IDENTIFICATION OF HOST FACTORS IN SWINE RESPIRATORY EPITHELIAL CELLS THAT CONTRIBUTE TO HOST ANTI-VIRAL DEFENSE AND INFLUENZA VIRUS REPLICATION

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ABSTRACT

Swine influenza viruses (SIV) are a common and an important cause of respiratory disease in pigs. Pigs can serve as mixing vessels for the evolution of reassortment viruses containing both avian and human signatures, which have the potential to cause pandemics. NS1 protein of influenza A viruses is a major antagonist of host defence and it regulates multiple functions during infection by interacting with a variety of host proteins. Therefore, it is important to study swine viruses and NS1-interacting host factors in order to understand the mechanisms by which NS1 regulates virus replication and exerts its host defense functions. Influenza A viruses enter the host through the respiratory tract and infect epithelial cells in the respiratory tract, which form the primary sites of virus replication in the host. Thus, studying SIV infection in primary swine respiratory epithelial cells (SRECs) would resemble conditions similar to natural infection.

The objectives of this study were to identify NS1-interacting host factors in the virus-infected SRECs and to understand the physiological role of at least one of the factors in influenza virus infection. The approaches to meet this objective were to generate a recombinant SIV carrying a Strep-tag in the NS1 protein, infect SRECs with the Strep-tag virus, purify NS1-interacting host protein complex from the infected cells by pull-down using strep-tactin resin and then study the physiological role of one of the NS1-interacting partners during influenza infection. Using a reverse-genetics strategy, a recombinant virus carrying the Strep-tag NS1 was successfully rescued and the SRECs were infected with this recombinant virus. The Strep-tag in the NS1 protein facilitated the isolation of an intact NS1-interacting protein complex and the proteins present in the complex were identified by liquid chromatography-tandem mass spectrometry. The identified proteins were grouped to enrich for different functions using

bioinformatics. This gave an insight into the different functions that NS1 may regulate during infection and the potential host partners involved in these functions.

Among the host proteins identified as potential interaction partners, RNA helicases were particularly of interest to study. Influenza being an RNA virus, RNA helicases could have important functions in the virus life cycle. Among the identified RNA helicases, DDX3 has been shown to regulate IFNβ induction and affect the life cycle of a number of viruses. However, its function in influenza A virus life cycle has not been studied. Hence, this study explored whether DDX3 has any role in the influenza A virus life cycle. Immunoprecipitation studies revealed viral proteins NP and NS1 as direct interaction partners with DDX3. DDX3 is a known component of stress granules (SGs) and influenza A virus lacking the NS1 gene is reported to induce SG formation. Therefore, the role of DDX3 in SG formation, induced by PR8 influenza A virus lacking NS1 (PR8 del NS1) was explored. The results from this study showed that DDX3 co-localized with NP in SGs indicating that DDX3 may interact with NP in the SGs. NS1 protein was found to inhibit virus-induced SGs and DDX3 downregulation impaired virus-induced SG formation. The contribution of the different domains of DDX3 to viral protein interaction and virus-induced SG formation was also studied. While DDX3 helicase domain did not interact with NS1 and NP, it was essential for DDX3 localization in virus induced SGs. Moreover, DDX3 downregulation resulted in the increased replication of PR8 del NS1virus, accompanied by an impairment of SG induction in infected cells.

Since DDX3 is reported to regulate IFNβ induction, the role of DDX3 in influenza A virus induced IFNβ induction was also examined. Using small molecule inhibitors and siRNA-mediated gene knockdown, the RIG-I pathway was identified as the major contributor of

influenza induced IFNβ induction in newborn porcine tracheal epithelial (NPTr) cells. DDX3 downregulation and overexpression also showed that DDX3 has an inhibitory effect on IFNβ expression induced by both influenza infection and low molecular weight (LMW) poly I:C treatment, which is also a RIG-I ligand. RNA competition assay to identify the mechanism of DDX3-mediated inhibition, showed that RIG-I binding affinity to its ligands LMW poly I:C and influenza viral RNA (vRNA) is much higher than that of DDX3. Furthermore, DDX3 downregulation enhanced titers of the PR8 del NS1 virus, while it did not affect the titers of the wild-type strains of PR8 and SIV/SK viruses. Overall, the results show that DDX3 has an antiviral role and the SG regulatory function of DDX3 has a profound effect on virus replication than the IFNβ regulatory function.

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ABBREVIATIONS

25-HC	25-Hydroxycholesterol
AA	Amino acid
AMP	Antimicrobial peptides
APC	Antigen presenting cell
ASC	Apoptosis-associated speck-like protein containing a caspase recruitment domain
BEGM	Bronchial epithelial growth medium
bp	base pairs
BPIC	Biotinylated LMW poly I:C
CARD	Caspase activation and recruitment domain
cDNA	Complementary deoxyribonucleic acid
CPSF	Cleavage and polyadenylation specificity factor
CPSF 1	Cleavage and polyadenylation specificity factor 1
CPSF 2	Cleavage and polyadenylation specificity factor 2
CPSF30	Cleavage and polyadenylation specificity factor 30
CRK/CRKL	v-CRK avian sarcoma virus CT10-homolog/v-CRK avian sarcoma virus CT10-homolog-like
CRM1	Chromosome region maintenance 1
cRNA	Complementary RNA

C_t	Cycle threshold
CTD	C-terminal domain
CTLs	Cytotoxic T lymphocytes
CV	Column volume
DAMP	Danger associated molecular pattern
DAPI	4',6-diamidino-2-phenylindole
DC	Dendritic cell
DDX	DEAD (Asp-Glu-Ala-Asp) box helicase
DDX1	DEAD (Asp-Glu-Ala-Asp) box helicase 1
DDX21	DEAD (Asp-Glu-Ala-Asp) box helicase 21
DDX3	DEAD (Asp-Glu-Ala-Asp) box helicase 3
DDX6	DEAD (Asp-Glu-Ala-Asp) box helicase 6
DHX9	DEAH (Asp-Glu-Ala-His) Box Helicase 9
Dlg1	Disks large homolog 1
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dsRNA	Double stranded RNA
DTT	Dithiothreitol
Dvl	Dishevelled
EDTA	Ethylenediaminetetraacetic acid

eIF	Eukaryotic translation initiation factor
eIF2α	Eukaryotic translation initiation factor-2alpha
eIF4A	Eukaryotic translation initiation factor 4A
eIF4E	Eukaryotic translation initiation factor 4E
eIF4GI	Eukaryotic translation initiation factor 4GI
EJC	Exon junction complex
FACS	Fluorescence activated cell sorting
FBS	Fetal bovine serum
FISH	Fluorescence *in situ* hybridization
FLB	Flag lysis buffer
FXR1	Fragile X mental retardation syndrome-related protein 1
G3BP	Ras GTPase-activating protein
GAS	Gamma activated sequences
GCN2	General control nonderepressible 2
GTP	Guanosine triphosphate
h.p.i.	Hours post infection
h.p.t.	Hours post transfection
HA	Hemagglutinin
HBV	Hepatitis B virus
HCV	Hepatitis C virus
HIV	Human immunodeficiency virus

hnRNP	Heterogenous nuclear ribonucleoprotein
hnRNP A2/B1	Heterogenous nuclear ribonucleoprotein A2/B1
hnRNPF	Heterogenous nuclear ribonucleoprotein F
hnRNPU	Heterogenous nuclear ribonucleoprotein U
HRI	Heme-regulated translation inhibitor
HRP	Horseradish peroxidase
HSP40	Heat shock protein 40
IFITM	IFN inducible transmembrane protein
IFN	Interferon
IFNAR	Interferon alpha receptor
IFNGR	Interferon gamma receptor
IFNLR	Interferon lambda receptor
IFNα	Interferon alpha
IFNβ	Interferon beta
IFNγ	Interferon gamma
IFNλ	Interferon lambda
IgG	Immunoglobulin G
IKK	Inhibitor of nuclear factor kappa-B kinase
IKKα	Inhibitor of nuclear factor kappa-B kinase alpha
IKKε	Inhibitor of nuclear factor kappa-B kinase epsilon
IL-10	Interleukin-10

IL10Rβ	IL-10 receptor beta
IL-12	Interleukin-12
IL-18	Interleukin-18
IL1-α	Interleukin 1-alpha
IL-1β	Interleukin-1 beta
IL28Rα	Interleukin-28 receptor alpha
IL-6	Interleukin-6
IL-8	Interleukin-8
ILF3	Interleukin enhancer binding factor 3
IP	Immunoprecipitation
IPS-1	IFNβ promoter stimulator protein 1
IRES	Internal ribosome entry site
IRF3	Interferon regulatory factor 3
IRF7	Interferon regulatory factor 7
ISG15	Interferon stimulated gene 15
ISGF3	IFN-stimulated gene factor 3
ISGs	Interferon stimulated genes
ISREs	Interferon stimulated response elements
JAK	Janus-activated kinase-signal transducer interferon regulatory factor
JEV	Japanese encephalitis virus
JMEM	Joklik’s modified minimum essential medium

JNK-ATF2	c-Jun N-terminal kinase-Activating transcription factor 2
KD	Knockdown
LC-MS/MS	Liquid chromatography-tandem mass spectrometry
LGP2	Laboratory of genetics and physiology 2
Lin7C	Lin-7 homolog C
LMW	Low molecular weight
LRR	Leucine rich repeat
M1	Matrix protein 1
M2	Matrix protein 2
M42	matrix protein 42
MAGI	membrane-associated guanylate kinase
MAVS	Mitochondrial antiviral signaling
MDA5	Melanoma differentiation-associated protein 5
MDCK	Madin-Darby canine kidney
MEM	Minimal essential medium
MHC	Major histocompatibility complex
MOI	Multiplicity of infection
mRNA	Messenger ribonucleic acid
Mx1	Myxovirus resistance 1
Mx2	Myxovirus resistance 2
Myd88	Myeloid differentiation primary response gene 88

NA	Neuraminidase
NaAs	Sodium arsenite
NCR	Non-coding region
NFκB	Nuclear factor kappa beta
NF-κB	Nuclear factor-kappa B
NK	Natural killer
NLR	Nod-like receptor
NLRP3	NOD-like receptor, pyrine domain containing protein 3
NLS	Nuclear localization signal
NOD	Nucleotide oligomerization and binding domain
NoLS	Nucleolar localization signal
NP	Nucleoprotein
NPTr	new born porcine tracheal epithelial
NS1	Non-structural protein 1
NS2/NEP	Non-structural protein 2/Nuclear export protein
NS3	Non-structural protein 3
NXF1	Nuclear RNA export factor 1
OAS	2’5’-oligoadenylate synthetase
ORF	Open reading frame
PA	Polymerase acidic
PABP1	Polyadenylate-binding protein 1

PAMP	Pathogen associated molecular pattern
PA-N155	Polymerase acidc-N155
PA-N182	Polymerase acidic-N182
PA-X	Polymerase acidic protein-X
PB1	Polymerase basic 1
PB1-F2	Polymerase basic 1- F2
PB1-N40	Polymerase basic 1-N40
PB2	Polymerase basic 2
PBM	PDZ-binding domain
PBS	Phosphate-buffered saline
PCR	Polymerase chain reaction
pDC	Plasmacytoid Dendritic cell
PERK	PKR-like endoplasmic reticulum kinase
PFA	Paraformaldehyde
PFU	Plaque forming unit
PI3K	Phosphatidylinositol 3-kinase
PKR	Protein kinase R
pol	Polymerase
PPP	Triphosphate
PRR	Pathogen recognition receptor
PYD	Pyrine domain

qPCR	Quantitative real-time PCR
RD	Repressor domain
RHAU	RNA associated with AU-rich element
RIG-I	Retinoic acid-inducible gene 1
RLR	RIG-I like receptor
RNA	Ribonucleic acid
RNP	Ribonucleprotein complex
RPL19	Ribosomal protein L19
rRNA	Ribosomal RNA
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SG	Stress granule
SiOT	Off-target siRNA
siRNA	Small interfering RNA
siRNA-OT	Off-target siRNA
SIV	Swine Influenza virus
SK	Saskatchewan
snRNA	Small nuclear RNA
SRECs	Swine respiratory epithelial cells
ssRNA	Single stranded RNA
STAT	Signal transducers and activators of transcription
Sw	Swine

TAP	Tip-associated protein
TBK1	Tank binding kinase 1
TBST	Tris buffered sodium chloride solution with 0.1% Tween-20
TC	Tetracysteine
THOC	THO complex
THOC4	THO complex 4
TIA-1	T-cell intracellular antigen-1
TIAR	TIA-1 related
TLR3	Toll-like receptor 3
TLR7	Toll-like receptor 7
TNFR	Tumor necrosis factor receptor
TNF-α	Tumor necrosis factor alpha
TRIF	TIR domain containing adaptor protein inducing IFNβ
TRIM	Tripartite interaction motif
tRNA	Transfer RNA
TYK	Tyrosine kinase
UAP56	ATP-dependent RNA helicase uap56
UTR	Untranslated region
UV	Ultraviolet
VACV	Vaccinia virus
vRNA	Viral RNA

vRNP	Viral ribonucleoprotein complex
WB	Western blot
WNV	West Nile virus
WT	Wild-type

CHAPTER 1: LITERATURE REVIEW

1.1 Influenza virus

1.1.1 Classification

Influenza viruses belong to the family of *Orthomyxoviridae.* This family is composed of RNA viruses, which are negative-sense, single, stranded and have a segmented genome. The viruses in this family are divided into six different genera, namely influenza A, B and C viruses, Thogotovirus, Isavirus and Quaranfilvirus. All influenza viruses are further classified based on the antigenic nature of their surface glycoproteins Hemagglutinin (HA) and Neuraminidase (NA) (461). Different subtypes of HA and NA are distinguishable serologically, in that the antibodies of one virus subtype do not react with another subtype (461). So far, 18 different HA subtypes (H1 to H18) and 11 different NA subtypes (N1 to N11) (155, 538, 586) have been identified for influenza A viruses, while influenza B HA and NA are each classified into two lineages, Victoria-like and Yamagata-like (49).

Different influenza viruses are named according to their genus, the species from which they were isolated (not included when isolated from humans), location of the isolate, the number of the isolate, the year of isolation and the HA and NA subtypes (only if it is an influenza A virus). For example, the nomenclature A/Sw/SK/18789/02 (H1N1) represents the type A influenza virus isolated from pigs (Sw-swine) in Saskatchewan (SK) as a virus isolate 18789, isolated in the year 2002 with the HA and NA subtype H1 and N1 respectively.

1.1.2 Virion and genome structure

Influenza virus is an enveloped virus and possess an outer layer lipid membrane derived from the host cell in which it multiples. The morphology of the virion is pleomorphic and can be

spherical (80-120 nm in diameter) or filamentous (up to several micrometers in length). Fresh clinical preparations are characterized by a significantly higher proportion of the filamentous virion (90), while multiple passages in egg and tissue culture renders the virion with a spherical morphology (87, 277). Reassortment and genetic studies in virus strains forming filamentous and spherical virions show that mutations within influenza A virus M1 and M2 protein affect the morphology of the virus particles (62, 125, 452). The surface glycoproteins HA, NA and Matrix protein 2 (M2) are embedded in the outer lipid membrane and project from the surface of the virion. The HA and NA projecting from the surface impart the virions the distinctive spikes that are readily observable in electron micrographs of negatively stained virus particles. The spikes are usually 10-14 nm long and are distributed at a ratio of 1: 4 (NA: HA) on the virion's envelope. Underneath the lipid membrane, Matrix protein 1 (M1), which is the most abundant structural component of the virion, forms a shell and associates with the ribonucleoprotein complex (RNP) (56, 393). The proteins M1 and M2 modulate the filamentous and spherical morphology of influenza A viruses (62, 71). The RNP complex forms the core of the virus particle and is made up of viral RNA (vRNA), nucleoprotein NP and polymerase proteins; polymerase basic 1 (PB1), polymerase basic 2 (PB2) and polymerase acidic (PA) (34). Nuclear export protein/non-structural protein 2 (NEP/NS2) has also been reported to be present in purified viral preparations (446) (Fig. 1.1).

The eight vRNA segments of the influenza A virus genome range in size from 890 to 2341 bases and contain conserved sequences at the 3' and 5' ends flanking the coding region (112). These conserved regions, comprising 13 nucleotides at the 5' end and 12 nucleotides at the 3' end, display partial and inverted complementarity and form the core promoter region, which is important for regulation of virus replication. The core promoter is associated with the

heterotrimeric RNA polymerase containing PB1, PB2 and PA in the virion (139, 282). Additionally, each vRNA segment is encapsidated by multiple copies of the NP protein forming a twisted rod-like structure (400). The secondary structure within the core promoter has been studied extensively, leading to the development of several models, which includes the panhandle model (221), RNA fork model (141) and corkscrew model (139). These secondary structures have been shown to be critical for polymerase binding, endonuclease activity and polyadenylation (67, 308, 309, 437).

The influenza A virus genome consists of 8 vRNA segments that encode up to 17 proteins. The vRNA segments and the proteins derived from each segment are illustrated in Fig 1.2. RNA segment 1 of the influenza A virus encodes the PB2 protein, which is an important component of the heterotrimeric virus polymerase complex and a major virulence determinant of influenza A viruses. The PB2 protein binds the 5'-cap of host pre-mRNA molecules and thus plays a key role in transcription initiation (186). The PB2 protein also plays a role in replication, since a single amino acid mutation has been reported to specifically affect replication but not transcription (160). The PB2 protein also affects virus pathogenicity and host range, as the amino acid at position 627 majorly contributes to this phenotype (295, 490, 507). Furthermore, PB2 regulates antiviral innate immunity by interacting with mitochondrial antiviral signaling protein (MAVS) and inhibiting MAVS-mediated beta interferon (IFNβ) expression (180).

The PB1 subunit of the RNA polymerase complex is encoded by RNA segment 2 of influenza A virus. The PB1 protein is responsible for the elongation of RNA during viral transcription and replication by catalyzing the sequential addition of nucleotides to the newly synthesized RNA chain (63, 175). The PB1 protein contains four highly conserved regions, each

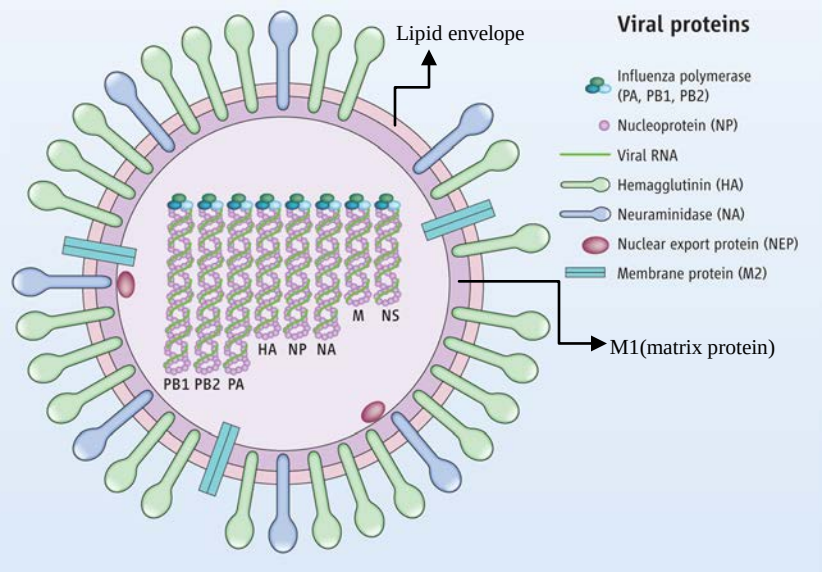

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Figure 1.1 Influenza A virus genome structure and organization. A schematic diagram of the structure of the influenza A virus particle is depicted. The eight single stranded RNA segments of antisense orientation encapsidated with NP comprise the genome. The genome is surrounded by a matrix made up of M1 protein. The viral polymerase complex (PA, PB1, PB2) are associated with the vRNA segments. The surface proteins HA, NA and the ion channel protein M2 are embedded in the lipid envelope of viral membrane and NEP is found associated with the virion.

ranging in size from 4-13 amino acids, which form a large functional domain important for polymerase activity (89). Additionally, PB1 is essential for the formation of the structural backbone and catalytic activities of the RNA polymerase (89). PB1 mRNA encodes two other proteins PB1-F2 and PB1-N40 by an alternate open reading frame (ORF) (83, 581). PB1-F2 is a multifunctional protein and varying sizes have been reported in different influenza strains (77). Its functions include direct or indirect roles in the apoptosis of immune cells via the mitochondrial pathway, exacerbation of pathogenicity in animal models, modulation of innate immune response in both *in vitro* and *in vivo* models, regulation of polymerase activity and enhancement of secondary bacterial infections (77). The PB1-N40 protein is translated from the fifth AUG codon of PB1 mRNA and lacks the first 39 AA of PB1 responsible for binding to the polymerase subunit PA (581); however, the precise function of PB1-N40 in influenza virus life cycle is unknown. The third RNA segment of influenza A virus encodes the third RNA polymerase subunit PA. The PA protein possesses the endonuclease activity of the viral polymerase, required to generate the capped primer necessary for viral mRNA transcription initiation (114, 604). Also the PA protein has been shown to possess proteolytic activity with two amino acid residues S624 and T157 present in the active site (194, 428, 458). RNA segment 3 encodes three additional proteins; PA-X by a ribosomal frameshift, PA-N155 and PA-N182 by using alternative translation initiation sites (234, 387). The PA-X protein possesses the endonuclease domain of the PA protein but lacks the C-terminal domain necessary for binding to other RNA polymerase subunits (234). Thus, the PA-X subunit is believed to be involved in host-shutoff through its endonuclease activity (273). However, the exact molecular mechanisms involved in PA-X-mediated host shut-off remains unknown and needs further study. PA-N155 and PA-N182 are N-terminal truncated forms of PA (387). Mutant viruses lacking PA-N155

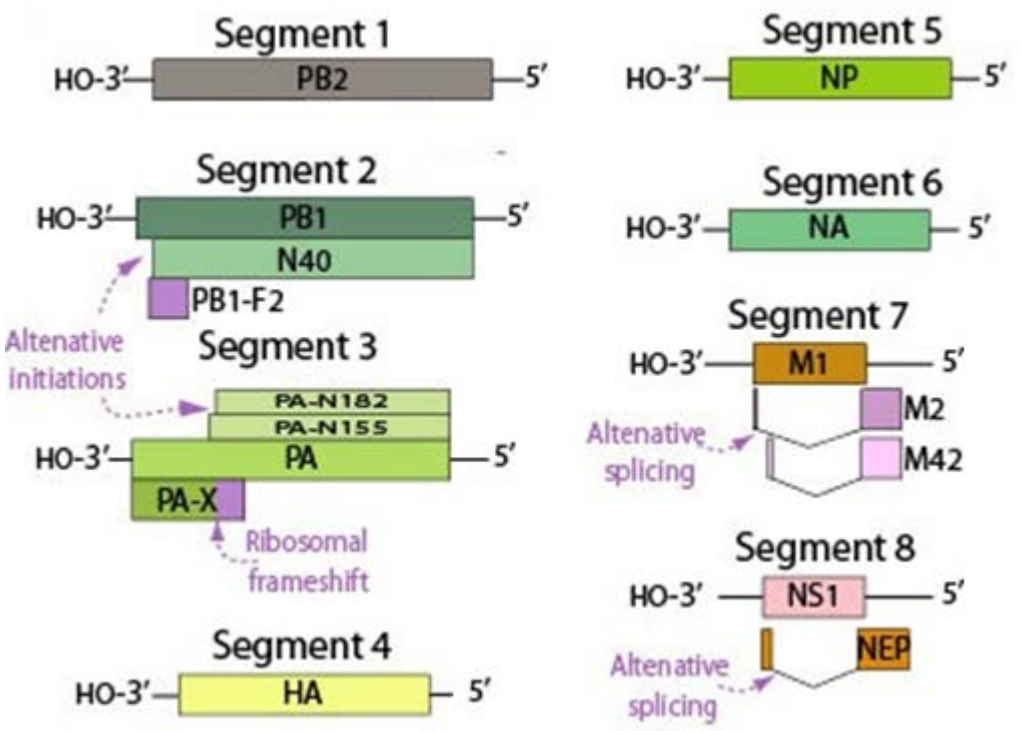

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Figure 1.2 Proteins encoded by 8 segments of the influenza A virus genome. RNA segment 1 encodes one protein, PB2. Segment 2 encodes three proteins, full-length PB1, PB1-F2 and PB1- N40 by alternate open reading frame (ORF). Segment 3 encodes the full-length PA, PA-N182 and PA-N155 by alternate ORF, PA-X by ribosomal frame shift. Segments 4, 5 and 6 each encode one full-length protein namely HA, NP and NA respectively. Segment 7 encodes full-length M1, two additional proteins M2 and M42 via alternative splicing. Segment 8 encodes three proteins, full-length NS1 protein, NEP protein by alternative splicing and NS3 also generated by alternative splicing (not described in this figure).

demonstrated slower replication kinetics and less pathogenicity in mice, while mutant viruses lacking PA-N182 replicated as efficiently as the wild-type virus (387). Even though PA-N155 affects virus replication, the exact function and mechanism are unknown. Hence, the exact function of the N-terminal truncated PA proteins, PA-N155 and PA-N182, warrants further study and understanding.

