects of introducing MxA back into MxA-knockdown cells upon virus genome import. A549 cells (**B**), MxA knockdown cellss (**C**), and shMxA knockdown cells over-expressing wobble MxA (**D**), pre-treated (+IFN) or left untreated (-IFN) with 1000u/ml IFN, were either mock-infected or infected with FLUAV at a very high MOI of 500 pfu/cell in the presence of 50µg/ml cycloheximide on ice for 45min to allow virus binding to cells. Subsequently, virus inoculum was removed and unbound viruses were washed away with ice-cold PBS and virus infection was synchronized by adding pre-warmed FBS-free medium onto the cells. At 2 h p.i., cells were fixed and double-stained with monoclonal anti-NP antibody and rabbit anti-MxA antibody.

(A)



(B)

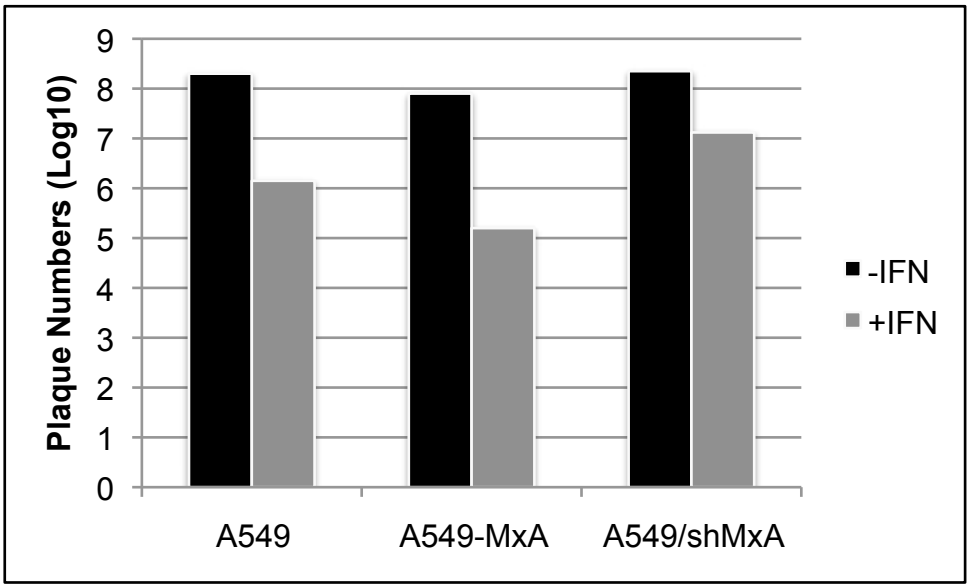


Fig. 3.18. Role of MxA in plaque reduction assay. Naïve A549 cells or cells with MxA knockdown (shMxA) or overexpression (A549-MxA) were treated with 1000u/ml IFN for 16 hours or left untreated. Monolayer of cells were infected with influenza A virus and the viruses were allowed to form plaques under 1% agarose. Plaque were visualized by immunostaining with polyclonal antibody against influenza A virus. Viral plaques were shown in Panel A, and the plaque numbers were plotted (Panel B).The number on the top left corner of each well in Panel A indicates the folds of dilution of original virus stock for infection.

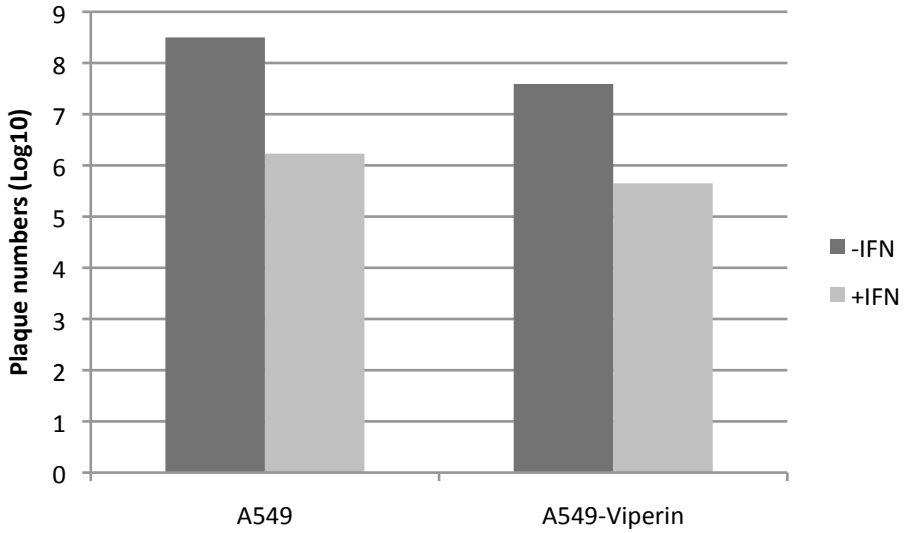


Fig. 3.19. Role of viperin in plaque reduction assay. Naive A549 cells or viperin over-expressing A549 cell lines were treated with 1000u/ml IFN for 16 hours or left untreated. Monolayer of cells were infected with influenza A virus and the viruses were allowed to form plaques under 1% agarose. Plaque were visualized by immunostaining with polyclonal antibody against influenza A virus.