The major surface glycoprotein HA of influenza A virus is encoded by RNA segment 4. The primary function of the HA protein is attachment to the sialic acid receptors and fusion activities. In virus-infected cells, HA is co-translationally translocated across the rough endoplasmic reticulum membrane and forms a precursor protein called HA0 (164). Viruses with the HA0 precursor remain non-infectious until being cleaved at the monobasic cleavage site by host trypsin-like proteases, found in the respiratory and gastrointestinal tracts, into HA1 and HA2 (94, 303). Some avian viruses of the H5 and H7 subtype possess multiple basic amino acids at the cleavage site, which is susceptible to cleavage by ubiquitous proteases, resulting in systemic spread and high pathogenicity in the host (132, 503, 558). Once the HA protein binds to the receptor, the virus is endocytosed and the low pH of the endosome triggers a dramatic conformational change. The cleaved HA facilitates a pH-mediated fusion of the endosomal and viral membrane, releasing the contents of the virion into the cytoplasm (494). The HA protein through its transmembrane domain mediates lipid raft association on the cell membrane, which is important for virus budding and replication (454). Influenza HA recognition of the sialic acid receptor is a key determinant of host range (427). Besides functioning in receptor binding and fusion, HA is a major antigenic determinant of influenza A viruses. Mutations in the HA protein caused by antigenic drifts and antigenic shifts can lead to the emergence of novel strains resulting in annual epidemics and sometimes global pandemics (528).

RNA segment 5 of influenza encodes the NP protein. The NP protein is the structural unit of RNPs, covering the entire vRNA except at the ends. The NP protein is important for nuclear and cytoplasmic trafficking of RNPs. After uncoating, the RNPs have to be transported to the nucleus for transcription (210). This is achieved through recognition of the NP nuclear localization signal (NLS) by the cellular cargo protein karyopherin α (404). NP also interacts with M1 to form the NEP-M1-RNP complex necessary for CRM1-mediated nuclear export of vRNP to the cytoplasm (3). Newly synthesized NP is important for the regulation of the switch between transcription and replication (560). Furthermore, amino acids associated with MxA resistance have been identified in the NP protein of pandemic viruses, implicating NP function in evading innate immunity (354).

The second major surface glycoprotein on the influenza A virus envelope is the NA protein, encoded by RNA segment 6. The primary role of NA is to release newly synthesized viral particles from the host cell by cleaving the sialic acid receptors from the cell surface, thereby preventing virus aggregation and permitting virus spread (561). Additionally, NA cleaves the sialic acids from respiratory tract mucins and helps the virus reach and bind to the target cells during infection (364).

RNA segment 7 encodes three proteins; the matrix protein M1 and the ion channel proteins M2 and M42. M1 is the most abundant protein in influenza A virions and forms a shell surrounding the core RNP. Meanwhile, M2 is a proton channel that opens in response to the low pH of the endosome upon virion endocytosis, allowing proton influx into the virion and triggering dissociation of the viral RNP from the matrix proteins (218). M1 facilitates the export of newly synthesized RNPs into the cytoplasm by interacting with both RNP and NEP (3).

Several studies have shown the importance of M1 protein in virus budding. M1 interacts with both the plasma membrane and the RNPs facilitating vRNP recruitment into the budding virions (454). M1 may also crosslink the cytoplasmic tails of HA and NA mediating their incorporation into the budding virion. In addition, M1 interaction with the cytoplasmic tails of HA and NA allows M1 to associate with lipid rafts and triggers its polymerization at the virus budding site (174, 465).

In addition to binding HA, NA and NP, M1 also binds to the M2 protein and the M2 cytoplasmic tail plays an important role in virus assembly and budding (454). The M1-M2 interaction is important for filament formation of the virion and mutations in the M2 cytoplasmic tail result in the impaired recruitment of vRNPs to the budding virion (454). Besides these functions, membrane scission and the release of budding virion from the host cell membrane also requires the M2 protein (453). The M42 protein is expressed by alternative splicing and is a M2 variant synthesized during infection with M2-null viruses (582). The M42 protein functionally complements the M2 protein, supporting efficient replication of the M2-null viruses (582).

RNA segment 8 encodes three proteins, NS1, NS2 (NEP) and NS3. Alternative mRNA splicing generates NEP and NS3 proteins. The NS1 protein is produced abundantly throughout the whole replication cycle and is encoded by the unspliced mRNA derived from RNA segment 8. The NS1 protein is a major virulence factor and is a multifunctional protein interacting with a variety of host cell factors (189). It is a key protein used by influenza A viruses to counteract host antiviral defence mechanisms and is a well-studied type I IFN antagonist (189, 355). Through its multiple interactions with cellular and viral proteins, the NS1 protein exerts several functions in the infected cell, all of which favour virus replication. These multiple functions

include IFN antagonism, regulation of viral replication and polymerase activity, enhancement of viral mRNA translation, regulation of apoptosis, regulation of splicing and mRNA export (355).

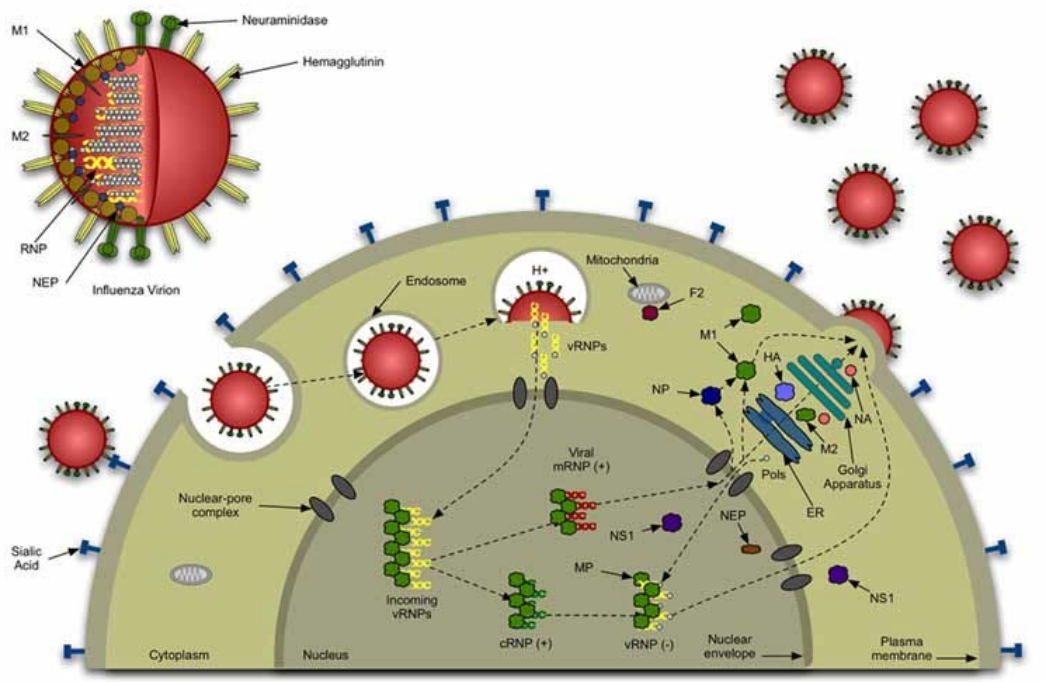
The NEP protein is expressed by the alternative splicing of segment 8 mRNA. It interacts with the M1 protein and functions in the export of vRNP complexes from the nucleus to the cytoplasm (3). NS3 is another protein encoded by segment 8 mRNA via alternative splicing. The NS3 protein is not expressed by all the influenza A virus strains and the specific mutation which is responsible for the expression of NS3 protein has been found in only 33 natural influenza A strains (476). It is speculated that the NS3 protein could be associated with adaptation to mice and other hosts, but it has not been proven yet (476).

1.1.3 Influenza A virus life cycle

Influenza A virus life cycle (Fig. 1.3) starts with the attachment of the HA protein on the virion to some of the sialic acids on the cell surface. Linkages between the sialic acids and the carbohydrates in the cell surface glycoproteins can be of two types: α (2, 3) or α (2, 6). The HAs of human viruses preferentially bind to sialic acids with the α (2, 6) linkage, while HAs of avian viruses preferentially bind to sialic acid with the α (2, 3) linkage (93). Pigs contain both sialic acid linkages, which makes pigs an ideal mixing vessel for the reassortment of human and avian viruses (348). However, this specificity is not absolute, since avian and human cells have been reported to contain both sialic acid linkages (363). Recent studies suggest that HA receptor specificity may not be as simple as differentiating between two types of sialic acid linkages. Several other factors such as chain length, sulfation and fucosylation may also play a role in HA interaction with the receptor (85, 502).

After binding receptors on the cell surface, influenza A virus is taken into the cell via receptor-mediated endocytosis. Clathrin-mediated endocytosis has been the most studied and is considered the major entry pathway of influenza A viruses (362, 423). However, recent studies show that influenza A virus can also enter through a non-clathrin mediated pathway resembling micropinocytosis, which is the main route for non-selective uptake of extracellular fluid by cells (109). The endosome has a low pH of approximately 5-6, which aids in the fusion process. The low pH of the endosome causes a conformational change in the cleaved HA, which exposes the fusion peptide of HA2 subunit. This fusion peptide inserts itself into the endosomal membrane, bringing the viral and endosomal membranes into contact with each other, resulting in fusion (188, 197). In the endosome, another important transformation occurs inside the virion due to low pH. The transmembrane domain of the M2 protein on the lipid envelope forms an ion channel, which allows the influx of protons into the virion from the endosome (432). This acidifies the viral core releasing the vRNPs from M1 interactions (433). Fusion of the viral envelope with the endosomal membrane and acidification of the core results in uncoating of the virion and releases the vRNPs into the cytoplasm.

Influenza A viruses are one of the few RNA viruses, where genome replication and transcription take place inside the nucleus (210). Hence, the vRNPs must get into the nucleus for normal virus replication to proceed. The vRNPs are considered too large to allow for passive diffusion into the nucleus and hence must rely on active nuclear import mechanisms. The NLS on NP has been found to be both essential and sufficient for vRNP nuclear import. This transport occurs through the recognition of NP NLS by a cargo protein called importin α (96).



Squires, R Burke. (2009) Influenza Life Cycle, http://www.reactome.org/figures/influenza_life_cycle_overview.jpg (18th December 2015).
See Appendix II for documentation of permission to republish this material

Figure 1.3 Influenza A virus life cycle. The life cycle of influenza A virus begins with the surface protein Hemagglutinin (HA) binding to sialic acid receptors on the host cell. The virion is internalized in the endosome and the low pH of endosome cause the fusion of the viral and endosomal membrane and the viral RNP is released into the cytoplasm. The viral RNP is transported into the nucleus and transcription and replication take place in the nucleus. The viral mRNA is exported from the nucleus and is translated to make new viral proteins by the host translational machinery. The vRNA is exported from nucleus and is assembled along with the viral proteins including the surface proteins inserted into the budding lipid envelope. Budding proceeds and the virus is released after the NA on the virion surface cleaves the inhibitory sialic acids on the host cell surface.

`Once in the nucleus, vRNA is initially used as a template for virus mRNA and cRNA synthesis (379). Viral mRNAs are incomplete copies of the template, transcription of which is dependent on the availability of 5'-capped cellular pre-mRNAs. The polymerase PB2 protein binds to the 5'-cap of cellular pre-mRNAs and the endonuclease function of PA protein cleaves the pre-mRNAs approximately 10 to 13 nucleotides from their 5'-caps (114, 130). This 5'-capped segment is used as the primer and the PB1 protein catalyzes chain elongation and continues until it reaches a stretch of uridines approximately 16 nucleotides before the 5'end of vRNA, where it stutters resulting in the addition of poly(A) tail (63, 322). In contrast to mRNAs, cRNAs are uncapped full-length copies of vRNA without a poly(A) tail. Hence, the mechanism of transcription has to be different. Nevertheless, how the polymerase switches and coordinates exactly between the two modes of transcription and replication is not fully understood. Several mechanisms have been proposed to explain the switch, but it has been suggested that the availability of soluble NP might be an important trigger for the switch (38, 560). During the early stages of influenza A virus life cycle, synthesis of NP and NS1 mRNAs is favoured, which might lead to the accumulation of NP and trigger the switch from transcription to replication (203, 479).

Next, newly synthesized vRNA is coated with NP and the polymerase complex. The interaction of vRNPs with NEP and M1 facilitates their export from the nucleus to the cytoplasm (3). The HA and NA proteins are post-translationally modified and are directed onto lipid rafts on the apical plasma membrane (454). M2 binds to the raft periphery and brings together several other raft domains. M2 and M1 association with the vRNPs result in the accumulation of all the viral components to the plasma membrane (454). This initiates the process of budding and the viral envelope separates from the cell membrane. Finally, the enzymatic activity of the NA

protein removes the sialic acid receptors from the host cell surface preventing virus aggregation (491) and the virus is released to infect another viable cell and continue its spread.

1.1.4 Evolution and genetics of influenza A viruses

RNA viruses possess low-fidelity RNA polymerases lacking the exonuclease proofreading ability and hence are inherently error-prone (216, 500). This results in very high mutation rates at the nucleotide level, but these high mutation rates may not always result in changes at the amino acid level. This is because of different selective pressures and evolutionary constraints on both the virus genes and the virus species. For example, although the nucleotide mutations occur at a similar frequency in all hosts, the amino acid mutations are very low in avian viruses infecting wild aquatic birds and thus they evolve very slowly, since they are well adapted to their hosts (37, 575). However, the evolutionary rates in human and mammalian viruses are much higher and the viral proteins continuously accumulate amino acid substitutions (575). Evolutionary rates among different proteins of the same virus species may also differ. For example, the M1 and M2 genes of human viruses have been shown to evolve very differently, with the M2 gene evolving much more rapidly when compared to the M1 gene, which showed almost no accretion of amino acid changes over a 55-year period (229, 575). The rate of evolution varies depending on the nature of the selective pressure and the evolutionary constraints. Surface proteins such as HA and NA might be subject to selective pressure by the host immune system, while internal proteins such as NP may not face any selective pressure by the immune system, but undergo host-specific adaptive evolution (152, 176, 276).

The genetic changes that drive evolution of influenza A viruses may occur by three different mechanisms: 1) mutations due to RNA polymerase errors, 2) reassortment and

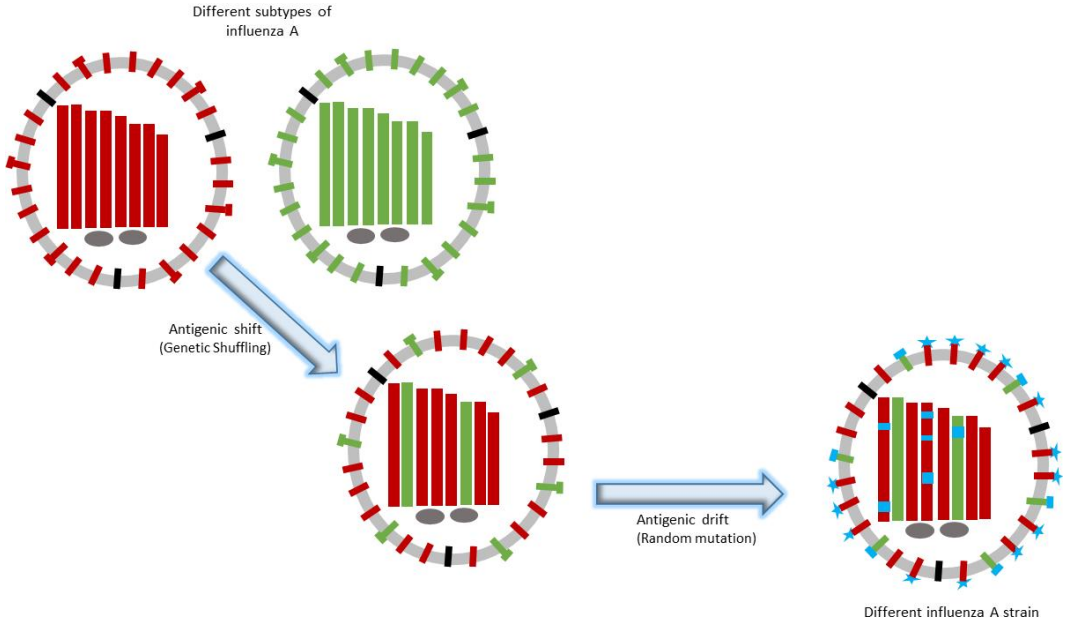


Figure 1.4 Influenza A virus antigenic shift and antigenic drift. Antigenic shift. When two or more influenza A viruses co-infect a host, there is a potential for the gene segments to be exchanged (reassortment), which could result in a novel virus with a new set of HA and NA genes. When reassortment results in a virus with a new set of HA and NA subtypes, it is called antigenic shift and has the potential to cause pandemic, when the vast majority of the host has not encountered this new virus subtype previously. **Antigenic drift.** As influenza A viruses replicate, the genes undergo random mutations due to the error prone viral polymerase. When these random mutations occur in the surface proteins, it results in new antigenically distinct strains of the existing subtype and is called antigenic drift. When these mutations are selected by the host favourably for virus replication, it has the potential to cause epidemics.

3) recombination. As discussed earlier, influenza A virus RNA polymerase is highly error prone with mutation rates ranging from approximately 1×10^{-3} to 8×10^{-3} substitutions per site per year (82). Hence, with each round of replication more and more point mutations are introduced into the replicated genome, which may have a neutral, positive or negative effect on the replication and survival of the virus. Many of these changes may not be viable, but those that are advantageous could become dominant due to various selective pressures encountered by the virus. The effect of these gradual changes are more apparent when these mutations accumulate in the antigenic domains of surface glycoproteins HA and NA, since these proteins are under a strong selective pressure from the host immunity (82). When these mutations cause positive selection of the HA and NA variants by escaping from neutralizing antibodies, it results in the generation of a new antigenic variant, which is able to evade host immunity. This phenomenon of change in the antigenicity of the surface glycoproteins due to gradually accumulated mutations is called antigenic drift (74) (Fig. 1.4). Consequently, as a result of antigenic drift the virus can no longer be neutralized by the host antibodies raised against the parent strain, allowing the virus to spread more readily among the population, resulting in epidemics (572). Mutations in the HA or NA amino acid sequence occur at a frequency of less than 1% per year and the antigenic drift occurs on average every 2-8 years (74). While minor changes in the antigenicity of viruses is detectable at any time, changes large enough to reflect on hemagluttination-inhibition assays accumulate over several years (106, 368, 556).

When compared to the slow and gradual changes that occur during antigenic drift, reassortment causes abrupt changes in the genome content of the virus. When two or more viruses co-infect a host or cell at the same time, there is a potential for the viruses to exchange gene segments. This phenomenon of rearrangement of viral gene segments occurring when a cell

is infected with two or more genomic variants of influenza viruses is called reassortment (Fig. 1.4) (513). From co-infection with two different viruses, reassortment can result in 2^8 or 256 gene variants, which shows that this process can result in significant genetic diversity. These variants under the right selective pressure could be selected to evade immunity or adapt to a new host resulting in major pandemics. When reassortment results in the introduction of a HA or/and NA subtype that are antigenically distinct from the circulating influenza variant into a population, it is called antigenic shift (Fig. 1.4) (499). The importance of gene reassortment in the evolution of novel viruses is highlighted by the past and recent pandemics. Viruses responsible for Asian influenza in 1957 and Hong Kong influenza in 1968 were reassortant viruses, containing avian origin HA, PB1 and NA segments and avian origin HA and PB1 segments in a human genetic background respectively (265, 304, 469). The 2009 pandemic H1N1 virus was also a result of reassortment. It was the result from reassorment of a triple reassortant virus with the Eurasian avian-like swine viruses. The triple reassortant viruses were a result of genetic exchange between the North American H3N2 virus and H1N2 swine viruses. As a result, the 2009 pandemic virus possessed PB2 and PA genes of North American avian virus origin, a PB1 gene of human H3N2 virus origin, HA (H1), NP, and NS genes of classical swine virus origin, and NA (N1) and M genes of Eurasian avian-like swine virus origin (395).

Besides point mutations and reassortment, the viral genome can also evolve by recombination. Recombination can occur between two virus genomes or between the virus genome and the host. In influenza viruses, recombination occurs by template switching, where the polymerase jumps from one template to the other and then continues transcribing, thus producing RNA with genetic information combined from two different segments. Although recombination in influenza is rare, both types of recombinations have been reported and have

resulted in an evolutionary advantage for the virus. For example, insertion of 54 nucleotides of 28S rRNA into an avian influenza virus HA gene and a 60-nucleotide insertion sequence from NP gene into a seal influenza virus HA gene, increased HA cleavability (274, 410). Other examples include conversion of a low pathogenic avian influenza virus to a high pathogenic variant after insertion of 21 nucleotides of the M segment or 30 nucleotides of the NP segment into the HA segment (421, 506). Viruses produced by recombination events can cause pandemics. For example, a recent phylogenetic analysis of the HA gene from the 1918 pandemic virus suggested that it may have been generated by recombination, where the sequence corresponding to the stalk region originated from a human-lineage influenza virus, while the globular domain of HA1 polypeptide originated from a swine virus (168).

1.2 Overview of innate immunity and virus evasion strategies

Innate immune response provides the first line of immunological defence against infection. Innate immunity provides a broad range of protection using receptors and molecules that are non-specific to a particular pathogen but recognize unique patterns in microbial or viral components common to most pathogens known as pathogen-associated molecular patterns (PAMPs). The host recognizes these PAMPs using membrane bound or soluble germline-encoded receptors called pattern-recognition receptors (PRRs). Three main classes of PRRs encoded by vertebrate hosts are Toll-Like Receptors (TLRs), Retinoic acid inducible gene-I (RIG-I)-like receptors (RLRs) and Nucleotide oligomerization and binding domain (NOD)-like receptors (NLRs). Additionally, lectin receptors also serve as a PRR in activating innate-immunity during infection with certain viruses containing glycoproteins on the virion surface (332). Some of the major PRRs, ligands recognized and their cellular distribution are listed in Table 1.1.

1.2.1 Pathogen recognition receptors (PRRs) and pathogen associated molecular patterns (PAMPs)

1.2.1.1 Toll-like receptors (TLRs)

Among the PRRs known today, TLRs were the first to be identified and were first recognized in *Drosophila* to be important for defense against fungal infections (315). Subsequently several mouse and human homologs of the *Drosophila* Toll receptor were discovered, 10 in humans (TLR1 – TLR10) and 12 in mouse (TLR1-TLR9 and TLR11-TLR13) and hence were called Toll-like receptors (TLRs) (263, 519). The TLRs are type I transmembrane proteins that are composed of an amino-terminal leucine-rich repeat-containing ectodomain responsible for PAMP recognition, a transmembrane domain and a cytoplasmic carboxy-terminal Toll-interleukin-1 receptor homology (TIR) domain that relays downstream signalling events (5). TLR1, TLR2, TLR6 and TLR10 reside on the plasma membrane and recognize microbial cell wall and membrane components such as lipoprotein and peptidoglycans (263). TLR4 and TLR5 also localize to plasma membrane and recognize bacterial lipopolysaccharide (LPS) and flagellin respectively (263). TLR2, 4 and 6 recognize fungal components such as zymosan, mannan and β-glucan as well (263). TLR3, TLR7, 8 and 9 are localized in the endosomes and recognize different nucleic acid ligands. TLR3 recognizes double-stranded RNA (dsRNA), TLR7 and 8 recognize single stranded RNA (ssRNA) and TLR9 engages unmethylated CpG DNA (6, 8, 263). TLR11 and TLR12 form a heterodimer to sense profilin form the parasite *Toxoplasma gondii* (18), while TLR13 detects bacterial 23S ribosomal RNA (323, 407). Differences in the signal transduction pathway exist among the TLRs, being either myeloid differential primary response 88 (MyD88)-dependent or TIR-domain containing

Pattern Recognition Receptor	Microbial ligand recognised	Cellular distribution
TLR2/TLR1, TLR2/TLR6	Lipoproteins and glycolipids of bacterial origin, viral envelope proteins and viral proteins released into extracellular space	Plasma membrane
TLR3	dsRNA, poly I:C	Endosome
TLR4	Lipopolysaccharide (LPS), flagellin, viral glycoproteins	Plasma membrane
TLR5	Flagellin	Plasma membrane
TLR7/8	viral ssRNA	Endosome
TLR9	Bacterial and viral unmethylated CpG DNA	Endosome
TLR10	Unknown	Plasma membrane
RIG-I	RNA containing dsRNA regions of approximately 20 nt with blunt ends containing a 5'-ppp moiety, low molecular weight poly I:C, short (25 bp) and long dsRNA (>200 bp) with 3' or 5' monophosphate	Cytoplasm
MDA5	High molecular weight poly I:C, long dsRNA (1-2 kb) and short dsRNA (100 nt)	Cytoplasm
NLRP3	leakage of ions from intracellular compartments, reactive oxygen species (ROS),activation by dsRNA through indirect mechanisms	Cytoplasm
Mannose-binding Lectins	High Mannose and Fucose	Secreted
SP-A, SP-D	Mannosylated and glycosylated proteins	Secreted
Galectins	Glycans containing β-galactoside	Secreted

Table 1.1 Pattern recognition receptors (PRRs) critical during viral infection, their ligands and cellular distribution.

adaptor inducing interferon-β (TRIF)-dependent (4, 261). All the TLRs except TLR3 use the MyD88-dependent pathway, which signals only through TRIF (588). TLR4 is unique in that it uses both the MyD88 and TRIF-dependent pathways for signalling (261, 518, 588).

Viral components are known to be sensed by several TLRs during influenza infection. However, TLR3 and TLR7 are the predominant TLRs important for sensing viral RNA. As discussed above, TLR3 senses dsRNA in endosomes. However, several studies have shown that no dsRNA is generated during influenza virus replication (431, 573) and that this is due to the helicase activity of cellular protein UAP56 (583). Therefore it is speculated that TLR3 recognizes unidentified RNA structures present in the dying infected cells, which are phagocytosed by dendritic cells (DCs) (233, 474). During Influenza A virus infection, activation of TLR3 has been shown to be more proinflammatory than antiviral in that, more IL-6, IL-8 and less IFNβ was induced (307). Indeed $TLR3^{-/-}$ mice have been shown to have a survival advantage when compared to wild-type (WT) mice following lethal influenza virus infection, in spite of having higher viral loads and lower viral clearance (306). This shows that these proinflammatory cytokines are essential for the host to efficiently clear the virus. Similar to $TLR3^{-/-}$ mice, $TLR4^{-/-}$ mice have also been shown to survive better than the WT mice upon influenza A virus infection (226, 397). Interestingly, the virus does not produce the ligand sensed by TLR4 but the infected cell does. It was shown that oxidative phospholipid OxPAPC (226) and S100A9 (543) protein produced in response to acute lung injury caused by influenza A virus infection can act as a non-PAMP or as a danger associated molecular pattern (DAMP) and stimulate the TLR4-TRIF pathway to induce proinflammatory cytokines.

Plasmacytoid dendritic cells (pDCs) produce high levels of IFNα upon infection with the WT virus (68, 75, 143, 236, 389). In pDCs, TLR7 recognizes ssRNA genome in the endosome after the virus is taken up (115, 344). Endosomal acidification is required for this recognition and does not require viral replication. Signalling through this pathway induces production of type I IFN and pro-inflammatory cytokines in a MyD88-dependent manner (115, 344).

TLR10 is the only known TLR receptor in the family without a known agonist, function or signalling pathway. However, a role for TLR10 as an innate immune sensing receptor in influenza A virus infection has recently been reported (314). It was found that H5N1 virus infection induced robust TLR10 expression when compared to infection with a seasonal H1N1 virus A/HK/54/98 and that both the virus replication and de novo protein synthesis is required for TLR10 induction (314). H5N1 and H1N1 infection of primary human macrophages and a monocyte cell line induced robust expression of type I and III IFNs and proinflammatory cytokines IL-6 and IL-8 in a TLR10-dependent manner (314). The authors also reported enhanced vRNP-induced IL-8 induction when TLR10 was exogenously expressed.

1.2.1.2 RIG-I (retinoic acid inducible gene-I) like receptors

RIG-I like receptors (RLRs) are cytosolic sensors of viral RNA. Three central members of the RLR family are RIG-I, melanoma differentiation factor 5 (MDA5) and laboratory of genetics and physiology 2 (LGP2). They are present in the cytoplasm of most cell types and are strongly induced by IFNs (521). The RLRs are very similar to TLR3 in that both detect viral RNA and induce type I IFN and proinflammatory cytokines (8, 474). However, the fundamental difference between the two is that the TLR3 and other endosomal TLRs recognize viral nucleic acids that have reached the endosomes through endocytosis (518), while the RLRs being located

in the cytosol detect intracellular viral RNA from actively replicating viruses (260). The other difference is that the RLRs are active in most cell types, while the TLRs are restricted to specialized immune cells such as macrophages and dendritic cells (254).

The RLRs belong to the DExD/H-box (Asp-Glu-x-Asp/H) family of helicases and are characterized by a central ATPase containing a DExD/H box helicase domain (345). RIG-I and MDA5 contain a N-terminal CARD (caspase activation and recruitment domain) domain which mediates downstream signalling, while LGP2 lacks a CARD domain (599, 600). All three receptors have a C-terminal domain (CTD); RIG-I and LGP2 harbor a repressor domain (RD) in the CTD, while MDA5 does not. Because of the RD, RIG-I adopts a closed auto-inhibited conformation where the CARDs are sterically unavailable for signal transduction (91, 135, 289). Hence, its overexpression in the absence of an activating ligand does not result in signalling. MDA5 on the contrary does not have a RD and hence is believed to adopt an open conformation (44).

Even though RLRs share similar structural domains, they differ in their ligand binding specificities. The RIG-I ligand comprises an RNA molecule with a 5’ triphosphate (PPP) moiety and blunt-ended base-paired region of approximately 20 nt. The base pairing at the 5’ end could be due to secondary RNA structures such as hairpin or panhandle conformations on individual ssRNA (219, 431, 462, 463). Although 5’PPP-ssRNA is a ligand for RIG-I, RNAs lacking a 5’PPP have also been reported to act as RIG-I agonists. Both short (25bp) dsRNA with a 3’ or 5’ monophosphate group and long (>200bp) dsRNA lacking terminal triphosphates have been reported to activate RIG-I (50, 517). Also, low molecular weight poly I:C (300-1000bp) and short dsRNA generated by T7 polymerase induces IFN in a RIG-I-dependent manner (255). The

requirement for 5’PPP moiety is believed to provide a mechanism of self-discrimination by RIG-I. This is because the 5’PPP in cellular transcripts is either masked by a 7-methyl-guanosine cap (in case of mRNA) or is removed before export from the nucleus (in case of tRNA and rRNA). Distinct from RIG-I, MDA5 detects high molecular weight poly I:C (1000bp-8000bp), long dsRNA (1-2 kb) and short dsRNAs (approx. 100nt) when present in large quantities (44, 45, 133, 426, 585). It has been reported that MDA5 similar to RIG-I is able to discriminate self-RNA from non-self RNA using a specific molecular signature at the 5’ end. The 5' cap structures of eukaryotic mRNAs have ribose 2'-O-methylation and many viruses that replicate in the cytoplasm of eukaryotes have evolved 2'-O-methyltransferases to modify their mRNAs. It was shown that coronavirus mutants deficient in 2’-O-methyltransferases induced higher type I IFN expression in a MDA5-dependent manner (611). This observation suggests that RNA modifications such as 2'-O-methylation provide a molecular signature for the discrimination of self and non-self mRNA.

As observed with the differential recognition of RNA species, RIG-I and MDA5 detect mostly non-overlapping groups of viruses. RIG-I has been reported to induce innate immune signalling in response to infection by negative strand RNA viruses such as paramyxoviruses, orthomyxoviruses, rhabdoviruses such as vesicular stomatitis virus, Ebola virus and EBER RNA carried by Epstein-Barr virus (72, 256, 457, 598, 611). MDA5 senses positive strand RNA viruses such as picornaviruses (171, 256), while certain flaviviruses induce signalling via both RIG-I and MDA5 (79, 147, 256, 337, 508).

The third RLR LGP2, which lacks the CARD domain, binds dsRNA independent of 5’-PPP and also differentially regulates RIG-I and MDA5 signalling (435). In another study, the

binding specificity of LGP2 was studied using surface plasmon resonance (516). It was reported that dsRNA and 5'PPP ssRNA both bound to LGP2, while ssRNA without a 5'PPP bound LGP2 with a lower affinity (516). While LGP2 lacking the tandem CARD domain was initially believed to be a negative regulator, gene disruption studies show that it may be a positive regulator (553, 600). Hence, the exact ligand and the role of LGP2 in innate immunity to virus infection are still unclear.

Among the RLRs, RIG-I is important for influenza detection in epithelial cells, macrophages and conventional DCs (254). RIG-I has been shown to detect full length 5'PPP ssRNA viral genome (444), preferentially binding to shorter genomic segments as well as subgenomic defective interfering particles (35). Interestingly, a smaller U/A-rich region within the 3' UTR of influenza genomic RNA induces IFN in a 5'PPP-independent manner (103). Despite these findings, it remained unknown whether the RIG-I ligand were naked RNA products generated during virus replication or whether the nucleocapsid encapsidated RNA could be recognized by RIG-I. Recent studies showed that RIG-I indeed interacts directly with the panhandle structure of incoming viral nucleocapsids, undergoes a conformational switch, oligomerizes and triggers the activation of IRF3 inducing IFN production (328, 574).

1.2.1.3 NOD like receptors (NLRs)

NLRs comprise a large receptor family of 22 members in humans and are characterized by a conserved nucleotide oligomerization and binding domain (NOD) motif (198, 228). The proteins in this group share a common domain organization containing one of several amino-terminal protein-protein interaction domains such as the CARD, pyrin domain (PYD), and baculovirus inhibitor repeat (BIR) domain, followed by an intermediary NOD domain. The

carboxyl terminus consists of a varying number of leucine-rich repeat (LRR) motifs with proposed functions of detecting PAMPs and danger associated molecular patterns (DAMPs), thus leading to NLR activation.

NLRs are subdivided into four subfamilies namely NLRA, NLRB, NLRP and NLRC based on the N-terminal effector domains (537). The NLRP and NLRC subfamilies comprise the larger number of members of the NLR family (20 proteins in humans). The NLRC subfamily consists of six members: NLRC1, NLRC2, NLRC3, NLRC4, NLRC5 and NLRX1. The proteins in this subfamily have one or two N-terminal CARD domains, which recruit caspase-1 or kinases to mediate downstream signalling. However, NLRC3, NLRC5 and NLRX1 are grouped into this subfamily due to their phylogenetic relationship, homology and have undefined N-terminal domains (385). NLRC1 and NLRC2 are stimulated following bacterial infection and they sense a specific muropeptide (diaminopimelic acid) and muramyl dipeptide from peptidoglycan respectively (169, 170). NLRC3 inhibits TLR4-dependent activation of NF-κB by interacting with TRAF6 to attenuate Lys63-linked ubiquitination of TRAF6 and activation of NF-κB (466). NLRC4 senses bacterial flagellin to activate caspase-1 and IL-1β (146, 377). NLRC5 is induced by IFNγ, LPS and Poly I:C (43). NLRC5 has a role in MHC-I expression and acts as a negative modulator of inflammatory pathways (43, 283). NLRX1 has been shown to regulate mitochondrial antiviral immunity (9, 383). Nevertheless, MAVS-dependent type I IFN response to poly I:C and sendai virus infection in NLRX1 knockout mice was similar to WT mice (23, 443, 495). Thus, the exact role of NLRX1 in MAVS-mediated innate immunity is still under investigation.

The NLRP subfamily comprises 14 proteins in humans, NLRP1-14 and is characterized by the presence of a N-terminal Pyrin domain (385, 537). Few members of this group have been studied in depth. Several NLRP family members such as NLRP1, NLRP2, NLRP3 and NLRP6 are key components of caspase-1 activated complexes called inflammasomes. They activate caspase-1 through the recruitment of ASC (Apoptosis–associated speck-like protein containing a caspase recruitment domain) to the pyrin domain of NLRPs, leading to the processing and release of IL-1β, IL-18 and secretion of leaderless proteins like pro IL1-α and Fibroblast growth factor (FGF)-2 (124, 270, 358, 360). However, many NLRPs also have a negative role in immune response. NLRP2 and NLRP12 suppress NFκB signaling (11, 142). NLRP4 negatively regulates type I IFN signalling by targeting TBK1 for degradation and suppresses autophagy by associating with beclin-1 (98, 245). NLRP10 has been shown to have anti-inflammatory activity, where it inhibited the auto-processing of caspase-1, caspase-1-mediated IL-1β processing and suppressed the aggregation of ASC (227). Besides this role, NLRP10 is also an essential receptor in initiating adaptive immunity by DCs (123). NLRP5, NLRP17 and NLRP14 are associated with functions in development and reproduction (292), while NLRP1, NLRP2, NLRP9 and NLRP11 play a role in autoimmune diseases (242, 351, 515).

NLRP3-mediated inflammasome activation has been demonstrated to be important for innate defence against influenza A virus infection. NLRP3 deficient mice were more susceptible to pathogenic virus infection when compared to wild-type mice (10, 532). The enhanced morbidity in NLRP3 deficient mice correlated with a decrease in immune cell recruitment, cytokine and chemokine induction, while there was no defect in the control of virus replication (10, 532). These studies suggest that NLRP3-mediated inflammasome is not involved in antiviral resistance, but increases disease tolerance through cellular recruitment and induction of tissue

repair in infected tissues (532). Inflammasome activation and cytokine production requires two signals. Signal 1 activates NFκB which induces the transcription and translation of pro-IL-1β, pro-IL18 and NLRP3 genes (510). This is called priming. Activation of NFκB is mediated by the ligands of TLR, IL-1 receptor and Tumor Necrosis Factor Receptor (510). Signal 2 is induced by signals resulting from host damage and this activates cleavage of caspase-1 leading to the secretion of mature IL-1β and IL-18 (510). It is believed that PAMP signals from commensal bacteria provide the signal 1 for inflammasome activation in the context of influenza A virus infection. Intact microbiota in mice provided signals for the expression of pro-IL-1β and pro-IL18 mRNA at steady state and mice treated with antibiotics displayed delayed viral-clearance and exhibit impaired innate and adaptive immune response against influenza A virus infection (1, 225). Several stimuli have been reported to provide the signal 2 for inflammasome activation during Influenza A virus infection. Influenza virus ssRNA, proton flux through virus encoded M2 ion channel in the trans-Golgi network and high-molecular weight PB1-F2 fibrils in the phagosomes of cells that have taken up dying virus-infected cells have all been reported to provide the signal 2 to activate the release of IL-1β and IL-18 (224, 367, 532).

1.2.1.4 Lectin receptors

Lectins belong to a highly diverse group of proteins that recognize carbohydrates on glycoproteins and glycolipids and are involved in protein modulation, cell growth and homeostasis (165, 338). Mammalian lectins are categorized into many families based on their carbohydrate recognition domains (CRDs), as C-type, I-type, L-type, M-type, P-type, R-type, F-box, chitinase-like lectins, galectins and intelectins (552). Some lectins, such as C-type lectins and galectins function as PRRs as they recognize viral components that are highly modified by

oligosaccharides and thus function in viral innate immunity (332). Mannose-binding lectin (MBL) belongs to the collectin family, which is a subgroup of C-type lectins. Characteristic of the collectin family MBL contains a cysteine-rich region, a collagen-like domain, a neck region and a CRD (529). MBL is biologically active in a tetramer form and functions as a soluble receptor in the complement cascade by recognizing carbohydrate patterns on the virus surface, leading to resistance against many viral infections (217, 484). Virus eradication does not occur by formation of membrane-attacking complex on the viral surface and is believed to be mediated by three mechanisms namely: 1) Virus neutralization by MBL-activated complement C3 and C4 deposition on the viral surface, 2) Direct virus neutralization by MBL and 3) Prevention of interaction between viruses and its receptors (148, 238, 498, 596). Surfactant proteins SP-A and SP-D are also members of the collectin family and defend the viral invasion of the respiratory system as they are primarily secreted in the respiratory tract and lungs (207, 349, 559).They prevent infection by influenza A viruses by binding to glycosylated HA and NA on the viral surfaces (199, 200, 530). Mechanisms of SP-A and SP-D mediated antiviral activity against influenza include, inhibition of viral entry by viral aggregation and enhancement of viral clearance by recruitment of macrophages and neutrophils (199, 200, 530). The surfactant proteins also bind to the respiratory syncytial virus (RSV) glycoproteins F and G on the surface of the virion and enhances opsonisation of the virions and modulates host immune response against RSV infection (31, 166, 182).

Galectins are a group of 15 proteins that are secreted and associate with specific cell surface glycans containing beta-galactosides (30). Galectin-1 is a well-studied member of this family and is secreted by immune cells such as T-cells and stromal cells surrounding B-cells (327). It has been reported to bind to the envelope glycoproteins F and HA of Nipah virus (NiV)

and influenza A virus respectively (159, 591). Binding of galectin-1 to NiV-F leads to reduced NiV-induced syncytium formation, while galectin-1-HA association leads to inhibition of hemagglutination activity (159, 591). However, galectin proteins also function as a proviral factor facilitating the viral entry of some viruses (157, 375, 584).

1.2.2 Effectors of innate immunity

1.2.2.1 Physical barriers of innate immunity

The principle components of innate immunity comprise of the physical barriers (skin, mucosal epithelia), the chemical barriers (antimicrobial peptides, cytokines), the innate immune cells (macrophages, DCs) and the components of humoral immunity such as complement factors. All of these are the effectors of innate immunity.

The mucosal epithelia can be considered as any tissue that is able to secrete mucus and antimicrobial products across an epithelial layer and is fortified by both innate and adaptive components of the immune system (370). The mucosal epithelial tissue is present in several sites including the respiratory tract, gastrointestinal tract, urogenital tract and eye conjunctiva and the epithelial cells present in these mucosal tissues form the main physical barriers providing innate defence.

Skin is the largest organ of the human body and provides an effective barrier to external assaults. Structurally, skin is made up of two layers, the outermost epidermis and inner dermis separated by a basement membrane. The uppermost layer of the epidermis, stratum corneum is comprised of keratinocytes, tightly linked by desmosomes in a hydrophobic cellular matrix forming the durable outer layers of the skin (2). Keratinocytes produce large quantities of the

protein keratin which forms the tough watertight surface of the skin and are also the main source of antimicrobial peptides (AMP) in the skin (2). Several types of AMP are produced in the skin and include cathelicidins, β-defensins, dermicidin and psoriasin, which have both antimicrobial and immunomodulatory functions (2). During a pathogenic insult or injury, recruited neutrophils, mast cells and other leukocytes produce the majority of AMP. Innate immune cells such as langerhan cells (specialized DC), macrophages, myeloid and plasmacytoid DCs and NK cells are also present in the lower parts of the epidermis (525).

1.2.2.2 Cells of innate immunity

The innate immune cells can be divided into two categories. Those that are phagocytic, such as neutrophils, macrophages and dendritic cells and those containing enzymatic granules such as eosinophils, basophils, mast cells and natural killer (NK) cells. Neutrophils are relatively short-lived lasting only 6-8 hrs in circulation, but are the most abundant and important circulating leukocytes (286). They are typically the first responders recruited to the site of infection. Upon infection, stimulated endothelial cells close to the sites of inflammation expose a class of molecules called selectins. The Neutrophils are captured by the selectin molecule and they roll along the endothelial wall following a chemokine gradient, which is then followed by integrin-mediated firm adhesion (286). The neutrophils then traverse through the endothelium and arrive at the site of inflammation, where they unleash their antimicrobial arsenal to eliminate the invading pathogen. Neutrophils exert their antimicrobicidal activity by releasing granules containing enzymes and AMP such as myeloperoxidase, NADPH oxidase, defensins, lysozyme, several proteases, cathepsins and lactoferrin (557). Neutrophils can also phagocytose pathogens through receptor-mediated endocytosis via PRRs or through opsonisation (550). Neutrophils are

also known to kill microbes through NETosis, where they undergo active cell death releasing decondensed chromatin containing histones, antimicrobial granular and cytoplasmic proteins into extracellular space (13, 284). These structures are thought to kill microbes by exposing them to high local concentrations of antimicrobial compounds (13).