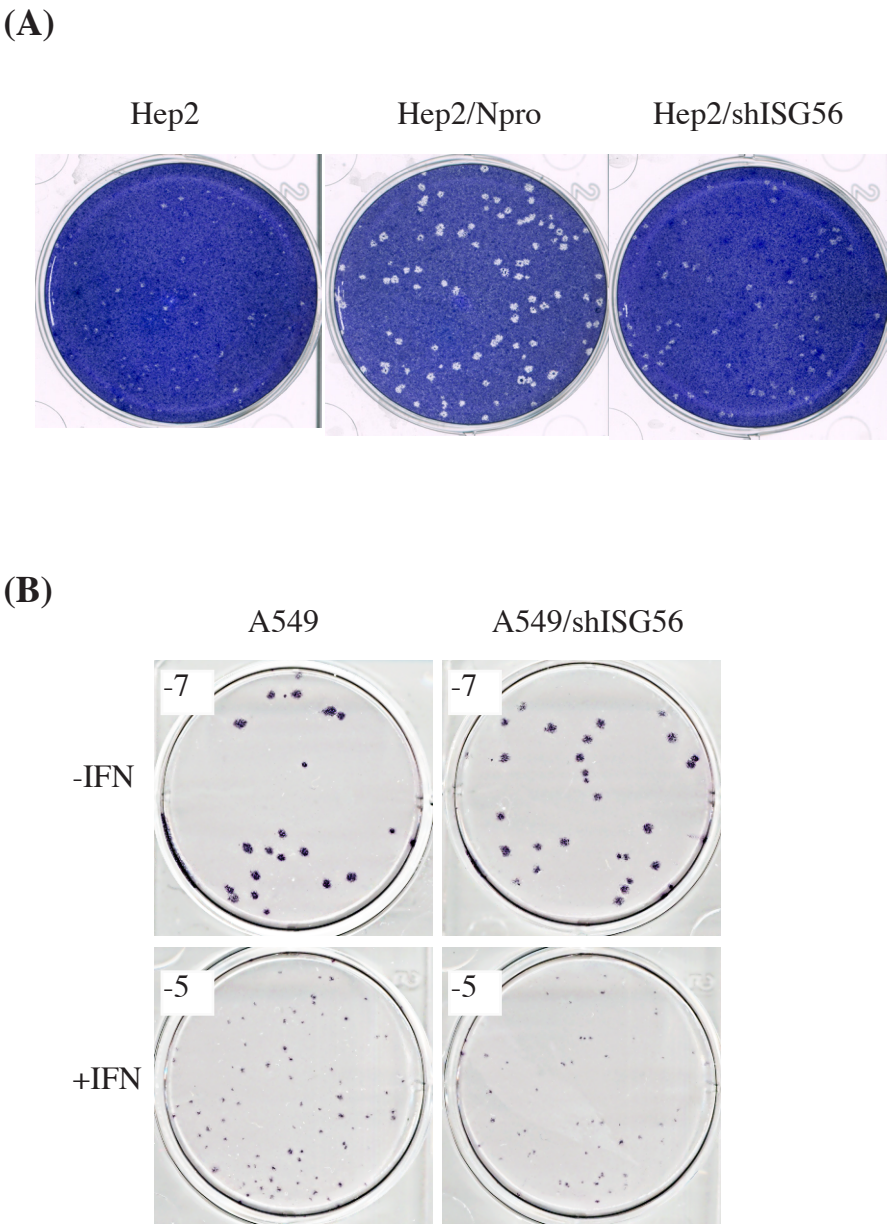


Fig. 3.20. Effect of ISG56 knockdown on plaque formation of PIV5 and FLUAV. (A) Plaques of PIV5 formed on monolayers of Hep2, Hep2/Npro, and Hep2/shISG56. Note Hep2/Npro cells cannot produce IFN in response to virus infection as BVDV-Npro targets IRF-3 for degradation (Hilton et al., 2006Down).Hep2/shISG56 was generated by lentivirus expressing shRNA against ISG56. Cells were fixed at 6 days p.i., and plaques were visulized by staining with crystal violet. (B) FLUAV plaque formation on naïve A549 cells or cells with ISG56 knockdown (A549/shISG56) were treated with 1000u/ml IFN for 16 hours or left untreated. Monolayer of cells were infected with FLUAV and the virus were allowed to form plaques under 1% agarose. Plaque were visualized by immunostaining with polyclonal antibody against FLUAV. The number on the top left corner of each well in Panel B indicates the folds of dilution of original virus stock for infection.