The macrophage is an important cell-type involved in innate immunity. Depletion of alveolar macrophages in pigs infected with influenza virus has been reported to result in 40% mortality and severe respiratory signs when compared to infected control pigs (278). Upon infection, infected epithelial cells increase the production of CCL2, which facilitates monocyte and macrophage recruitment through their CCR2 receptor (209, 325). Activated macrophages secrete proinflammatory cytokines such as IL-6, IL-8 and TNFα (39). The alveolar macrophages can phagocytose virus particles and apoptotic infected cells. They also modulate the adaptive immune response to influenza virus infection (202, 223, 578).

NK cells destroy virus-infected cells without the need for previous antigen stimulation. They recognize the weakened expression of MHC class I molecules on the surface of infected cells, which normally bind to inhibitory receptors on the surface of NK cells (301, 334, 541). Additionally, NK cells expressing the main triggering receptors NKp46 and NKp44 have been reported to recognize the HA protein expressed on the cell surface of influenza-infected cells (22, 352). NK cells have also been shown to lyse influenza virus-infected cells by recognition of HA through NKp46 (352). NK cells can also mediate antibody-dependent cell-mediated cytotoxicity by recognizing the Fc portion of antibodies bound to virus-infected cells through CD16 receptor (201, 509). The importance of the NKp46 receptor and NK cells during influenza

infection is evident in a study, where mice deficient of NKp46 receptor equivalent NCR-1 displayed increased morbidity and mortality upon influenza virus infection (161).

DCs are the professional antigen presenting cells (APCs). During infection, they form an important bridge between the innate and adaptive immunity by presenting viral antigens to naïve and memory B and T lymphocytes (28, 185). During infection, DCs can acquire the antigen for presentation by two distinct mechanisms. First, DCs can be infected directly with influenza virus and the viral proteins can then be processed inside the cell, loaded onto MHC class I molecules and presented to virus specific $CD8^+$ cytotoxic T cells (CTLs) (28, 47, 595). The second mechanism is when DCs acquire antigens by phagocytosing virus particles and infected apoptotic cells, where the antigenic peptides are processed and loaded onto MHC class II (MHC-II) molecules and presented to $CD4^+$ helper T cells and $CD8^+$ CTLs by cross-presentation (549). Thus, DCs serve an important function in the innate immune response during viral infection.

1.2.2.3 Chemical mediators of innate immunity

IFNs are important chemical mediators of innate immunity and elicit distinct antiviral responses. They are grouped into three classes called type I, II and III IFNs, according to their amino acid sequence (442). In humans, type I IFNs comprise of multiple IFNα subtypes, one IFNβ gene and other genes of less well-defined roles such as IFN-ω, IFN-ε, IFN-τ, IFN-δ and IFN-κ, while IFNγ is the only member of type II IFN. IFNα/β are the major type I interferons secreted by cells in response to infectious agents, particularly viral pathogens. They have several major functions during infection. They are known to induce an antimicrobial state in neighbouring and infected cells via the induction of interferon stimulated genes (ISGs), modulate the innate immune response in a balanced manner and activate the adaptive immune system by

promoting antibody production and effector T cell responses (32). The Type III IFN gene family consists of three genes IFN-λ_1, λ_2 and λ_3 and is more closely related in structure and sequence to the cytokine IL-10 (117).

Type I IFNs can be produced by almost any type of cell in the body in response to stimulation, whereas haematopoietic cells, particularly pDCs specialize in its secretion (512). The first step in the production of type I IFNs is the recognition of microbial pathogens by cellular PRRs. Several PRRs including RLRs, TLRs and NLRs sense different microbial components as described previously. Pathogen sensing by the PRRs initiates a signalling cascade, which culminates in the activation of transcription factors IRF-3, IRF-7 and NFκB. The transcription factors then translocate to the nucleus, bind to the IFN gene promoter and induce transcription of the IFN gene. IRF3 and IRF7 are phosphorylated by cellular kinases and translocate to the nucleus after dimerization (262), whereas the IKK complex, consisting of IKK-α, IKK-β and IKK-γ, phosphorylates the NFκB inhibitor IκB leading to its proteasome-dependent degradation and release of functional NFκB to translocate to the nucleus (251). The type I IFNs can also be induced by host factors and cytokines such as TNF, which signals via IRF1 rather than through IRF3 and IRF7 (592). IFN-α/β exerts the antiviral effect in neighbouring and infected cells by binding and signalling through a heterodimeric transmembrane receptor termed the IFNα receptor (IFNAR), which is composed of two subunits IFNAR1 and IFNAR2. Type I IFN binding to IFNAR activates the receptor-associated protein tyrosine kinases Janus kinase I (JAK1) and Tyrosine kinase 2 (TYK2), which then phosphorylate the transcription factors signal transducer and activator of transcription 1 and 2 (STAT1 and STAT2) (27, 113). Phosphorylated STAT1 and STAT2 dimerize and translocate to the nucleus, where they assemble with IRF9 to form a trimolecular complex called IFN-stimulated gene

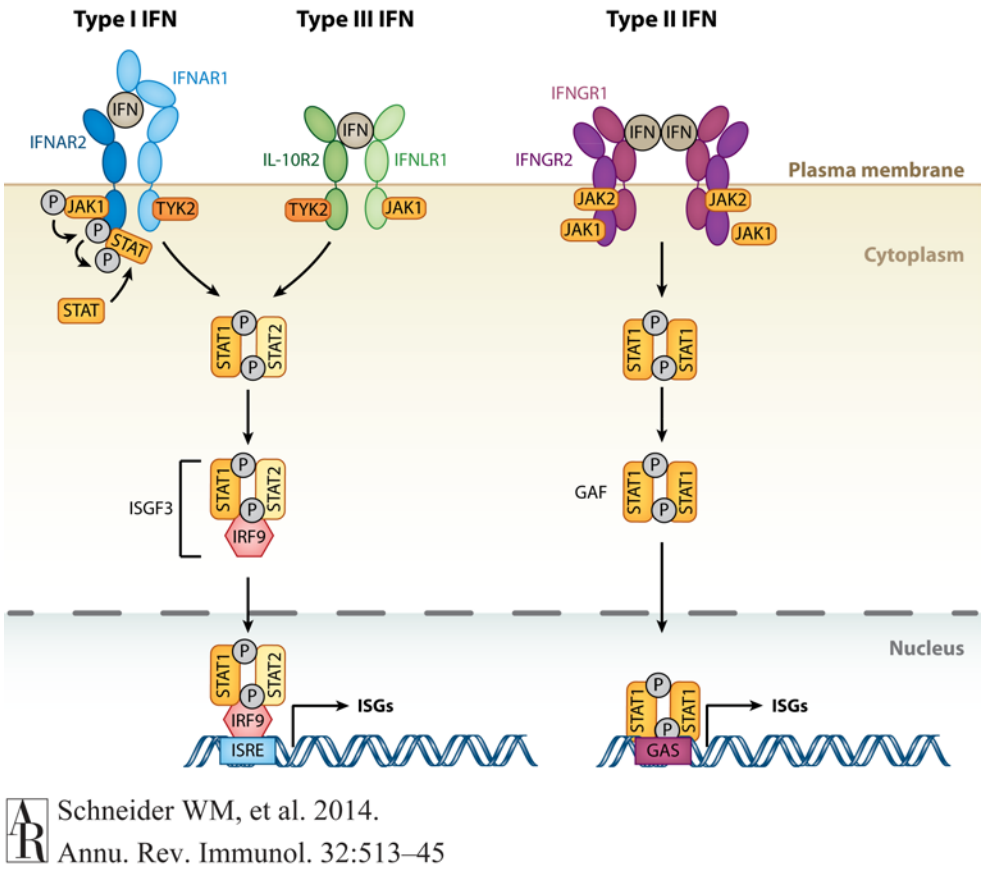


Figure 1.5 Receptors of type I, II and III IFNs. Distinct receptors are used by the three classes of IFNs. Type I IFNs signal through IFNα receptor (IFNAR), a heterodimer comprised of subunits IFNAR1 and IFNAR2. Type II IFNs signal through IFNγ receptor (IFNGR) comprised of subunits IFNGR1 and IFNGR2. Type III IFNs signal through IFNλ receptor (IFNLR), a heterodimer comprised of IL10Rβ and IFNLR1 subunits. Binding of the IFNs to their respective receptors trigger phosphorylation of the JAK and TYK kinases, which in turn phosphorylate the receptors at specific tyrosine residues leading to recruitment of STAT proteins and their phosphorylation. The STAT proteins dimerize and recruit other transcription factors to form a complex, which translocates to the nucleus and triggers transcription of genes regulated by IFN stimulated response elements (ISRE) and gamma-activated sequence (GAS) promoter sequences.

factor 3 (ISGF3). ISGF3 binds to DNA sequences known as IFN-stimulated response element (ISREs), activating the transcription of ISGs (Fig. 1.5) (468). The ISGs are primarily responsible for the antiviral effects of type I IFNs.

IFN-gamma (IFNγ) is the lone type II IFN and is known to be induced by many cell types such as macrophages, DCs, NK cells, NKT cells, $CD4^+$ T cells, $CD8^+$ T cells and B lymphocytes (371). The production of IFNγ is controlled by the cytokines IL-12 and IL-18, which are secreted by the APCs in response to infection (470). Even though IFNγ production is largely restricted to immune cells, IFNγ receptors (IFNGR) comprised of IFNGR1 and IFNGR2 chains are expressed by most cell types and hence are capable of responding to IFNγ (546). The IFNGR is made up of two ligand-binding IFNGR1 chains associated with two IFNGR2 chains that is involved in signal transduction (548). Upon IFNγ binding to IFNGR, kinases JAK1 and JAK2 bind to IFNGR1 and IFNGR2 subunits respectively and become tyrosine phosphorylated (371). Activated JAKs phosphorylate the IFNGR tails, which recruit the STAT1 monomers, which are again phosphorylated by the JAKs leading to STAT1 dissociation from the receptors and STAT1 homodimerization (371). The STAT1 homodimer translocates to the nucleus, binds to γ-activated sequence (GAS) elements to induce transcription of IFNγ responsive genes (Fig. 1.5) (371). Many of the transcribed genes are transcription factors and the major one is interferon response factor 1 (IRF1) (55). IFNγ secretion has many different immunomodulatory effects. It is involved in the upregulation of MHC-I molecules and in increasing the quality, quantity and repertoire of peptides loaded onto MHC-I (371). Other functions of IFNγ include upregulation of MHC-II molecules for efficient antigen presentation, development of a Th1 response and the activation of the microbicidal functions of macrophages (371). Type I and type II IFN are known

to induce the expression of a common set of ISGs as well as a distinct set of ISGs in order to exert its antiviral and immunomodulatory functions (331).

Type III IFNs or IFNλ consists of four proteins IFNλ_1 (IL-29), λ_2 (IL-28A), λ_3 (IL-28B) and λ_4. In contrast to type I IFNs, IFNλ was fairly recently discovered and shares many biological functions with type I IFN (288, 483). IFNλ expression is induced by the stimulation of the same PRRs that induce type I IFN expression, with one difference being the Ku70 DNA sensor that activates IFNλ but not the type I IFNs (305, 606). The IFNλ receptor (IFNLR) is a unique heterodimeric receptor which shares one subunit with the IL-10 family of cytokines called IL10Rβ and a second subunit specific to IFNλ called IFNLR1 or IL28Rα (Fig. 1.5) (288, 483). In contrast to IFNAR which is expressed by almost all cell-types, IFNLR expression is restricted to epithelial cells and hepatocytes and because of this, IFNλ provides the therapeutic benefits of type I IFN and yet avoid the side-effects that come with type I IFN treatment (305). In terms of the transcription factors that induce IFNλ expression, it has been reported that IRF and NFκB can induce IFNλ expression independently, which is different from the concerted action of several transcription factors required for type I IFN induction (536). The IFNLR signals through a similar JAK-STAT pathway as the IFNAR complex and induces a subset of the same ISGs (467). However the magnitude of IFNλ stimulated response is generally lower than type I IFNs and lasts for a longer duration with a delayed peak response (357).

1.2.3 Mode of action of IFNβ in inducing antiviral state

As discussed previously, IFNβ binding to its receptors triggers the transcription of a set of genes called ISGs. IFNβ inhibits virus replication and establishes an antiviral state through the

antiviral activity of these ISGs. ISGs affect various stages of a virus life cycle including virus entry, virus replication, virus translation, virus release and exit (Fig. 1.6).

1.2.3.1 Inhibitors of virus entry

Murine myxovirus resistance 1 (Mx1) and murine myxovirus resistance 2 (Mx2) proteins belong to a small family of dynamin-like large guanosine triphosphatases (GTPases) closely related to the dynamin GTPase family. The human proteins related to the murine Mx1 and Mx2 proteins are MxA and MxB respectively (425). They have inhibitory activity against several different viruses and act by blocking the early steps of viral replication cycle (190). The mouse Mx1 protein is localized in the nucleus and inhibits primary transcription of influenza viral mRNAs and exhibits higher inhibitory activity towards the longer transcripts corresponding to the viral polymerases PB2, PB1 and PA (424). In contrary, the human MxA protein is localized in the nucleus and does not affect viral mRNA transcription and polyadenylation. However, it interferes with intracytoplasmic transport of viral mRNAs, viral protein synthesis and translocation of newly synthesized viral proteins to the cell nucleus (424). A recent study reported that Mx1 interacts with the viral proteins PB2 and NP and interferes with PB2-NP interaction, thereby reducing the viral polymerase activity (555). Furthermore, the human MxA protein has also been reported to interact with the viral protein NP (544). The Mx2 protein on the other hand does not have any known antiviral activity towards influenza viruses (333), but inhibits Human immunodeficiency virus -1 (HIV-1) and HIV-2 (178, 249, 333). Mx2 prevents the nuclear entry of the reverse transcribed genome, thereby ultimately inhibiting chromosomal integration of the HIV genome, which is a key step essential for HIV replication (178, 249, 333).

Cholesterol 25-hydroxylase is an enzyme that catalyzes the secretion of oxysterol 25-Hydroxycholesterol (25-HC) and is upregulated in response to TLR activation and is an IFN inducible gene that promotes resistance to a variety of viral infections (36, 52, 330). 25-HC broadly inhibits the growth of several enveloped viruses by directly modifying the cell membrane and thereby blocking membrane fusion between the virus and the cell (330). 25-HC has also been shown to act in both autocrine and paracrine fashion in macrophages infected with influenza virus (52). In addition, 25-HC is involved in prenylation of proteins, an important modification affecting the function of several viral and cellular proteins (467).

The IFITM family of proteins consists of five members, IFITM1, IFITM2, IFITM3, IFITM5 and IFITM10 (26, 492). Among these IFITM1, 2 and 3 have been shown to be IFN inducible and are known to affect the replication of a variety of viruses including influenza A viruses, severe acute respiratory syndrome coronavirus, flaviviruses, dengue virus and West Nile virus (64, 222, 239, 468). The IFITM proteins are able to inhibit such a diverse array of viruses because of their localization in different compartments including the plasma membrane, the endosome and the lysosomes (131, 222, 319, 602). IFITM3 is the most potent IFITM family member in restricting influenza virus replication and a model has been suggested where IFITM3 prevents virus fusion trapping the virions in the endosomes, ultimately leading to their destruction in the lysosomes and autolysosomes (64, 131). Basal levels of IFITM3 restricted initial virus infection until IFN expression further reinforced the restriction by upregulating IFITM3 expression (64). Although the mechanism of restriction is unclear, a recent study showed that the entry of non-enveloped viruses such as reovirus that utilize the endosome-dependent entry mechanism was also inhibited by the action of IFITM3 (25). This study showed that acidification and subsequent proteolysis of the reovirus capsid was affected in IFITM3

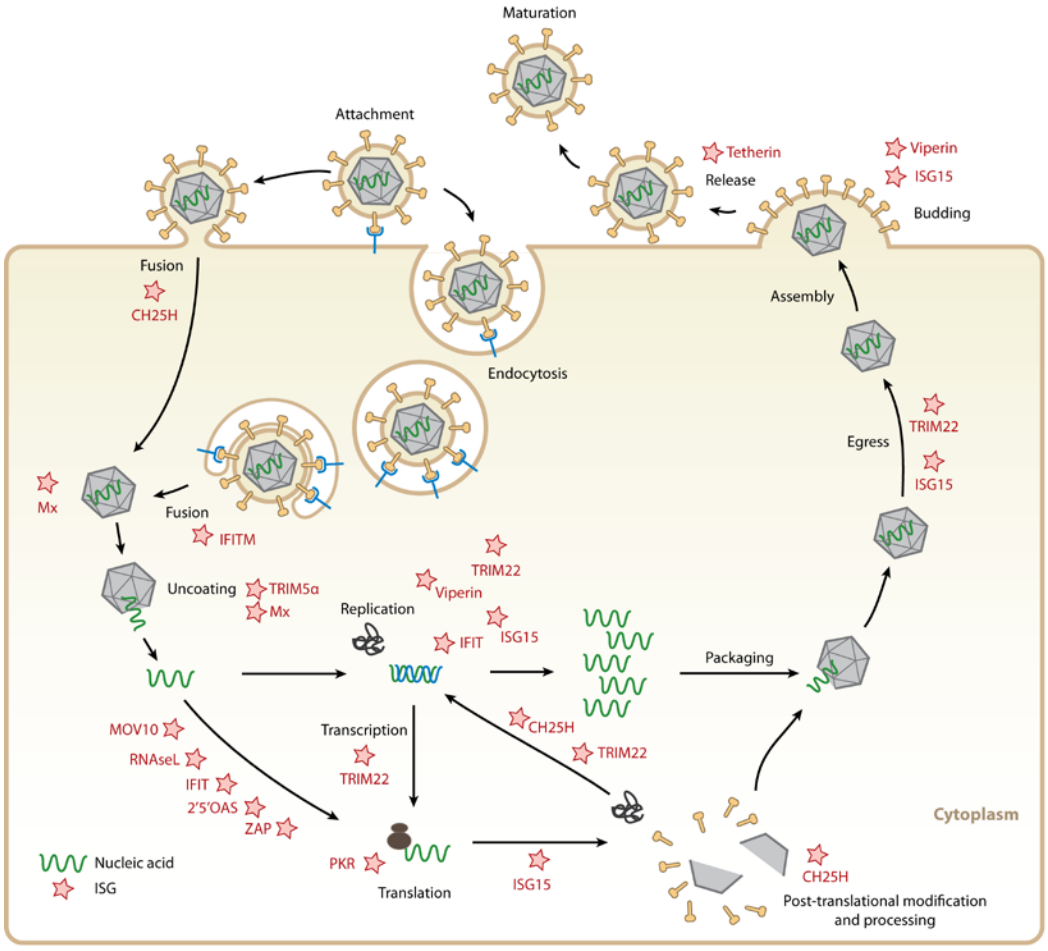
expressing cells leading to failed membrane penetration or subsequent lysosomal degradation of the virus particle. The IFITM proteins have also been suggested to alter the curvature and fluidity of the membrane, thereby inhibiting steps prior to membrane hemifusion (319).

The TRIM family constitutes a large family of proteins with diverse cellular activities, including E3 ubiquitin ligase activity (415). A well-known member of this is TRIM5α, which inhibits the early stages of HIV-1 life cycle (504). This study showed that TRIM5α might restrict HIV-1 replication by directly binding and ubiquitinating the capsid protein, adversely affecting the uncoating process. Another member of the TRIM family, TRIM22 also restricts HIV-1 by interfering with the trafficking of HIV-1 gag protein to the plasma membrane, leading to decreased virus particle production (32). TRIM22 also restricts influenza A virus replication by interacting with NP, leading to polyubiquitination and proteosomal degradation of NP (113).

1.2.3.2 Inhibitors of virus translation and replication

Protein kinase R (PKR) is a major sensor of dsRNA in the cytoplasm. It binds to dsRNA produced from infection in the cytoplasm, leading to its activation. Activated PKR phosphorylates the translation initiation factor eIF2α, resulting in the shutdown of viral and cellular protein synthesis leading to the inhibition of virus replication (27, 456). PKR has also been shown to be instrumental in activating the NFκB pathway and in maintaining the integrity of IFNα and IFNβ mRNA during infection with certain viruses (293, 475).

2’-5’ oligoadenylate synthetase (OAS) is an IFN stimulated gene (455) and is activated by the dsRNA, which is produced during both RNA and DNA virus infections. Activation of OAS results in the production of 2’-5’ oligoadenylates from ATP (493). 2’-5’ oligoadenylates



Schneider WM, et al. 2014.
Annu. Rev. Immunol. 32:513–45

Figure 1.6 Antiviral activities of interferon stimulated genes. Interferon stimulated genes (ISGs) affect different stages of the life cycle of viruses starting from entry of the virus until virus exit and release.

then activate the ubiquitous cellular endoribonuclease RNaseL, which cleaves the viral RNA preferentially over cellular RNA (493). During influenza virus infection, RNaseL activity has a major contribution to IFN antiviral activity (380). It has been reported that mutant viruses with the NS1 R38A mutation are highly susceptible to IFN antiviral activity, while this inhibition is relieved upon the knockdown of RNaseL or in its absence in RNaseL$^{-/-}$ cells (380).

Interferon stimulated gene 15 (ISG15) is an ubiquitin like protein that is rapidly upregulated upon induction by type I IFNs (111). Upon induction, Herc5 the major E3 ligase for ISGylation mediates the ISGylation of more than 300 proteins co-translationally (121). The function of ISGylation of a majority of these proteins is unknown. However, ISG15 deficient mice have been shown to have increased susceptibility to infection by a number of viruses such as influenza, herpes and sindbis viruses, which underscores its important antiviral function in the host (316). While ISG15 exerts its antiviral function through targeting the influenza A NS1 protein, a recent study has shown that ISG15-mediated protection of mice against influenza virus infection is through non-antiviral mechanisms, possibly through playing a role in lung repair after virus infection (384, 608).

1.2.3.3 Inhibitors of virus release and exit

Viperin is a widely induced ISG and it has been reported to contribute to the antiviral activities against a number of viruses including Hepatitis C virus (HCV), sindbis virus, HIV, influenza and human cytomegalovirus (514). It exerts its antiviral function by inhibiting an enzyme in isoprenoid biosynthesis called farnesyl diphosphate synthase, which alters membrane fluidity. Since, influenza A virus and HIV-1 bud from lipid rafts, alterations in these rafts affects the budding and release of the virus particles from infected cells (391, 567).

Tetherin is an important ISG and restricts virus release by inserting its N- and C-terminal domains simultaneously into both the virus envelope and the plasma membrane thereby retaining the virion to the plasma membrane (554). While its restrictive activity is well studied and well understood in HIV and has also been reported to have antiviral activity against several enveloped viruses, its antiviral role in influenza virus infection is not completely understood (511). While some influenza virus strains have been shown to be sensitive to tetherin, several strains have been shown to be resistant to tetherin-mediated antiviral activity (172). The mechanism of tetherin antagonism in influenza viruses is believed to be mediated by the HA and NA proteins and may involve interference with tetherin expression (172).