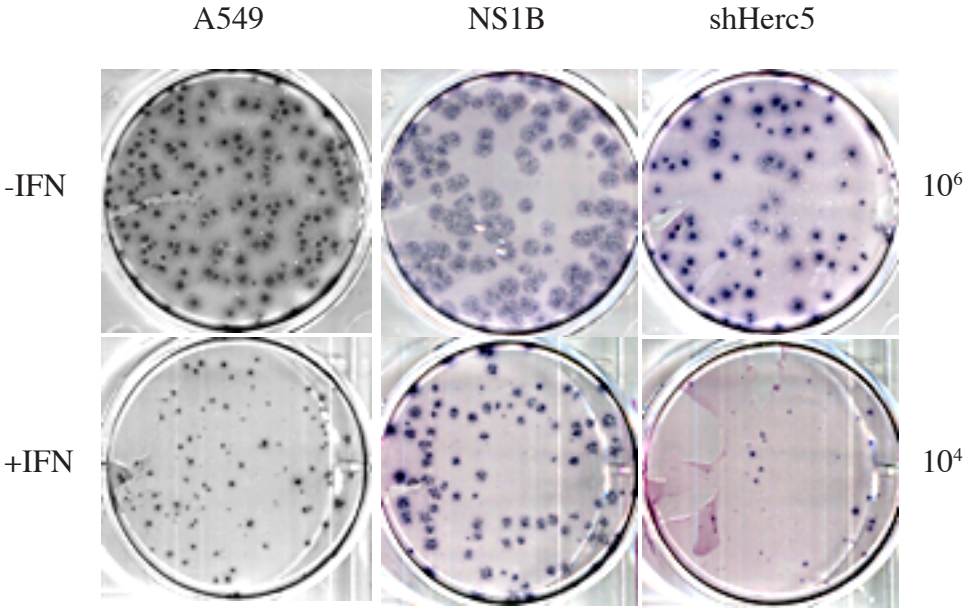


Fig. 3.21. Effects of ISGylation on IFN-mediated plaque reduction. Naïve A549 cells or cells knocking down E3 (shHerc5) of ISGylation pathway or cell line overexpressing influenza B NS1 gene (which prevent ISGylation) were treated with 1000u/ml IFNα for 16 hours or left untreated. Monolayer of cells were infected with FLUAV and the virus were allowed to form plaques under 1% agarose. Plaque were visualized by immunostaining with polyclonal antibody against FLUAV.

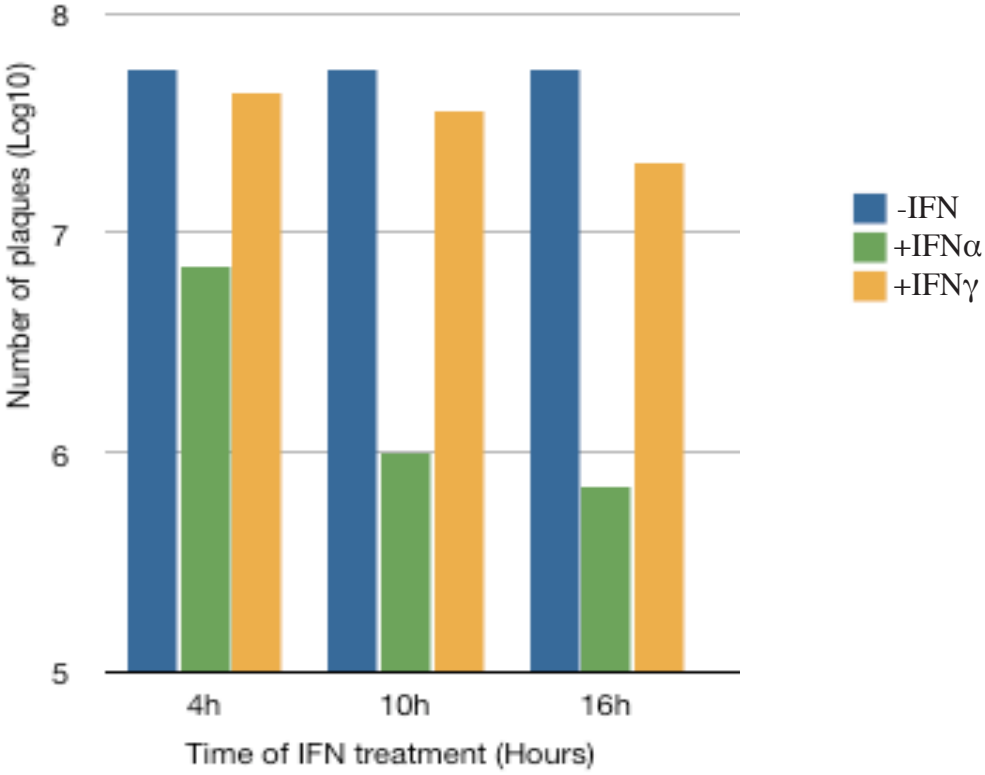


Fig. 3.22. IFNγ does not have an obvious effect on influenza virus plaque reduction in contrast to IFNα. Naïve A549 cells were treated with 1000u/ml IFNα, IFNγ for the indicated time or left untreated. Monolayer of cells were infected with influenza A virus and the virus were allowed to form plaques under 1% agarose. Plaque were visualized by immunostaining with polyclonal antibody against influenza A virus. Plaques numbers were counted and and plotted.