1.2.4 NS1 interaction with host proteins and its function in influenza virus life cycle

While the mechanisms of antigenic drift and antigenic shift help the virus in evading the adaptive immune response raised by the host, influenza A virus relies on the interaction of viral proteins to various components of the innate immune system to aid in evading the innate immune response (549).

NS1 is a multifunctional protein affecting a variety of host functions and is an important virulence factor for influenza A viruses (189, 290, 355). Influenza A viruses encoding NS1 with functional defects have been shown to be highly attenuated in various animal models (128, 156, 447, 523). Recombinant viruses lacking the NS1 gene are severely attenuated in IFN competent systems but replicate well in IFN-deficient systems such as in Vero cells and 6-day old embryonated eggs (156, 523). Moreover, infection with influenza virus lacking the NS1 gene leads to enhanced expression of IFN and ISGs, when compared to WT virus infection (162).

These findings show that NS1 and its functions could serve as ideal targets for the development of effective and new antiviral drugs. Indeed knowledge on the cellular interaction partners of NS1 and other viral proteins has resulted in the development of small molecule inhibitors with potent antiviral activity. For example, a fragment of cleavage and polyadenylation specificity factor 30 (CPSF30), which binds to the CPSF30 binding site on the NS1 protein has been shown to prevent NS1 binding to CPSF30 and abrogates the NS1 inhibitory function on 3'end processing of cellular pre-mRNAs (545). Studies using siRNA-based approaches have identified many cellular factors involved in different steps of the influenza A virus life cycle. Drugs targeting some of these cellular factors have been screened and show potent antiviral activity against influenza A virus (105, 570). Thus, the identification of NS1 cellular interaction partners will provide valuable data for the development of future antiviral drugs and boost the knowledge on cellular mechanisms modulated by influenza NS1 to promote virus replication. Some of the well-known interactors of NS1 and their functions in influenza virus replication are discussed below (Fig. 1.7).

One of the well-known pathways activated during influenza virus replication is the PI3K/Akt signalling pathway (122). The PI3K/Akt pathway is regulated by influenza NS1 protein through its direct interaction with the SH3 and C-terminal SH2 domain of p85β, which is the regulatory subunit of PI3K (489). The SH3 binding motif 1 of NS1 is required for this interaction and the PI3K/Akt pathway activation (487). The PI3K/Akt pathway activation inhibits virus-induced apoptosis through caspase-9 phosphorylation and treatment of cells with a PI3K specific inhibitor has been reported to reduce viral protein expression, viral RNA synthesis and vRNP nuclear export (487, 488). NS1 proteins of the 1918 pandemic virus and many avian

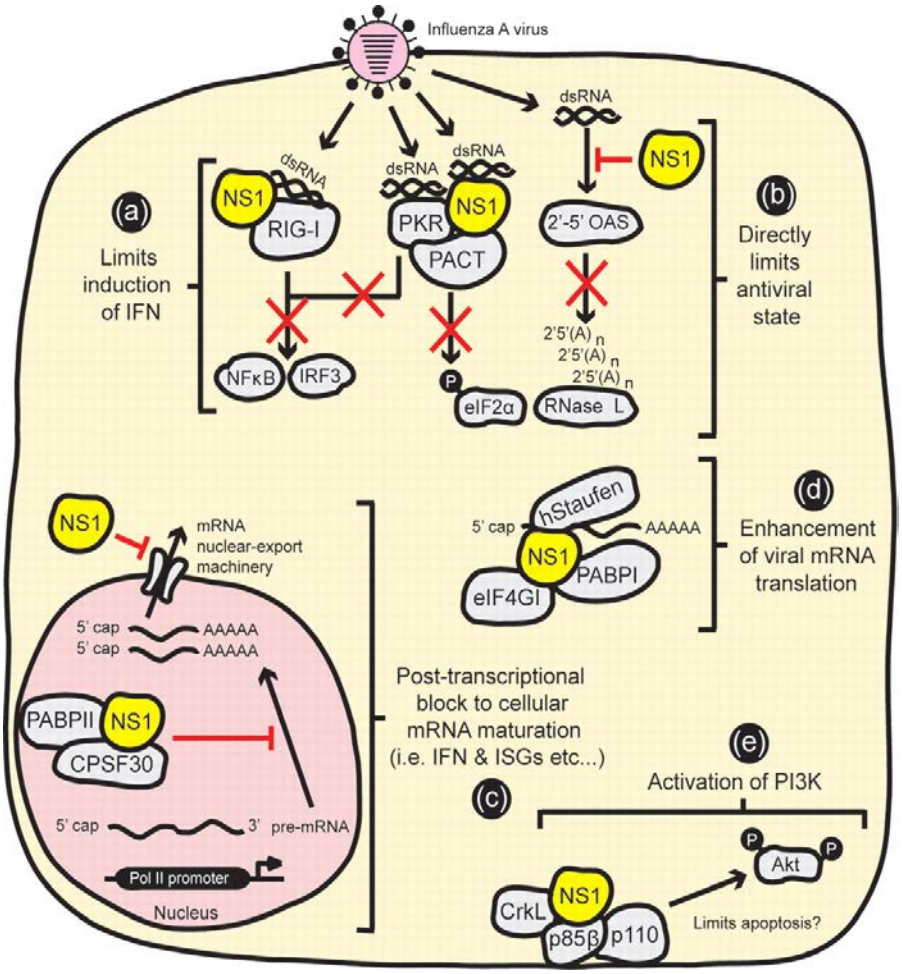


Figure 1.7 The antiviral functions of influenza A NS1. Influenza A NS1 interacts with dsRNA and several host proteins to exert its antiviral function. By binding dsRNA and interacting with RIG-I and PKR, it has a direct effect on limiting the induction of IFN. NS1 inhibits cellular mRNA maturation by interacting with CPSF30 and PABPII, while interaction with components of the nuclear export machinery blocks mRNA export. NS1 interaction with p85β and CrkL activates the PI3K signalling pathway, which favours viral replication.

influenza A viruses contain a consensus class II SH3-binding sequence, which is necessary for binding to the N-terminal SH3 domain of two related adaptor proteins Crk/CrkL (206). While binding to these adaptor proteins did not influence the ability of NS1 to suppress IFNβ expression, it was found to be associated with enhanced PI3K signalling (206). Several studies have shown that activation of the PI3K signalling pathway has many beneficial effects on viral replication, which includes delayed apoptosis induction in infected cells (340, 341, 597).

Another important cellular interaction partner of NS1 is CPSF30, which is an essential component of the 3'end cellular processing machinery of host pre-mRNAs (29). The NS1 protein binds CPSF30 and prevents its binding to the RNA substrate, which inhibits the 3'end cleavage and polyadenylation of host pre-mRNAs (394). Inhibition of this process also results in nuclear retention of cellular pre-mRNAs and restricts translation of these mRNAs (394). Through this mode of inhibition, NS1 prevents the post-transcriptional processing of several cellular antiviral pre-mRNAs including IFNβ pre-mRNA (399). While cellular pre-mRNAs are affected by this interaction, viral mRNAs are not since they obtain their poly(A) tail by the stuttering of viral polymerase on the vRNA template (609). NS1 also inhibits cellular mRNA export by binding to various components of the mRNA export machinery including NXF1/TAP, p15, Rae1 and E1B-AP5, which form a protein complex (144, 460). The majority of host mRNA export is mediated through the heterodimeric transport receptor NXF1-p15, which interacts with both the mRNA and various nuclear pore complex (NPC) proteins called nucleoporins (505). Rae1 and E1B-AP5 are also involved in nuclear export (24). They aid in the interaction of NXF1 to mRNAs and in the recruitment of Nup98 to NXF1 respectively to mediate mRNA export through the NPC (24, 54). NS1 prevents the interaction of a complex containing these proteins with other members of the mRNA export machinery by either rearranging the NXF1/p15/Rae1/E1B-AP5 mRNA export

complex or by masking the binding sites of this complex (460). One of the well-established functions of NS1 is its selective enhancement of translation of viral mRNAs. NS1 achieves this through its interaction with the translation initiation factor eIF4GI and PABP1. Mapping studies showed that the eIF4GI and PABP1 interacting domains correlate to residues 81-113 and 1-81 of the NS1 protein respectively (70). The NS1 protein promotes viral mRNA translation by binding to the 5' UTR of viral mRNA as well as with eIF4GI and PABP1 and aiding in the specific recruitment of the 43S complexes on the viral mRNA (70).

The NS1 protein of influenza A virus exerts its IFN antagonism by targeting several components of the innate immune system (Fig. 1.7). The NS1 protein is divided into an N-terminal RNA-binding domain (AA 1-73) which binds to dsRNA and a C-terminal effector domain (AA 74-230) which binds to several host proteins (189). Expression of the dsRNA-binding domain alone is sufficient to inhibit IFNβ induction, while substituting the amino acids of the dsRNA-binding domain (R38/K41) to alanine resulted in a reduction in the IFN inhibitory effect (568). Thus, dsRNA binding is important for the IFN antagonistic function of the NS1 protein. The RIG-I protein is a major PRR responsible for IFN induction during influenza viruses (600). Upon binding viral RNA, RIG-I exposes the N-terminal CARD domain, which binds to another CARD containing protein MAVS. This results in the activation of a signalling cascade leading to the phosphorylation of transcription factors such as IRF3 and IRF7. The activation of these transcription factors cause the induction of IFNβ promoter (264, 587, 600). Moreover, the E3 ubiquitin ligase activity of TRIM25 induces K63-linked ubiquitination of the CARD domain, which has been shown to facilitate RIG-I interaction with MAVS and enhance IFNβ induction (151). The NS1 protein inhibits the RIG-I signalling pathway by directly interacting with both RIG-I and TRIM25. The NS1 protein binds directly to the second CARD domain of RIG-I and

inhibits RIG-I function (246, 378); meanwhile, amino acids E96/E97 and R38/K41 required for RNA binding activity in NS1 are necessary for interaction with TRIM25 (150). Mutation of these amino acids resulted in the loss of NS1's ability to inhibit TRIM25-mediated RIG-I ubiquitination, RIG-I CARD-induced IRF3 phosphorylation, dimerization and IFNβ promoter activation (150). Thus, the NS1 protein is able to efficiently block the RIG-I signalling pathway. An important host defence pathway is the PKR pathway, which is activated upon binding dsRNA and leads to the phosphorylation of the translation initiation factor eIF2α (27, 456). eIF2α phosphorylation in turn results in the disruption of protein translation and prevents viral replication, since the virus depends on the host machinery for viral mRNA translation. NS1 inhibits PKR activation by binding to and sequestering its ligand dsRNA and by binding directly to PKR (204, 343, 524). Residues 1-230 of PKR is required for direct interaction with NS1, while the dsRNA binding mutant of NS1 is also able to interact with PKR and inhibit its activation (321). This shows that NS1 is able to inhibit PKR by a mechanism independent of dsRNA binding. Thus, NS1 exerts its multifunctional nature and affects various stages of the virus life cycle by interacting with several host proteins.

NS1 being a multifunctional protein, new host interaction partners are being discovered routinely and hence the NS1 protein is believed to interact with additional host proteins that are yet to be identified (189, 355).

1.3 DDX3 and virus life cycle

1.3.1 Biological functions of DDX3

DDX3 belongs to the DEAD box RNA helicase family and harbors ATPase and RNA helicase activities (145, 594). DDX3 has two homologues, DDX3X and DDX3Y, which are

located on the X and Y chromosomes respectively (280, 296, 419). DDX3X is ubiquitously expressed in most tissues while DDX3Y expression is restricted to the male germline tissues (116). Like most other DEAD box helicases, DDX3X or DDX3 has been implicated in a number of functions related to RNA metabolism, including transcriptional regulation, translation, splicing, mRNA export and ribosome biogenesis (471). DDX3Y on the other hand is associated with male fertility and is essential for spermatogenesis in both humans and mice (134, 302).

Results from several studies show that DDX3 is a multifunctional protein (496) and its characteristic as a nucleocytoplasmic shuttling protein might be important for this pleiotropic function (594). The multiple functions of DDX3 protein in cellular RNA metabolism are discussed below.

1.3.1.1 DDX3 in transcription

DDX3 has a role in the transcriptional regulation of genes from different promoters including IFNβ, E-cadherin and P21 $^{waf1/cip1}$ promoters (59, 80, 472, 497). However, the mechanism by which DDX3 regulates these promoters appears to be different. Chromatin immunoprecipitation studies have shown direct association of DDX3 with the IFNβ and E-cadherin promoters (59, 497). However, the IFNβ promoter is upregulated by DDX3, while the E-cadherin promoter is downregulated. Alternatively, DDX3 regulates the P21 waf promoter by binding to the transcription factor Sp1 (80). In this case, P21 waf promoter activity is enhanced and regulation is Sp1-dependent. While the effect of DDX3 on the IFNβ promoter activity is independent of its helicase or ATPase activity (472, 497), DDX3 regulation on the P21 waf promoter requires ATPase but not unwinding activity (80). Therefore, the mechanism of action of DDX3 on the regulation of different promoters might be different with varied regulatory

outcomes. More future studies, examining the mechanism of promoter recruitment and the exploration of other DDX3 promoter targets are needed. Based on the function of other DEAD-box helicases such as DDX5 and DDX17 acting as adaptors between promoter-specific transcription factors (149), it is speculated that DDX3 also gets recruited to specific promoters in a similar manner.

1.3.1.2 DDX3 and pre-mRNA splicing

Splicing is a key step in eukaryotic gene expression, where the introns are excised from pre-mRNA and the exons are ligated together to form a continuous reading frame catalyzed by the spliceosome complex (579). The protein composition of this spliceosome complex has been identified by mass spectrometry of purified mRNP complexes (445, 527). Among these proteins, the best characterized are the group of proteins belonging to the exon junction complex (EJC), which binds to spliced mRNA 20-24 nucleotides upstream of the exon-exon ligation site (526). In a study with affinity purified spliced mRNP complexes, DDX3 was identified as a novel mRNP protein and found to associate stably with the mRNPs via the EJC (376). Also, the association was splicing-dependent and DDX3 was found exclusively in the spliced mRNAs carrying an EJC (376). However, whether DDX3 knockdown or impairment has an effect on splicing is not yet known. Hence, the exact role of DDX3X in pre-mRNA splicing remains to be clarified.

1.3.1.3 DDX3 and nuclear RNA export

Tip associated protein (TAP) is considered as a major receptor for mRNA export from the nucleus to the cytoplasm (253, 505). DDX3X was identified as a TAP interacting protein and as a result associates with TAP-associated mRNPs (298). Depletion of TAP causes nuclear

accumulation of DDX3 but it does not alter the mRNA distribution in the cell (298). Therefore, even though DDX3 interacts with TAP and its export is at least partially TAP-dependent, it does not appear to be critical for the export of most cellular mRNAs (298). However, it is still possible that DDX3-TAP interaction is essential for the nuclear export of a small subset of mRNA.

Distinct from the TAP-mediated export pathway is the CRM1-dependent RNA export pathway. Several studies implicate DDX3X in the CRM1-dependent pathway. DDX3 was found to be essential for CRM1-mediated export of unspliced HIV RNA (594) and also as a component of the eIF4E-mediated mRNA export pathway (539). eIF4E enchances the mRNA export of genes that contain an approximately 50 nt element in the 3' UTR known as the eIF4E-sensitive element (99). DDX3 was found to associate with these eIF4E-sensitive mRNPs and its interaction with eIF4E was RNA-independent (539). However, the role of DDX3X in this pathway is not yet well defined.

1.3.1.4 DDX3 in translation and cytoplasmic stress granule formation

Even though several studies have investigated the role of DDX3 in translation, its function is still murky because of the contradicting results from these studies. In HeLa cells, DDX3 knockdown was found to dramatically decrease protein production from a β-globin reporter construct and the translation promoting function of DDX3 was determined to be mediated through its interaction with the translation initiation factor eIF3 (310). In another study, DDX3 depletion in Huh7 cells with the transfection of different reporter constructs showed downregulation of translation with both cap and IRES (Internal Ribosome Entry Site) containing mRNAs and that DDX3 assists 60S subunit joining process to assemble functional 80S ribosome

(163). Therefore, these studies concluded that DDX3 depletion has a negative role in general translation. However, a different study showed that DDX3 has an inhibitory effect on cap-dependent translation but enhances hepatitis C virus IRES-mediated translation (485). The study ascribed this translation regulatory effect of DDX3 to its interaction with eIF4E and their observation with cap affinity chromatography analysis suggests that DDX3 traps eIF4E in a translationally inactive complex by blocking its interaction with eIF4G (485). Besides these studies, DDX3X has also been reported to target complex 5'-UTR of a subset of mRNAs, specifically promoting the translation of cyclin E1 mRNA (297). Thus, it remains to be clarified, whether DDX3 has a general effect on translation or it has a specific effect on a subset of mRNAs and whether the translation regulatory effect is inhibitory or enhancing. Most of the studies mentioned above have found associations of DDX3 with several translation initiation factors such as eIF4A, eIF4E, eIF4G, eIF3 and PABP. Therefore, despite the controversial data regarding the effect of DDX3 on translation, it is quite clear that it has some role in translation initiation.

Stress granules (SGs) are non-membranous cytoplasmic foci containing aggregates of untranslated mRNA in cells exposed to adverse stress conditions such as heat shock, UV irradiation, virus infection, hyperosmolarity and oxidative stress (15). Assembly of the SGs during stress conditions helps the cells to direct the translation machinery for the production of stress response factors and heat shock proteins by sequestering housekeeping mRNAs into the SGs (417). SG formation is initiated by the phosphorylation of eukaryotic translation initiation factor 2α (eIF2α) (269). eIF2α phosphorylation results in the reduced availability of eIF2α-GTP-$tRNA_i^{Met}$ ternary complex which is required for translation initiation, thereby resulting in stalled translation initiation complexes (267). RNA-binding proteins such as TIAR and TIA-1 then bind

to these stalled translation initiation complexes and facilitate the formation of SGs (269). Phosphorylation of eIF2α can occur by 4 different kinases activated by distinct types of stress (589). Heme-regulated translation inhibitor (HRI) kinase is activated in response to oxidative stress, GCN2 kinase is activated in response to nutrient deprivation and UV irradiation, PKR kinase is activated in response to viral infections, and PKR-like endoplasmic reticulum kinase (PERK) signals in response to endoplasmic reticulum stress. Many viruses induce SGs through the activation of PKR kinase and in some cases GCN2 by detection of viral RNA in the cytoplasm (46, 350). Many viruses have been reported to inhibit SG formation by various mechanisms underscoring the antiviral role of SGs (335, 409).

Many RNA helicases such as DDX1, DDX3, DDX6, eIF4A and RHAU have been shown to localize in SGs (69). A recent study reported localization of DDX3 in SG, induced by a variety of cellular stress including sorbitol, arsenite, DTT, heat shock treatment and UV irradiation. DDX3 was found to be a SG-nucleating factor and DDX3-eIF4E interaction was found to be essential for SG formation (486). This observation was found to provide a functional link between the translation inhibitory effect of DDX3 and its SG inducing capability, because eIF4E binding defective DDX3 mutant was unable to repress translation (486). However, another study reported that DDX3 knockdown did not impair arsenite-induced SG formation, even though overexpressed GFP-DDX3 was found to localize in SGs (298). Nevertheless, it is clear that DDX3 is localized in SGs along with the translation initiation factors it is known to interact with. Thus, DDX3 recruitment to the SGs might be an important link to explain its translation inhibitory phenotype and association with the translation initiation factors. Besides that, recent reports show that infection with PR8 influenza virus lacking the NS1 gene induces formation of SGs in the infected cell (271, 272). Influenza virus-induced SGs have antiviral properties and

several viral proteins, such as NS1, PA-X and NP have been reported to counteract SG formation (271, 272, 409). However, the role of DDX3 in influenza virus-induced SG formation is unknown and might be an interesting area of study.

1.3.1.5 DDX3 in cellular signalling and IFNβ induction

DDX3 was identified in a genome wide siRNA screen for novel wnt-β catenin signalling regulators (97). The report suggests that DDX3 binds CK1ε, promoting its kinase activity and thereby enhances the phosphorylation of the scaffold protein dishevelled (Dvl). Antiviral PRRs such as TLRs and RLRs recognize different species of viral nucleic acids and induce type I IFNs (259, 534). Both the TLR3 and RIG-I pathway lead to the activation of IRF3, mediated by IKK-related kinases TBK1 and IKKε. IRF3 then translocates to the nucleus and promotes transcription from the type I IFN promoter (138, 243, 254, 403, 482). DDX3 interacts with both TBK1 and IKKε and contributes to IFNβ induction (472, 497). DDX3 interacts directly with IKKε and enhances autophosphorylation and the activation of IKKε (183). Activated IKKε in turn phosphorylates DDX3 and the phosphorylated DDX3 recruits IRF3, facilitating the phosphorylation of IRF3 by IKKε (183). Phosphorylated DDX3 has also been shown to bind directly to IFNβ promoter and promote its transcription (497) (Fig. 1.8). Another recent study also suggested that DDX3 interacts directly with HCV RNA and then triggers MAVS-dependent signalling enhancing IFNβ induction (413). Even though all the above studies point to a role for DDX3 as an IFNβ enhancer, the exact mechanism by which it fulfills this function is controversial. Nevertheless, DDX3 being a multifunctional protein, it could be contributing to IFNβ induction through multiple mechanisms. Moreover, several viruses appear to target DDX3 to counteract IFNβ induction, as discussed below in more detail.

1.3.2 DDX3 in virus infection

Several viruses such as HIV, HCV, Hepatitis B virus (HBV) and Vaccinia virus (VACV) either co-opt DDX3 as a cellular cofactor or inactivate the protein with specific viral factors to aid in viral replication. For example, DDX3 is a well-established cellular co-factor in HIV replication (388, 593, 594). Yedavalli et al. showed that DDX3 interacts with HIV rev protein and the cellular nuclear export protein CRM1 (594). Through this rev-DDX3 interaction, HIV was reported to target its partially spliced mRNAs for CRM1-mediated nuclear export (594). Based on these observations, DDX3 was reported to function as a cofactor essential for HIV-1 replication. Another study also showed that DDX3 interacts with HIV Tat and enhances Tat function during HIV replication (593). A combination of DDX3 with other helicases such as DDX1, DDX5, DDX6, DDX21 and DDX56 synergistically enhanced Rev function but not Tat function (593). Only DDX3 specifically enhanced HIV-1 Tat function (593) . The ATPase-dependent RNA helicase activity of DDX3 was required for both Rev and Tat function(593, 594) and the interaction with Tat localized DDX3 to cytoplasmic P-bodies and stress granules (593).

Multiple studies have shown DDX3 to affect HCV virus life cycle. DDX3 was first identified as a HCV core-interacting protein by yeast two-hybrid assay. In this study DDX3 and core were found to co-localize in distinct spots in the perinuclear region of the cytoplasm (414). Another evidence implying DDX3 in the HCV virus life cycle came from the observation that DDX3 is downregulated in Hepatocellular carcinoma specimens (78). Proteomic analysis of lipid droplets in core expressing hepatoma cell lines identified DDX3 along with another DEAD box helicase DDX1 as present in the lipid droplet fraction (459). This observation suggested that lipid droplets containing HCV core protein may participate in RNA metabolism thereby affecting

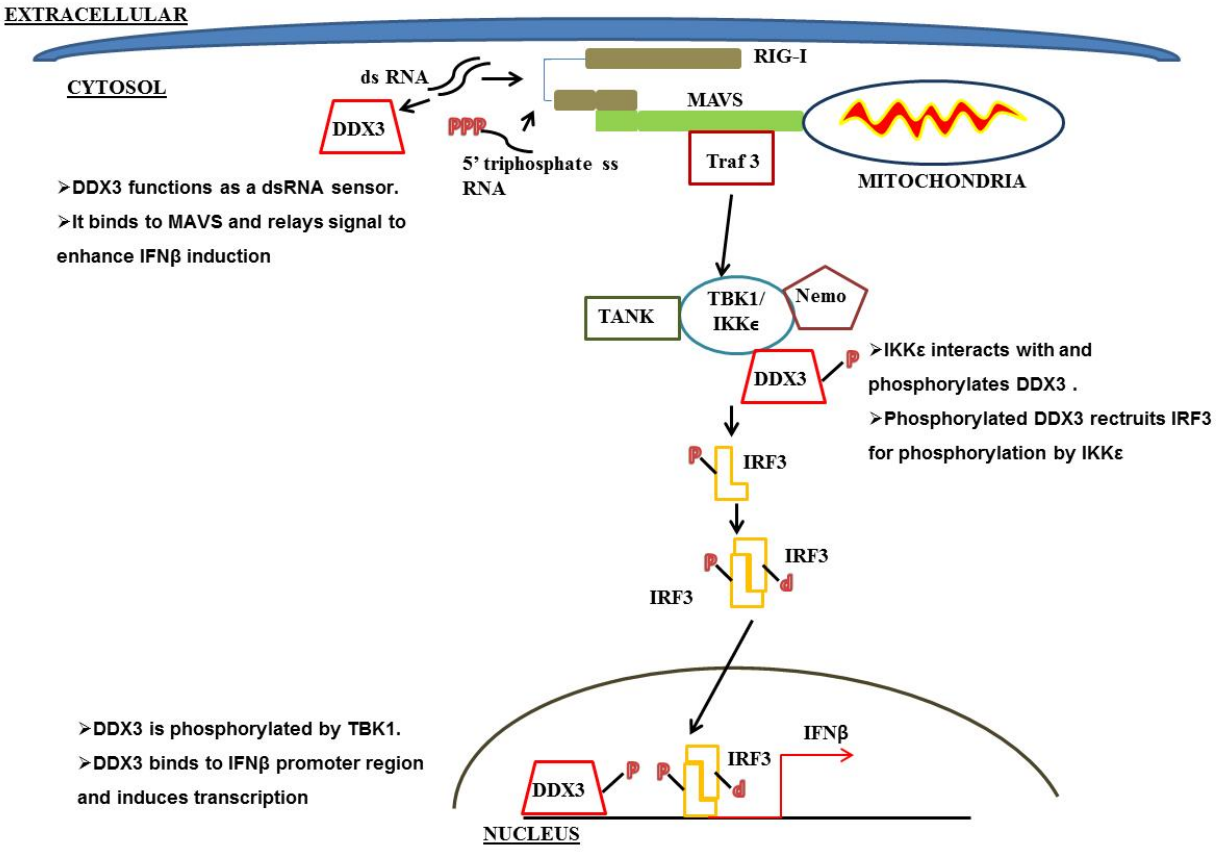


Figure 1.8 The role of DDX3 in IFNβ signalling pathway. Transcription factors and other proteins involved in RIG-I-mediated signalling cascade for IFNβ induction are shown in the figure. DDX3 has been reported to target the RIG-I-mediated IFNβ induction pathway by targeting the different components of the pathway as shown in the schematic. The multifunctional nature of DDX3 is believed to facilitate DDX3 interaction with these diverse components of the pathway.

HCV replication (459). Ariumi et al. demonstrated the reduced accumulation of both genome-length HCV RNA (HCV-O, genotype 1b) and replicon RNA in DDX3 knockdown cells (20). Moreover, RNA replication of JFH1 (genotype 2a) and the release of core protein into the culture supernatants was suppressed in DDX3 knockdown cells. These results suggested that DDX3 is required for HCV replication. Several studies have shown that DDX3 upregulates IFNβ induction through several mechanisms (413, 472, 497). A recent study showed that DDX3-core interaction, besides other mechanisms, could aid in HCV replication by suppressing DDX3 augmented IPS-1 signalling for IFNβ induction (411). Another mode of action of DDX3 in HCV replication has also been demonstrated. In this study, it has been shown that DDX3 interacts with the 3' UTR of HCV genomic transcript activating IKKα (320). IKKα then translocates to the nucleus and induces CBP/p300-mediated transcriptional program, which enhances core-associated lipid droplet formation to facilitate virus assembly (320).

DDX3 has also been implicated in the replication of other flaviviruses such as Japanese Encephalitis virus (JEV) and West Nile virus (WNV). DDX3 was shown to be required for JEV replication, because DDX3 knockdown inhibited JEV replication (317). DDX3 was shown to interact with JEV NS3, NS5 and bind to 5' and 3' UTR region (317). Also, DDX3 upregulated viral RNA translation and DDX3 helicase activity was found to be crucial for JEV replication (317). Many studies have shown that P-bodies are greatly diminished during WNV infection (126, 317). A recent study explored the fate of the P-body components after WNV infection (76). It was reported that many P-body components including DDX3 are recruited to virus replication sites and positively regulate viral replication (76).

VACV K7 protein has a potent inhibitory effect on TLR-dependent and independent IRF activation and consequently IFNβ promoter induction (472). On further exploration VACV infection was found to stimulate DDX3 and K7 interaction and this interaction was found to be necessary for the IFN inhibitory function of K7 (472). Expression of DDX3 led to enhanced TBK1/IKKe-dependent IFNβ promoter induction, whereas suppression of DDX3 expression by RNAi inhibited this as well as virus or poly I:C-induced IRF3 activation (472). This was the first study to reveal an IFN stimulating function for DDX3. HBV was also found to use a strategy similar to VACV to inhibit IFN induction. HBV pol protein was reported to interact with DDX3 and inhibit TBK1/IKKε activity by disrupting the interaction between IKKε and DDX3 (563). Even though DDX3-HBV pol interaction is beneficial for the virus because of reduced IFN induction, this might not be the only function. DDX3 has also been shown to inhibit HBV reverse transcription by binding to HBV pol and DDX3 was found to be incorporated into nucleocapsids (562). The ATPase motif and not the helicase motif of DDX3 was found to be essential for this polymerase inhibitory effect (562).

CHAPTER 2: RATIONALE, OBJECTIVES AND AIMS

2.1 Rationale

Influenza A viruses cause annual epidemics and recurring pandemics and are considered as a persistent global health issue. Additionally, sporadic cases of avian to human transmission of H5N1 and H7N9 avian influenza viruses have caused concerns regarding the pandemic potential of these viruses. Besides vaccines, antiviral drug therapy is an effective treatment measure for influenza-infected patients. Currently, FDA has approved two classes of drugs for prophylaxis and treatment of influenza virus infection, which include M2 ion channel inhibitors, such as amantadine, rimantadine and Neuraminidase inhibitors, such as oseltamivir and zanamavir. In addition, some countries have approved the use of several new drugs targeting additional viral functions. However, a major concern in using these drugs is the frequent emergence of drug-resistant strains. Therefore, there is an urgent need for the development of new antiviral drugs, especially those targeting non-viral targets. Influenza A virus depends on the host cellular machinery for its replication and spread. Therefore, host factors would serve as an ideal non-viral target for the development of new antiviral therapy. Some examples of antiviral drugs targeting host factors include the sialidase DAS181 targeting the influenza virus receptor sialic acids and maraviroc, which targets the HIV-1 co-receptor CCR5 for HIV-I antiviral therapy. Therefore, it is critical to identify novel host cellular factors involved in the influenza A virus life cycle. Besides enhancing the current knowledge of influenza virus life cycle, such novel host factors may also serve as novel targets for next generation antiviral therapy.

The NS1 protein of influenza A virus is a multifunctional protein. In addition to its well-known function to antagonise host defence mechanisms, NS1 is also involved in the regulation of viral polymerase activity, RNA export, splicing, translation and viral replication. NS1 exerts

its multifunctional nature through its interaction with a variety of host cell factors. Hence, identification of the host proteins that interact with NS1 during virus infection will provide an insight into the multiple functions regulated by NS1; leading to the identification of novel cellular pathways regulated by the influenza A NS1 protein. Additionally, some of the identified proteins could potentially be used as targets for future antiviral therapy. Even though many NS1 interacting host proteins have already been identified, it is widely believed that many more NS1 interactors are yet to be discovered. Therefore, there is a strong necessity for more studies to identify these NS1 interacting host factors. Epithelial cells in the respiratory tract serve as the primary site of replication during influenza infection. The respiratory epithelial cells also produce immunomodulatory cytokines and chemokines upon infection. Therefore, when compared to using continuous cell lines, studying influenza virus infection and NS1 host factor interactions on swine respiratory epithelial cells (SRECs) would resemble conditions similar to natural infection.

2.2 Hypothesis

I hypothesize that the NS1 protein of influenza A virus antagonizes host antiviral defence mechanisms and regulates different functions in the virus life cycle by interacting with multiple host factors during infection.

2.3 Objective and aims

The overall objective of my study is to identify the novel host proteins that interact with influenza A virus NS1 protein in physiological settings and to characterize the role of at least one of the identified cellular factors during influenza A virus infection. To achieve the objective, I propose the following aims and approaches.

2.3.1 Aim 1. Generation and characterization of a recombinant swine influenza virus (SIV) encoding Strep-tagged NS1

2.3.1.1 To generate a recombinant virus encoding Strep-tag NS1 using reverse genetics.

2.3.1.2 To characterize the phenotype of the virus in terms of growth property, NS1 localization and protein expression kinetics compared to the wild-type virus.

2.3.2 Aim 2. Isolation of primary SRECs and purification of the intact NS1 protein complex from infected SREC

2.3.2.1 To isolate SRECs from the trachea of healthy pigs and confirm the purity of the isolated cells using fluorescence-activated cell sorting (FACS) analysis.

2.3.2.2 To infect SRECs with the recombinant SIV encoding Strep-tag NS1 and purify the intact NS1 protein complex from the infected cell lysate using the streptactin resin.

2.3.3 Aim 3. Identification of proteins in the purified NS1 protein complex and bioinformatics analysis

2.3.3.1 To identify the host proteins co-precipitated with NS1 in the purified protein complex using liquid chromatography-tandem mass spectrometry.

2.3.3.2 To analyze the dataset containing the host proteins using DAVID bioinformatics resources, STRING database and Cytoscape software tool to understand the potential host functions regulated by NS1.

2.3.4 Aim 4. Validation and characterization of one of the viral interaction partners: DDX3

2.3.4.1 To confirm DDX3 interaction with NS1 by immunoprecipitation and to identify other potential viral proteins that interact with DDX3 by Western blotting from virus-infected cell lysate.

2.3.4.2 To characterize the RNA dependency of the interaction between DDX3 and viral proteins.

2.3.4.3 To identify the domains in DDX3 that are important for NS1 and NP interaction.

2.3.5 Aim 5. Validation and characterization of the role of DDX3 in influenza virus-induced SGs

2.3.5.1 To study the kinetics of SG formation in influenza A virus-infected cells and to investigate the localization of DDX3 and NP interaction.

2.3.5.2 To characterize the role of different domains of DDX3 in the recruitment of DDX3 to influenza A virus-induced SGs.

2.3.5.3 To assess the effect of siRNA-mediated DDX3 knockdown on SG formation by immunofluorescence staining and how DDX3 knockdown affects influenza A virus propagation.

2.3.6 Aim 6. Validation and characterization of the role of DDX3 in influenza A virus-induced IFNβ expression

2.3.6.1 To identify the major PRR responsible for influenza A virus-induced IFNβ expression using siRNA-mediated knockdown of the PRRs, RIG-I and TLR3.

2.3.6.2 To analyse the effect of DDX3 knockdown and overexpression on virus-induced IRF3 phosphorylation, IFNβ mRNA expression and IFNβ promoter activity.

2.3.6.3 To investigate the mechanism of IFNβ inhibition by DDX3 using an *in vitro* competition assay.

2.3.6.4 To test the effect of DDX3 downregulation on SIV/SK-WT and PR8 del NS1 virus replication by examining the virus titer using plaque assay.

CHAPTER 3: NETWORK OF HOST FACTORS THAT INTERACT WITH NS1 PROTEIN OF INFLUENZA A VIRUS

Relationship of this study to the dissertation.

Influenza A virus NS1 is a multifunctional protein with critical host defense antagonistic properties (189). We hypothesized that the multifunctional nature of the protein is exerted through its interactions with cellular proteins. Therefore, we sought to study NS1 interactions with host proteins in infected cells by infecting epithelial cells with a recombinant virus expressing Strep-tagged NS1 protein.

3.1 Abstract

Pigs are an important reservoir of influenza A viruses, due to their potential to host reassortment viruses with pandemic potential. NS1 protein is a key virulence factor of influenza A viruses and a major antagonist of innate immune responses. It is also involved in enhancing viral mRNA translation and regulation of virus replication. Being a protein with pleiotropic functions, NS1 has a variety of cellular interaction partners. Hence, studies on swine influenza viruses (SIV) and identification of swine influenza NS1-interacting host proteins is of great interest. Here, a recombinant SIV carrying a Strep-tag in the NS1 protein was constructed and primary swine respiratory epithelial cells (SRECs) were infected with this virus. The Strep-tag sequence in the NS1 protein enabled us to purify the intact NS1 protein and its interacting protein complex specifically. Cellular proteins present in the purified complex were identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and a dataset of these proteins was generated. Four hundred and forty five proteins were identified by LC-MS/MS and among them 192 proteins were selected by setting up a threshold based on the mass spectrometry

parameters. The selected proteins were analyzed by bioinformatics and were categorized in the involvement of different functional groups such as translation, RNA processing, innate immunity and apoptosis. Protein interaction networks were derived using these data. The novel proteins and the networks revealed in this study will be the potential candidates for targeted study of the molecular interaction of NS1 with the host proteins, which will provide insights into the identification of new therapeutic targets to control influenza infection and disease pathogenesis.

3.2 Introduction

Influenza A viruses are globally important human and animal pathogens responsible for seasonal, epidemic and periodically world-wide pandemic outbreaks. Among these viruses, swine influenza viruses (SIV) are a common and an important cause of respiratory disease in pigs (86, 416). Pigs are an important host of influenza A viruses, which could harbor viruses with pandemic potential (7, 213, 275), as they are known to support the replication of both human and avian viruses. Hence, they serve as a mixing vessel for the generation of virus re-assortments, which might result in more pathogenic and novel viruses, such as the pandemic 2009 H1N1 virus that originated from pigs (7, 213, 275). Viruses being obligate parasites use diverse cellular machinery for replication and spread in the host. It is now widely recognized that the next generation of antivirals should be developed targeting cellular machinery rather than viral machinery, because historically viral targets are easily mutated by the virus to develop resistance against the drug (570). Host machinery, which is not under any evolutionary pressure to mutate, would be difficult for viruses to develop resistance against (339). Therefore, identification of host factors affecting SIV infection in its natural host is of paramount importance both for the basic understanding of the virus life cycle and for developing new antiviral therapy.

The NS1 protein of influenza A viruses is involved in regulating splicing, mRNA export, translation and antagonizing host defenses. It fulfills these functions via interaction with multiple cellular partners (189, 355). Although a variety of NS1 interaction partners have been identified, there is still a great interest in discovering novel interaction partners (104, 478). In this study, a recombinant SIV carrying a Strep-tag in the NS1 protein was constructed and primary swine respiratory epithelial cells (SRECs) were infected with this virus. Studying SIV replication by

infecting the primary swine cells will resemble conditions close to natural infection and the Strep-tag sequence in the NS1 protein would enable us to purify the intact NS1 protein and its interacting protein complex specifically. Cellular proteins present in the purified complex were identified by liquid chromatography-tandem mass spectrometry (LC-MS/MS) and a dataset of these proteins was generated. The dataset was further analysed by bioinformatics and provided comprehensive networks of the host factors that interact with NS1. The novel proteins revealed in this study will be potential candidates for the targeted study of the molecular interaction of NS1 with host proteins.

3.3 Materials and Methods

3.3.1 Cells and viruses. Madin-Darby canine kidney (MDCK) cells were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS). SRECs were isolated and cultured as described elsewhere (590). Briefly, trachea from healthy 6-8 week old pigs were obtained after euthanization and washed in sterile Phosphate buffered saline (PBS). The trachea from each pig was transected longitudinally and the surface epithelium was pulled off the submucosa using a glass microscope slide. The epithelium was washed with Joklik's MEM (JMEM) (Sigma-Aldrich) containing glutamine (2mM), Dithiothreitol (0.5 mg/ml) (Sigma-Aldrich), Deoxyribonuclease (10 µg/ml) (Sigma-Aldrich) and antibiotics for 3 hrs at 4˚C, changing the media every 1 hr. The tissue was then digested with protease XIV (Sigma-Aldrich) in the above media for 18 hrs at 4˚C. After 18 hrs, the protease XIV was neutralized by adding FBS to a final concentration of 20%. The media was filtered through a 70 µm cell strainer and the cells were pelleted down and washed with JMEM supplemented with 20% FBS. The cells were then resuspended in Bronchial Epithelial Growth Medium (BEGM) (Lonza Group Ltd.)

with added growth factors from the bullet kit (Lonza Group Ltd.) and then plated on an uncoated dish for 2-3 hrs and left in an incubator at 37˚C. Contaminating fibroblasts attach to the plate, while the epithelial cells are only lightly attached and can be easily dislodged by gentle agitation. The floating cells were once again washed in BEGM media and plated onto a type IV collagen (Sigma-Aldrich)-coated cell culture flask. Once the cells reached 100% confluency, they were split on to type IV collagen-coated flasks and propagated further.

A/Sw/SK/18789/02 (H1N1) (SK02) virus was propagated in 11-day-old embryonated chicken eggs as described previously (487). Influenza virus titer was determined by plaque assay on MDCK cells.

3.3.2 Antibodies. Rabbit polyclonal NS1 and NP antibodies were generated in our laboratory as previously described (488). The other antibodies were purchased from different sources as follows: Strep MAB-Classic antibody conjugated to horseradish peroxidise (HRP) (IBA), Alkaline Phosphatase (AP) – Conjugated anti-rabbit IgG (Jackson ImmunoResearch), Mouse anti human cytokeratin (pan) (AbD Serotec), Goat F (ab')2 Anti-Mouse IgG1- FITC conjugate (Southern Biotech), mouse IgG1 negative control antibody (AbD Serotec), Goat anti-Rabbit IgG secondary antibody, Alexa Fluor 594 (Life Technologies).

3.3.3 Plasmid construction and generation of mutant viruses. The DNA sequence corresponding to the Strep-tag (TGGTCACACCCACAGTTCGAAAAA) was introduced into pHW-SK-NS (361) plasmid by overlapping PCR. Plasmid pHW-SK-NS-Strep-replacement (plasmid #544) encodes SIV/SK-NS1 with AA 77-84 replaced by the Strep-tag, while plasmid pHW-SK-NS-Strep-insertion (plasmid #545) encodes SIV/SK-NS1 with the Strep-tag inserted between AA 79 and 80.

The recombinant viruses encoding the Strep-tag NS1 were rescued using an eight-plasmid reverse genetics system (214). For the rescue of the recombinant viruses, plasmids pHW-SK-PB2, pHW-SK-PB1, pHW-SK-PA, pHW-SK-HA, pHW-SK-NP, pHW-SK-NA, pHW-SK-M (361) and one of either plasmids pHW-SK-NS-Strep-replacement or pHW-SK-NS-Strep-insertion was transfected onto co-cultured MDCK and 293T cell. The rescued viruses designated SIV/SK-544 and SIV/SK-545 were propagated in 11-day-old embryonated eggs.

3.3.4 Western blotting. A portion of the cell lysate (input), eluent or wash fractions from the Strep-tactin sepharose column and samples for protein expression kinetics were resolved by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting was performed as described elsewhere (488).

3.3.5 Immunofluorescent staining. MDCK cells were grown on glass chamber slides. After virus infection, the cells were washed with PBS, fixed and permeabilized with 1:1 acetone /methanol mixture at -20°C for 20 mins. The cells were washed in PBS, blocked in 5% goat serum in PBS for 45 mins and then incubated with the rabbit polyclonal NS1 antibody in blocking buffer for 2 hrs at room temperature or overnight at 4ºC. The cells were again washed in PBS and incubated with the Goat anti-Rabbit IgG secondary antibody, Alexa Fluor 594 (Life Technologies) secondary antibody in the blocking buffer for 1 hr at room temperature. After washing in PBS, the cells were mounted using Prolong Gold Antifade reagent (Life Technologies). Multiple images of different fields of view were captured using Leica TCS SP8 confocal laser microscope. A representative of the multiple images is presented in the results.

3.3.6 Purification of Strep-tagged NS1 protein complex. SRECs were infected with SIV/SK-544 at an MOI of 2.0. At 16 h.p.i., cells were harvested in cell lysis buffer (Cell Signaling Technology) with protease inhibitor (Complete protease inhibitor cocktail tablets – Roche Diagnostics Corporation). The lysate was sonicated and then clarified by centrifugation at 16200 ×g for 10 min at 4˚C in a standard micro centrifuge. The Strep-tactin sepharose resin (IBA) was washed 3 times with 4 column volume (CV) of cell lysis buffer, then added to the clarified lysate and incubated at 4˚C overnight. Next, the lysate-sepharose mixture was added to a polypropylene column (Qiagen) and washed extensively with wash buffer [100 mM Tris-Cl (pH 8.0), 150 mM NaCl, 1 mM EDTA] and the Strep-tag NS1 protein complex was then eluted from the sepharose resin with 3 CV of elution buffer (wash buffer with 2.5 mM desthibiotin). Protease inhibitor cocktail was added to the eluent to prevent degradation of the proteins and the proteins present in the complex were identified by LC-MS/MS at the University of Victoria-Genome BC Proteomics Centre.

3.3.7 Liquid chromatography-tandem mass spectrometry (LC-MS/MS) analysis of protein complex. The sample for LC-MS/MS analysis was first subjected to In-solution trypsin digestion. The sample was reduced with dithiothreitol (30 min at 37°C) and cysteine sulfhydryls were alkylated with iodoacetamide (30 min at 37°C in darkness). Twenty μg of trypsin (Promega) was added to each sample, at a sample to enzyme ratio of 50:1 and digested at 37°C for 16 hr. The sample was de-salted on a Waters HLB Oasis column, speed vac concentrated and then stored at -80°C prior to LC-MS/MS analysis. The above procedure and the LC-MS/MS analysis were performed at University of Victoria-Genome BC Proteomics Centre.

3.3.8 Analysis of LC-MS/MS data. The proteins identified by LC-MS/MS were enriched and grouped into different functional categories using the functional annotation tool of DAVID Bioinformatics Resources 6.7 (241). The proteins from our NS1-interacting complex were screened against the known NS1-host factor interactions from VirHostNet (Virus-Host Network) 2.0, which is a public knowledgebase dedicated to the management, analysis and integration of virus-host interactions (187, 392). Proteins annotated to be involved in innate immunity were screened using the Gene Ontology Analysis tool from InnateDB database (65, 346, 347).

3.3.9 Construction of protein-protein interaction networks. Interaction networks of the proteins were generated through the use of STRING database (version 10) (237) and Cytoscape software (version 3.2.0) (477) .

3.4 Results and Discussion

3.4.1 Construction and characterization of Strep-tagged NS1 mutants

The NS1 protein is made of a RNA-binding domain and an effector domain joined by a flexible and highly variable linker region (57, 438). Our lab had previously rescued mutant viruses having a TC tag insertion or a Strep-tag insertion in this variable region of NS1 (324, 326). Since the objective was to study SIV NS1-interacting partners in its natural host, a recombinant SIV possessing a Strep-tag sequence (WSHPQFEK) in the linker region of NS1 protein in the background of SK02 virus was rescued. The presence of the Strep-tag sequence would enable purification of the NS1 protein along with any associated proteins from the infected cell lysate. Two mutant viruses were rescued: SIV/SK-544 that encodes NS1 with a Strep-tag sequence replacing AA 77 to 84 and SIV/SK-545 that encodes NS1 with a Strep-tag sequence inserted between AA 79 and 80.

The replication potential of the recombinant viruses were first assessed by comparing the plaque size and multiple cycle growth kinetics to that of the wild-type (WT) virus. Even though plaque size of both mutants appear to be similar to the WT virus (Fig. 3.1A), SIV/SK-545 was attenuated in multi-cycle growth kinetics, while SIV/SK-544 displayed growth kinetics similar to the WT virus (Fig. 3.1B).

The phenotype of the mutant virus was further characterized to confirm that the introduction of Strep-tag sequence did not alter the function of NS1 protein. Since SIV/SK-544 mutant virus displayed similar plaque size and growth kinetics to that of the WT virus, I focused on the SIV/SK-544 mutant. Influenza NS1 predominantly localizes in the nucleus of infected

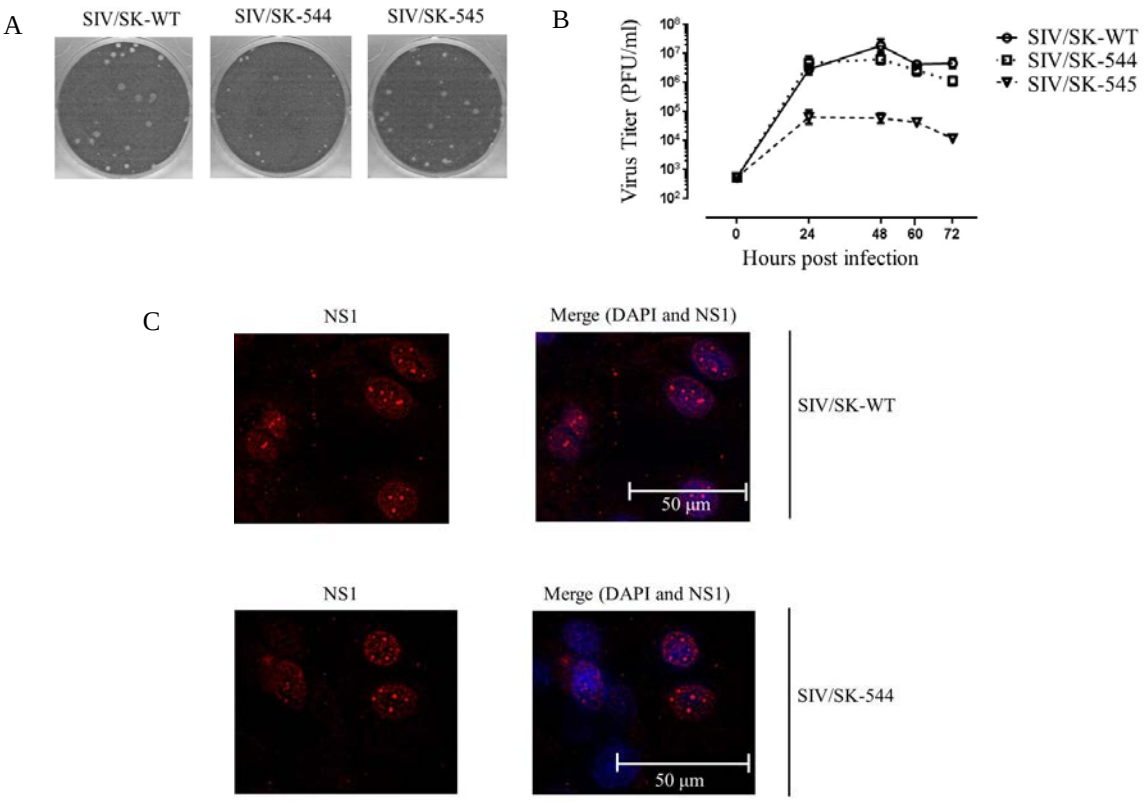


Figure 3.1 Characterization of recombinant Strep-tag NS1 viruses. A) MDCK cells were infected with either the WT virus or the recombinant Strep-tag NS1 viruses. At 48 h.p.i., plaques were visualized by staining with coomassie blue. B) MDCK cells were infected with either the WT virus or the recombinant Strep-tag viruses at an MOI of 0.001 in triplicates. Supernatant was collected every 24 hrs and the titer was determined by plaque assay on MDCK cells. A growth curve was plotted using the mean titer values at each time point and the associated standard deviation is displayed as error bar. C) MDCK cells were infected with either the WT virus or the mutant SIV-SK/544 virus at an MOI of 2 on chamber slides. At 7-8 h.p.i. the cells were fixed, permeabilized and stained with rabbit anti-NS1 serum followed by Alexa fluor 594-conjugated anti rabbit antibody (red) and mounted with a mounting media containing DAPI stain (blue). A representative image of multiple fields of view is shown in the figure.

cells (181, 396) and its intracellular distribution is vital for its multifunctional ability. Therefore, WT and SIV/SK-544 virus-infected cells were immunostained with antibodies specific for NS1 and the intracellular localization of NS1 protein in the infected cells was observed. As shown in Fig. 3.1C, NS1 localized to the nucleus and nucleolus in both SIV/SK-544 and WT virus-infected cells, revealing a similar distribution pattern. These results show that introduction of the Strep-tag sequence did not affect the function of the recombinant virus and hence SIV/SK-544 virus was used for further studies to isolate the intact NS1-interacting protein complex from the infected cells.

3.4.2 Identification of the cellular interaction partners of NS1 protein from infected primary SRECs

I believe that studying host-virus protein interaction in infected primary cells would result in the identification of novel interactors more relevant to the natural infection conditions. It has been reported that the primary site of replication in the host during influenza A virus infection is the respiratory epithelial cells in the trachea (353). Therefore, I was keen on isolating primary SRECs to study NS1-host protein interaction from these cells. Primary SRECs were isolated from the trachea of healthy 6-8 week old pigs and cultured on collagen-coated culture flasks. The cultured primary cells displayed typical cobble-stone morphology (data not shown) and almost all of the cultured cells stained positive for the epithelial cell marker cytokeratin (33) (Fig. 3.2A), confirming their epithelial nature.

To ensure that the virus can replicate efficiently in primary SRECs, the SRECs were infected with the WT and mutant viruses at an MOI of 1 and the cell lysates were collected at different time points. The cell lysates were then subjected to Western blotting with antibodies

against NS1, NP and Strep-tag. The protein expression kinetics was found to be similar for both the WT virus and SIV/SK-544 virus in infected SRECs (Fig. 3.2B). While the NS1 antibody detected both WT NS1 and Strep-tagged NS1, only the Strep-tagged NS1 was detected by the antibody against Strep-tag.

Next, the accessibility of the Strep-tag to Strep-tactin, a derivative of streptavidin, which binds to the Strep-tag specifically with high affinity was tested (464). SRECs were infected with either the WT or SIV/SK-544 virus and at 16 hours post infection (h.p.i.), the cell lysate was prepared and subjected to a pull-down assay with the Strep-tactin resin, followed by Western blotting to determine the presence of NS1 in the eluent. Only NS1 from SIV/SK-544 infected cells could be pulled down efficiently, while the WT NS1 protein did not bind to the resin and was lost during the washes (Fig. 3.2C).

The viral and cellular proteins present in the NS1 pull-down complex from the SIV/SK-544 infected cell lysate and the WT virus-infected cell lysate were then identified by LC-MS/MS. Only 5 cellular proteins were identified in the SIV/SK-WT eluent, whereas 445 cellular proteins were identified in the eluent from SIV/SK-544 infected cell lysate. The 5 cellular proteins identified in the SIV/SK-WT eluent were excluded from further analysis. While no viral proteins were identified in the SIV/SK-WT eluent, viral proteins PB2, PB1, PA, HA, NP and M1 were identified in SIV/SK-544 eluent. The proteins present in the complex were identified using a software package called Mascot from Matrix Science (www.matrixscience.com) that compares the observed mass spectra data to a database of known proteins, to determine the most likely matches. For each protein match, Mascot calculates an overall protein score, which reflects the

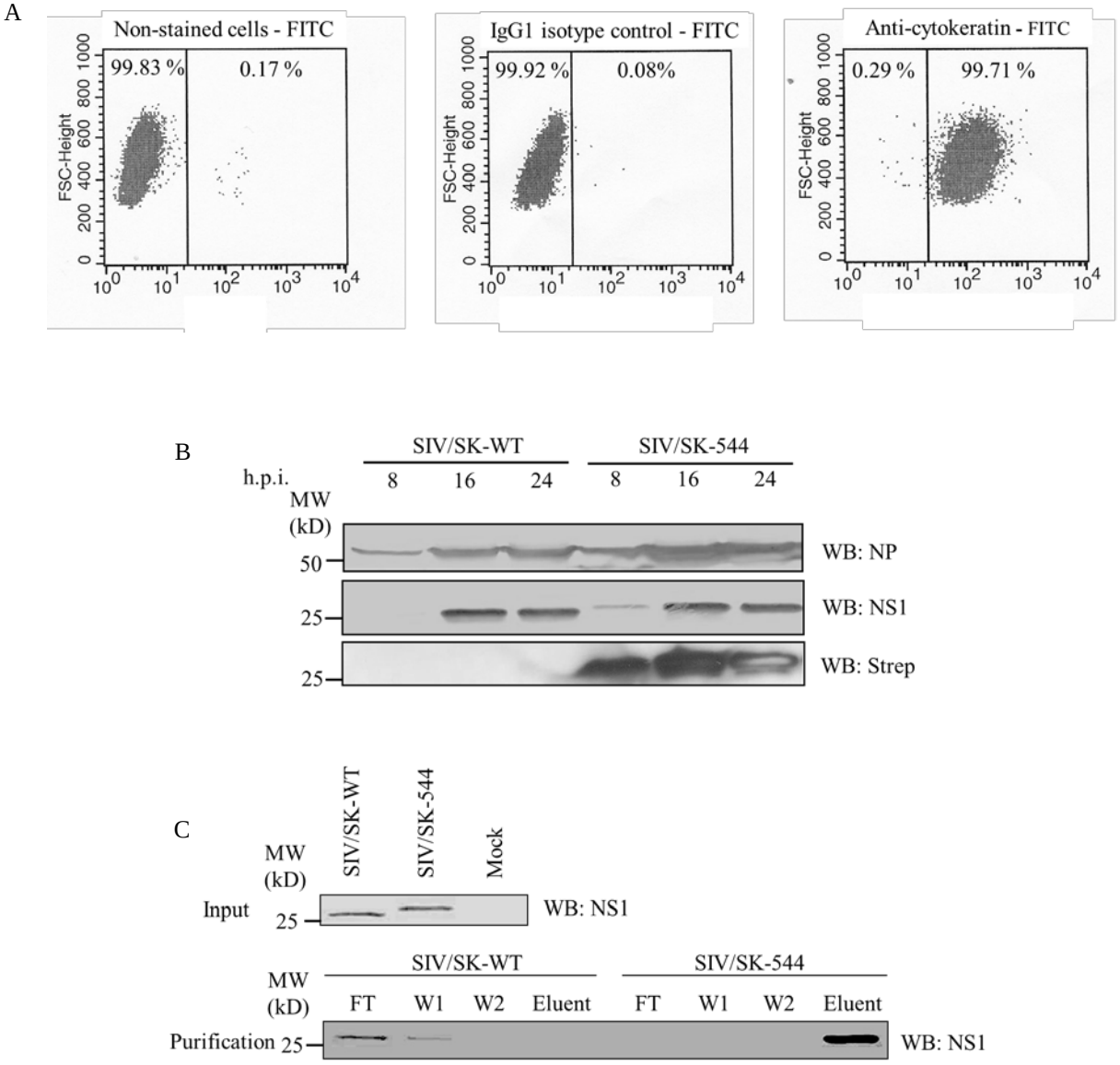


Figure 3.2 FACS staining of SRECs, protein expression kinetics and NS1 protein Strep-tactin pull-down from WT and SIV-SK-544 infected cells. A) SRECs were stained with an antibody against the epithelial cell marker cytokeratin and the purity of the cells were analysed using FACS machine. B) SRECs were infected with WT or SIV/SK-544 virus at an MOI of 1. Cell lysates collected at the indicated time points were subjected to Western blotting with antibodies specific for NP, NS1 and Strep-tag. C) SRECs were infected with either the WT virus or SIV/SK-544 virus at an MOI of 2. At 16 h.p.i., cell lysate was collected and subjected to a pull-down assay with Strep-tactin sepharose. The Flow through (FT), Wash fractions (W1, W2) and the Eluent were collected and subjected to Western blotting with the antibody against NS1.

combined score of all the observed mass spectra that can be matched to amino acid sequences within that protein. Therefore, a higher score indicates a more confident match. To be more stringent in the specificity of the proteins chosen for analysis, we set the threshold to be a Mascot protein score of 150 and above. Additionally, we also excluded those proteins identified with a single peptide match by LC-MS/MS. By setting up this threshold and exclusion, the analysis was restricted to the proteins with the highest confidence scores and thereby I ended up with 192 cellular proteins (43% of the total number, Table 3.1) for further analysis.

3.4.3 Bioinformatics analysis of the protein dataset

VirHostNet 2.0 database provides a high-confidence resource of manually curated interactions defined for a wide range of viral species including influenza. The influenza virus-host interaction database in VirHostNet 2.0 was screened against the 192 top scoring proteins identified in the LC-MS/MS analysis. I found that 92 out of the 192 proteins have been documented in the VirHostNet 2.0 database as NS1-interacting partners (Table 3.1). This analysis gave further proof that the proteins identified in this study are valid NS1 protein interaction partners.

NS1 exerts its multifunctional nature primarily by interacting with host proteins. Therefore, categorizing and grouping the proteins identified by LC-MS/MS based on their molecular functions in the host will reveal potential pathways that might be affected by NS1 expression during infection. The proteins in this dataset were therefore analysed using the DAVID bioinformatics resources (241) to enrich for different functional annotations (Table 3.2). These include protein translation, RNA processing and splicing, Cytoskeleton and microtubules

List of Proteins above a protein score of 150 in the purified NS1 protein complex

Uniprot ID	Gene Name	Peptides matched	Sequence coverage (%)	Protein Score
Q13085	ACACA	81	47.1	4631
Q00839	HNRNPU	7	12.4	2162
P05166	PCCB	29	73.8	2146
P07437	TUBB5	28	79.3	2031
P05217	TBB2	28	79	1915
P04350	TUBB4A	5	12.3	1903
P68371	TUBB4B	5	12.3	1903
Q9H4B7	TBB1	28	71.2	1861
P39023	RPL3	13	27	1706
Q14573	ITPR3	35	18.1	1606
P62841	RPS15	6	54.9	1508
P11940	PABPC1	29	42.7	1426
P62701	RPS4X	22	61.3	1413
P52272	HNRNPM	16	37.8	1331
P04264	KRT1	16	38.6	1303
Q9H4B7	TUBB1	17	41	1232
P68363	TUBA1B	21	64.7	1198
Q96FU6	ACTG1	21	69.7	1167
P61247	RPS3A	24	67.5	1158
P60709	ACTB	20	64.4	1140
P62753	RPS6	11	32.4	1132
Q9BUF5	TUBB6	17	45.2	1050
P67809	YBX1	12	65.9	1016
Q9BQE3	TUBA1C	20	59.8	1002
P62424	RPL7A	17	48.2	986
Q9Y224	C14orf166	10	39.8	950
Q92499	DDX1	19	32.9	948
P05141	SLC25A5	12	35.9	945
P68366	TUBA4A	15	46.3	939
P36578	RPL4	17	39.3	918
Q8NCA5	FAM98A	10	38.6	906
P52597	HNRNPF	10	36.8	902
P22087	FBL	13	58.4	886
Q12905	ILF2	11	44.2	881
P43243	MATR3	13	27.8	871
P12236	SLC25A6	14	51.6	863
Q6PEY2	TUBA3E	14	49.2	855
Q12906	ILF3	21	35.2	847
Q7Z417	NUFIP2	8	18	846
P62081	RPS7	11	55.2	833
Q13310	PABPC4	18	32.2	806
P68104	EEF1A1	12	47.5	803
P15880	RPS2	16	49.2	795
P12235	SLC25A4	8	30.2	785
Q6UN15	FIP1L1	13	37.7	780
P06576	ATP5B	10	35.2	751
P25705	ATP5A1	10	25.6	746

Uniprot ID	Gene Name	Peptides matched	Sequence coverage (%)	Protein Score
P46776	RPL27A	9	40.3	739
Q02878	RPL6	14	40.3	707
P11498	PC	10	13.4	701
P62241	RPS8	13	54.8	697
P62266	RPS23	6	42.7	667
Q10570	CPSF1	16	16.8	663
P62888	RPL30	7	51.8	661
P14618	PKM	8	26.7	648
P62829	RPL23	6	36	639
P23396	RPS3	17	69.3	639
P62263	RPS14	9	68.3	636
P62249	RPS16	12	52.5	618
P53621	COPA	13	15.8	610
P05165	PCCA	14	22.8	603
Q9Y2T7	YBX2	10	35.7	595
P61978	HNRNPK	9	35.4	587
O00571	DDX3X	8	17.2	572
P13645	KRT10	9	27.6	564
Q07020	RPL18	8	36.4	564
Q02543	RPL18A	9	36.6	564
P62917	RPL8	10	42.5	553
Q9NZB2	FAM120A	10	15.4	547
P27635	RPL10	8	35.6	547
P18124	RPL7	14	46.8	546
P62750	RPL23A	9	44.8	537
P51991	HNRNPA3	7	31.7	527
Q9H361	PABPC3	16	20.4	517
P18621	RPL17	13	51.4	514
O00567	NOP56	8	18.2	503
P11142	HSPA8	14	25.8	499
P26373	RPL13	13	44.6	488
P62277	RPS13	13	49.8	488
Q12959	DLG1	9	18	487
Q3ZCM7	TUBB8	10	22.1	473
P35527	KRT9	7	28.8	472
P62244	RPS15A	10	61.1	461
Q96CW1	AP2M1	11	34.6	454
P16402	HIST1H1D	7	23.1	450
P62269	RPS18	10	42.2	443
P04406	GAPDH	7	33.3	434
P62805	HIST1H4A	7	52.3	411
P55795	HNRNPH2	8	30.9	409
Q5SSJ5	HP1BP3	13	28.9	409
Q08211	DHX9	12	11	407
P14866	HNRNPL	6	23.5	407
P06899	HIST1H2BJ	5	38	391
P46782	RPS5	4	36.3	391

Uniprot ID	Gene Name	Peptides matched	Sequence coverage (%)	Protein Score
Q02978	SLC25A11	5	30.5	391
P07910	HNRNPC	5	19.5	381
P62913	RPL11	7	36.5	376
P62979	RPS27A	3	19.8	371
Q92974	ARHGEF2	8	13.3	367
P84098	RPL19	5	21	367
P46778	RPL21	9	37.2	361
Q07065	CKAP4	8	17.3	357
P62847	RPS24	6	31.1	353
Q9Y2X3	NOP58	4	11.9	349
O60506	SYNCRIP	6	12.6	346
P26599	PTBP1	4	18.3	342
O43390	HNRNPR	7	12.4	337
Q9Y6M1	IGF2BP2	7	16.4	337
P16401	HIST1H1B	6	18.5	329
P62280	RPS11	9	46.7	327
Q9C0J8	WDR33	12	16	326
P10412	HIST1H1E	4	12.5	324
P83731	RPL24	5	25.8	323
P05455	SSB	5	11	322
P46781	RPS9	12	31.1	319
Q92841	DDX17	8	21	313
P61313	RPL15	10	36.7	313
Q3KQU3	MAP7D1	5	6.8	300
Q8WWM7	ATXN2L	13	14.4	287
Q13435	SF3B2	6	12	277
P22492	HIST1H1T	3	8	276
O15523	DDX3Y	5	10.6	272
Q9BQG0	MYBBP1A	6	8.1	272
P62910	RPL32	5	33	270
Q9HCE1	MOV10	3	3.5	269
P08106	HSPA2	6	12.7	266
Q9NR30	DDX21	7	12	260
Q1KMD3	HNRNPUL2	7	9.8	258
P50914	RPL14	5	21.6	258
P62899	RPL31	7	44.9	258
P16104	H2AFX	4	37.8	256
Q14204	DYNC1H1	9	2.9	254
P62158	CALM1	3	49	252
P27482	CALML3	3	49	252
Q6NZI2	PTRF	6	20	252
P23528	CFL1	5	47.4	244
P45880	VDAC2	3	18.5	241
Q15717	ELAVL1	4	24.9	240
P68431	HIST1H3A	2	29.1	239
P0DMV9	HSPA1B	6	13.3	238
P04792	HSPB1	4	38.8	236
O60884	DNAJA2	4	18.8	234

Uniprot ID	Gene Name	Peptides matched	Sequence coverage (%)	Protein Score
P51116	FXR2	5	11.3	232
P62851	RPS25	4	23.9	228
P84103	SRSF3	4	26.5	225
Q13084	MRPL28	4	27.3	224
P47914	RPL29	3	18.8	224
P42766	RPL35	4	24.9	223
O76094	SRP72	8	18.9	223
Q9P2I0	CPSF2	4	7.1	222
P46109	CRKL	9	35.6	218
P02511	CRYAB	5	51.9	218
P17858	PFKL	6	16.2	218
P36542	ATP5C1	4	15.9	216
P62854	RPS26	3	42.2	210
Q13405	MRPL49	2	14.9	207
O43143	DHX15	5	10.1	206
Q9BUJ2	HNRNPUL1	4	10.3	200
O95793	STAU1	6	12.1	197
Q96A33	CCDC47	3	10.4	196
Q13509	TUBB3	5	15.1	195
P61513	RPL37A	4	40.9	193
P51114	FXR1	7	18.4	190
P11387	TOP1	5	7.5	190
P83881	RPL36A	4	25.9	186
Q9Y3U8	RPL36	6	36.7	185
Q00325	SLC25A3	4	16.6	183
P38159	RBMX	6	15.9	181
Q8TCJ2	STT3B	4	6.9	179
P84243	H3F3A	2	29.3	174
Q6PKG0	LARP1	4	3.9	173
P61254	RPL26	8	32.5	173
Q8WXX5	DNAJC9	2	11.7	172
Q9Y3Y2	CHTOP	3	17.5	171
O14910	LIN7A	3	15.6	171
Q9NUP9	LIN7C	5	33.6	171
P52815	MRPL12	2	12.8	171
P49411	TUFM	6	17.3	169
P32969	RPL9	2	15	166
P40429	RPL13A	8	30.3	165
P07305	H1F0	3	18.4	162
P42677	RPS27	5	38.2	162
P0DMV8	HSPA1A	3	7.4	158
P08107	HSPA1A	6	13.3	158
Q86V81	ALYREF	5	40	157
Q86V81	THOC4	5	40	157
Q99459	CDC5L	2	3.2	156
P67936	TPM4	4	18.1	155
Q9UJS0	SLC25A13	2	5.2	152
Q99729	HNRNPAB	3	11	151

Uniprot ID	Gene Name	Peptides matched	Sequence coverage (%)	Protein Score
Q9H0A0	NAT10	3	4.1	151
Q13283	G3BP1	2	7.4	150

Table 3.1 List of proteins above a protein score of 150 in the purified NS1 protein complex. The table represents the list of all proteins in the purified protein complex, identified using LC-MS/MS with a protein score of 150 and above. The proteins documented in the VirHostNet 2.0 database as known NS1-interacting partners are highlighted with a dark tan colour.

and apoptosis. These are known to be important host functions regulated during influenza infection.

To understand the interaction between the identified proteins, interaction networks of the proteins grouped in each of the enriched functional categories mentioned above were generated using STRING database and Cytoscape software (Figures 3.3-3.6). Here, the role of NS1 in affecting these functions, known NS1-host interaction partners involved in these functions and how the identified proteins fit in the context of influenza A virus infection and NS1 function are discussed.

3.4.4 Protein translation and viral replication

There are several reports supporting a role for NS1 in the selective translation of viral mRNAs over cellular mRNAs (107, 158, 356, 420). It is widely believed that NS1 interacts with the 5'UTR of viral mRNA, eIF4GI and PABP1 and facilitates the recruitment of 40S ribosomal subunit bound to eIF3, thereby enhancing the selective translation of viral mRNA (189, 355). NS1 protein has also been shown to interact with hStaufen (STAU1) causing its redistribution. Co-localization of both proteins in the ribosomal and polysomal fraction in influenza virus-infected cells have also been reported (129). As STAU1 is known to contribute towards the microtubular transport of cellular mRNAs to polysomes, this interaction is also thought to enhance the selective translation of viral mRNA by NS1. Besides regulating translation, NS1 is also known to regulate virus replication by interacting with the viral RNP and RNA motifs in positive-strand viral RNAs (356, 448). In addition, NS1 also fulfills this function by interacting with cellular proteins such as DDX21, hnRNPU, hnRNPA2/B1 and hnRNPF proteins

Translation	
Uniprot ID	Gene Name
P49411	TUFM
P68104	EEF1A1
P27635	RPL10
P62913	RPL11
P26373	RPL13
P40429	RPL13A
P50914	RPL14
P61313	RPL15
P18621	RPL17
Q07020	RPL18
Q02543	RPL18A
P84098	RPL19
P46778	RPL21
P62750	RPL23A
P83731	RPL24
P61254	RPL26
P46776	RPL27A
P46779	RPL28
P47914	RPL29
P62888	RPL30
P62899	RPL31
P42766	RPL35
Q9Y3U8	RPL36
P83881	RPL36A
P61513	RPL37A
P39023	RPL3
P36578	RPL4
Q02878	RPL6
P62424	RPL7A
P62917	RPL8
P32969	RPL9
P46783	RPS10
P62280	RPS11
P62277	RPS13
P62263	RPS14
P62841	RPS15
P62244	RPS15A
P62249	RPS16
P62269	RPS18
P15880	RPS2
P62266	RPS23
P62847	RPS24
P62851	RPS25
P42677	RPS27
P62979	RPS27A
P23396	RPS3
P61247	RPS3A
P62701	RPS4X
P46782	RPS5
P62753	RPS6
P62081	RPS7
P62241	RPS8
P46781	RPS9
P62910	RPL32
P52815	MRPL12
Q13084	MRPL28
Q13405	MRPL49
Q13310	PABPC4
P40429	RPL13A

RNA Processing	
Uniprot ID	Gene Name
Q99459	CDC5L
Q92499	DDX1
Q92841	DDX17
O43143	DHX15
Q08211	DHX9
Q6UN15	FIP1L1
Q9HCE1	MOV10
O00567	NOP56
Q9Y2X3	NOP58
P05455	SSB
Q86V81	THOC4
P67809	YBX1
Q10570	CPSF1
Q9P2I0	CPSF2
P22087	FBL
P51991	HNRNPA3
P07910	HNRNPC
P52597	HNRNPF
P61978	HNRNPK
P52272	HNRNPM
O43390	HNRNPR
Q00839	HNRNPU
Q9BUJ2	HNRNPUL1
Q13310	PABPC4
P11940	PABPC1
P26599	PTBP1
P62913	RPL11
P50914	RPL14
P61254	RPL26
P83881	RPL36A
P62263	RPS14
P62841	RPS15
P62249	RPS16
P62847	RPS24
P62753	RPS6
P62081	RPS7
P38159	RBMX
Q13435	SF3B2
P84103	SRSF3
O60506	SYNCRIP
P14866	HNRNPL

Splicing	
Uniprot ID	Gene Name
Q99459	CDC5L
Q92499	DDX1
O43143	DHX15
Q08211	DHX9
Q86V81	THOC4
P67809	YBX1
Q10570	CPSF1
Q9P2I0	CPSF2
P51991	HNRNPA3
P07910	HNRNPC
P52597	HNRNPF
P61978	HNRNPK
P52272	HNRNPM
O43390	HNRNPR
Q00839	HNRNPU
Q9BUJ2	HNRNPUL1
P11940	PABPC1
P26599	PTBP1
P83881	RPL36A
P38159	RBMX
P14866	HNRNPL
Q13435	SF3B2
P84103	SRSF3
O60506	SYNCRIP

Microtubule & Cytoskeleton	
Uniprot ID	Gene Name
Q08211	DHX9
Q3KQU3	MAP7D1
Q92974	ARHGEF2
P62158	CALM1
Q9Y224	C14orf166
P02511	CRYAB
Q14204	DYNC1H1
P04792	HSPB1
P68363	TUBA1B
O95793	STAU1
Q9BQE3	TUBA1C
Q6PEY2	TUBA3E
P68366	TUBA4A
Q9H4B7	TBB1
P05217	TBB2
Q13509	TUBB3
P04350	TUBB4A
Q9BUF5	TUBB6
Q3ZCM7	TUBB8
P07437	TUBB5

Apoptosis	
Uniprot ID	Gene Name
P23528	CFL1
P02511	CRYAB
P04792	HSPB1
P08107	HSPA1A
P62979	RPS27A
P16989	YBX3
P23396	RPS3
P61247	RPS3A
P05217	TBB2
P07437	TUBB5
Q92974	ARHGEF2
P62913	RPL11
P62753	RPS6

PDZ domain containing	
Uniprot ID	Gene Name
Q12959	DLG1
O14910	LIN7A
Q9NUP9	LIN7C

Innate Immunity	
Uniprot ID	Gene Name
P62158	CALM1
P46109	CRKL
P02511	CRYAB
Q92499	DDX1
Q9NR30	DDX21
O00571	DDX3X
Q08211	DHX9
Q15717	ELAVL1
P51114	FXR1
P14866	HNRNPL
Q12906	ILF3
Q14573	ITPR3
Q9HCE1	MOV10
Q15365	PCBP1
Q15366	PCBP2
P84098	RPL19
P49411	TUFM
P67809	YBX1

Table 3.2 Enriched functions as analyzed by DAVID resources and InnateDB. This table represents the different enriched functional categories identified using DAVID bioinformatics resources and contains the proteins belonging to each functional category. Proteins related to innate immunity were grouped using InnateDB database.

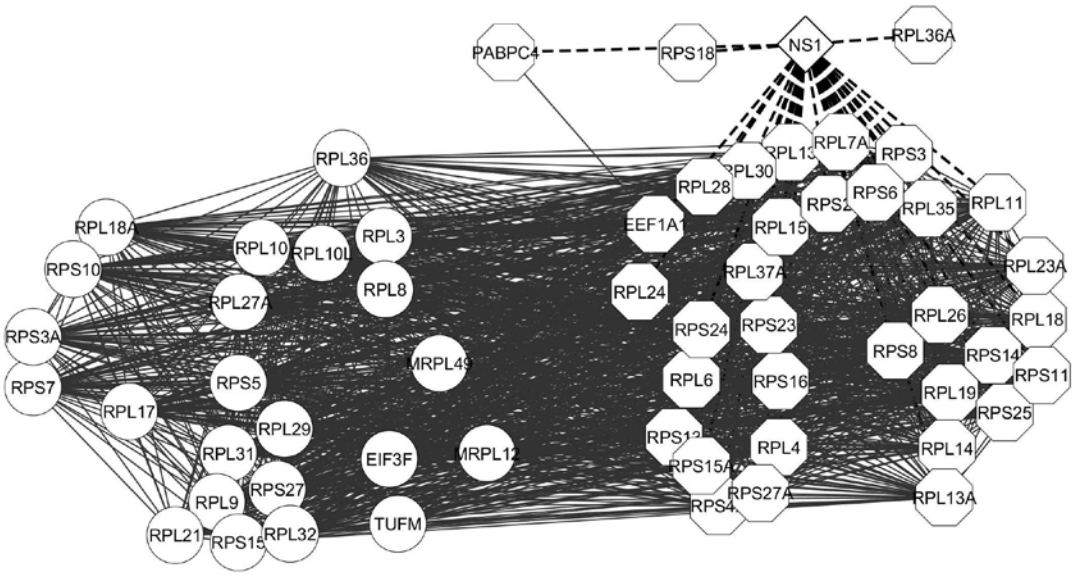


Figure 3.3 Interaction network of proteins involved in translation. The proteins classified as related to the translation machinery (Table 3.2) were analyzed by STRING database and an interaction network was generated using Cytoscape. The edges connecting NS1 and known NS1-interacting host proteins are represented by dashed lines and the NS1-interacting host protein nodes are highlighted by an octagon shaped border. Nodes represent the proteins in the network. Edges represent the interaction connecting the two nodes.

(81, 311, 430, 569). In line with these observations, PABP1, STAU1, eIF3F, DDX21, hnRNPU and hnRNP proteins were identified in the NS1-interacting complex in this study (Table 3.1). Among the NS1-associating host proteins, 59 proteins were grouped by DAVID to have a function in translation with a P value equal to 4.8×10^{-56} (Fig. 3.3) (Table 3.2).

3.4.5 Splicing and nuclear export of viral mRNA

Once the vRNP is in the cytoplasm, it is transported into the nucleus by the nuclear import machinery in association with the viral proteins (96, 564). Once in the nucleus, genomic replication, transcription and pre-mRNA processing takes place. Influenza viruses are unique among RNA viruses in that the whole replication cycle takes place inside the nucleus and not in the cytoplasm (210), since the virus needs the host splicing machinery to splice the NS1 and M mRNA into smaller NEP and M2 mRNA respectively (299, 300). Additionally, viral transcripts are exported through the NXF1 protein which is recruited to the viral transcripts by a complex of proteins made up of THO complex (THOC1-7), UAP56 and Aly/REF (601).

NS1 protein affects several functions in RNA processing. Influenza NS1 is reported to increase the splicing of viral M mRNA but does not appear to affect the splicing of its own mRNA (449, 450). Contrary to this enhancing effect, influenza NS1 protein is known to inhibit cellular pre-mRNA splicing by associating with spliceosomes and U6 snRNA, contributing to host's shut-off (342). hnRNP proteins are abundant nuclear proteins known to be bound to pre-mRNAs in the nucleus (119) and they function in the splicing and nuclear export of pre-mRNA in eukaryotic cells (88, 119). Influenza NS1 protein is proposed to have a function in the nuclear export of viral mRNAs and has been shown to interact with several proteins involved in nuclear export including NXF1. Its binding to the cellular protein CPSF inhibits polyadenylation of host

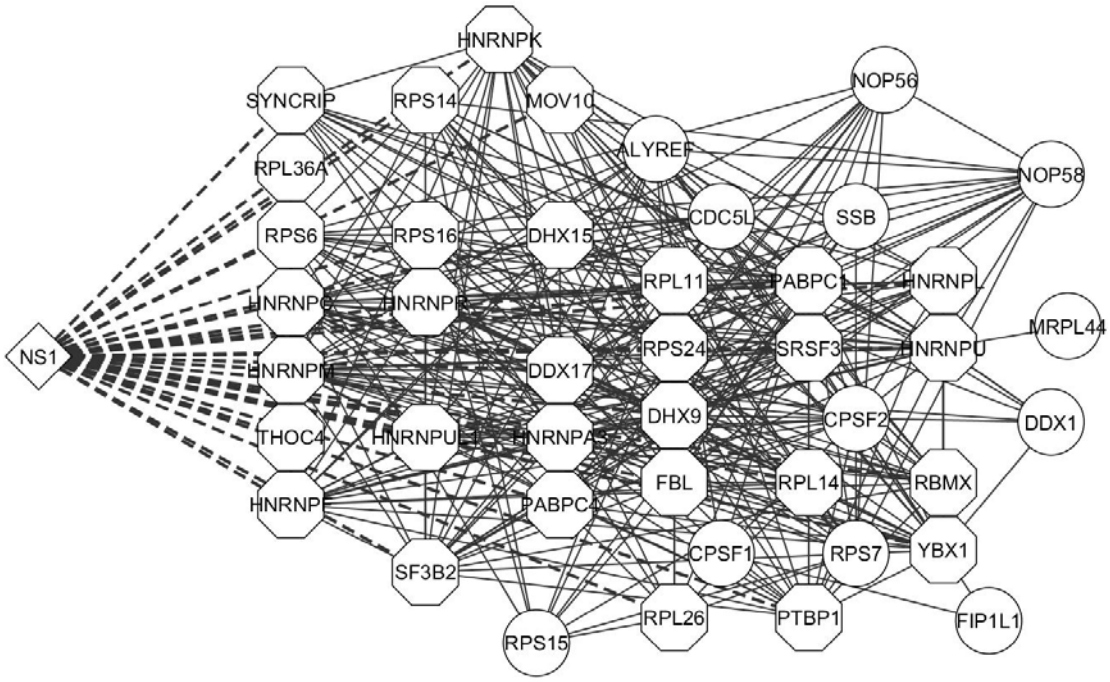


Figure 3.4 Interaction network of proteins involved in RNA processing. The proteins classified as related to the RNA processing machinery (Table 3.2) were analyzed by STRING database and an interaction network was generated using Cytoscape. The edges connecting NS1 and known NS1-interacting host proteins are represented by dashed lines and the NS1-interacting host protein nodes are highlighted by an octagon shaped border. Nodes represent the proteins in the network. Edges represent the interaction connecting the two nodes.

pre-mRNAs and prevents their nuclear export (394, 460). Among the NS1-associating host proteins, 41 proteins and 24 proteins were grouped by DAVID to be involved in RNA processing ($P = 2.6\times10^{-21}$) and RNA splicing ($P = 7.4\times10^{-13}$) respectively, including CPSF1, CPSF2, 8 out of the 20 known hnRNP proteins and THOC4 (Fig. 3.4) (Table 3.2).

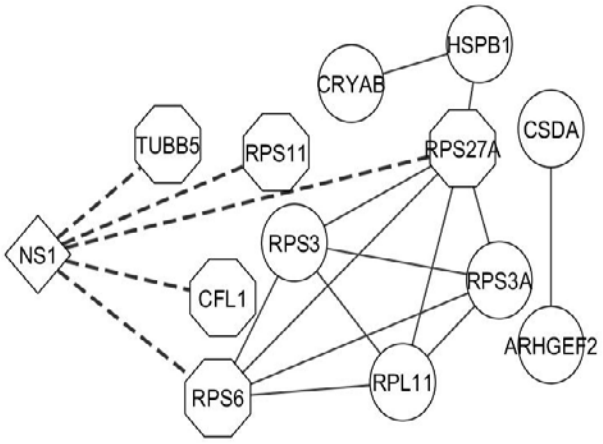
3.4.6 Apoptosis and Innate immunity

Even though proteins from my dataset were weakly enriched for the apoptosis function by DAVID ($P = 0.12$), it is an important pathway affecting both the virus and host during influenza A virus life cycle (Fig. 3.5A) (Table 3.2). Hence, I was keen on exploring the potential contribution of the proteins in the dataset to the apoptosis pathway.

Apoptosis is an important innate immune mechanism to maintain homeostasis in the host and is a major pathway regulated during influenza virus infection (212, 522). Influenza A virus has been documented to modulate apoptosis to favour efficient virus replication by interacting with several host proteins through its viral proteins, including NS1 (208, 232). A recent study showed that NS1 interaction with β-tubulin disrupts the cellular microtubule network and thereby commits the cell to apoptosis (192). NS1 interaction with the p85 regulatory subunit and CRK/CRKL prevents premature cell-death and facilitates enhanced virus replication (122, 220, 489). Additionally, the microtubule and cytoskeleton network also contributes to trafficking of vRNPs from the cytoplasm to the cell periphery for assembly and budding of the virus (12). Thus, the microtubule network and apoptosis pathways are key players in the influenza A virus life cycle. Furthermore, 20 proteins in the dataset belong to the microtubule and cytoskeleton network, as analyzed by DAVID bioinformatics resources ($P = 1.6\times10^{-4}$) (Fig. 3.5B) (Table 3.2).

NS1 is a known antagonist of innate immune response. InnateDB is a manually curated

A



B

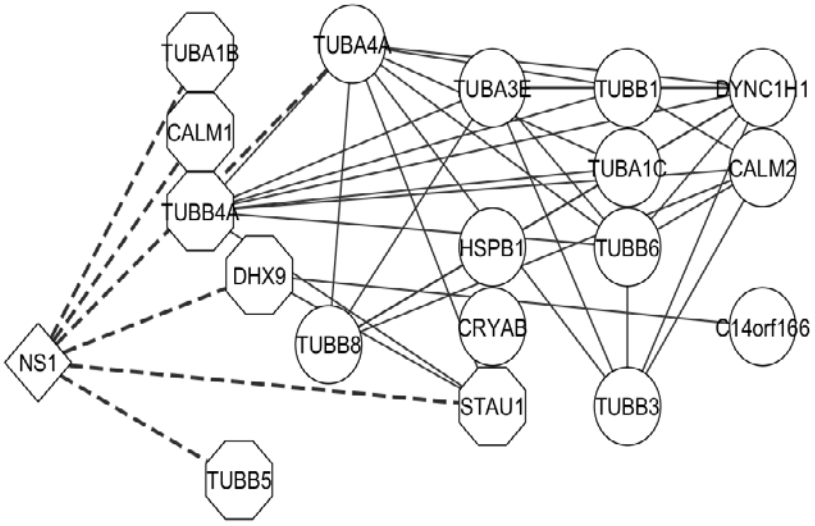


Figure 3.5 Interaction network of proteins involved in the Apoptosis & Cytoskeleton network. The proteins classified as belonging to the a) apoptosis pathway and b) cytoskeleton network (Table 3.2) were analyzed by STRING database and an interaction network was generated using Cytoscape. The edges connecting NS1 and known NS1-interacting host proteins are represented by dashed lines and the NS1-interacting host protein nodes are highlighted by an octagon shaped border. Nodes represent the proteins in the network. Edges represent the interaction connecting the two nodes.

knowledgebase of the genes, proteins, interactions and signalling responses in mammalian innate immunity (65). Therefore, I screened the dataset against the genes present in the innateDB database, which have been annotated to have a role in innate immune response. Eighteen proteins from the dataset were identified, which belonged to this database (Table 3.2). Several proteins listed in the table are well known interactors of NS1. DHX9 and DDX21 interact with NS1 and regulate virus replication (81, 326), CRKL interaction with NS1 induces enhanced PI3K signalling (597), while other proteins such as FXR1 and ILF3, have known functions during influenza A virus infection but have not been reported to interact with NS1 (565, 610). Hence, NS1 being an antagonist of innate immune responses, it might be interesting to study the interaction of these cellular proteins with NS1 and their function. A protein interaction network of these host factors revealed strong associations between these proteins (Fig. 3.6).

3.4.7 SIV/SK-NS1 polymorphisms and their potential contribution to interaction partners

It is well documented that significant polymorphisms exist in the NS1 sequences among various strains (355). SK02 virus is a wholly avian virus isolated from pigs (250) and because of this the NS1 protein has some unique sequences and motifs. NS1 protein of influenza A viruses are known to have a PDZ-binding motif (PBM) at the last four amino acid residues (406). As the name suggests, PBMs confer binding to proteins containing a characteristic structure called the PDZ domain. PDZ domain-containing proteins act as scaffolds to assemble protein complexes and function in cell signalling and cell polarity (196, 401). Human influenza A virus isolates contain a PBM with varied sequences depending on the strain and the NS1 of H5N1 avian influenza A virus isolates from human infections generally have a PBM with the sequence ESEV (173). Some studies have shown that avian NS1 with the ESEV PBM can bind the PDZ proteins

Scribble, Dlg1, MAGI-1, MAGI-2, MAGI-3 and an indirect association with Lin7C (173, 329, 531). The SK02 virus used in this study for infections has the same avian signature ESEV PBM (AA 227-230) in the NS1 protein. Therefore, I was interested in finding out, whether any of the above mentioned proteins have been identified in my pull-down complex. Consistent with the literature, Dlg1 and Lin7C were indeed present in the dataset (Table 3.2). This interaction decreases tight junction integrity of the epithelial cells and benefit virus replication (173, 329, 531). Other PDZ containing proteins identified in my complex but not included in gene ontology analysis include Pdlim5 and Pdlim7 proteins, which could have a role in cytoskeleton organization (247). SK02 virus also has a partial nucleolar localization signal (NoLS) comprising the basic amino acids in positions 219, 220 and 224 as per the consensus NoLS identified by Melen et al. (374) and nucleolar localization of SK02 NS1 was also observed in this study (Fig. 3.1C).

It has been observed that most avian influenza A viruses have a class II SH3 domain in the NS1 protein with the sequence PPLPPK at AA 212-217, while this motif is rarely seen in human influenza A viruses (206). NS1 proteins with this sequence have been shown to bind to the cellular adaptor protein CRK/CRKL, while the NS1 proteins of human influenza A viruses do not bind to this sequence (206, 220). Consequently, NS1-CRK/CRKL interaction inhibits the virus-induced activation of JNK-ATF2 pathway, which in turn prevents premature cell death and thereby facilitates enhanced virus replication (220). The NS1 protein of SK02 also possesses a class II SH3 binding motif and CRK/CRKL protein was identified in the pull-down complex (Table 3.1). All these unique interactions could have a significant effect on the pathogenesis of the virus at the cellular level.

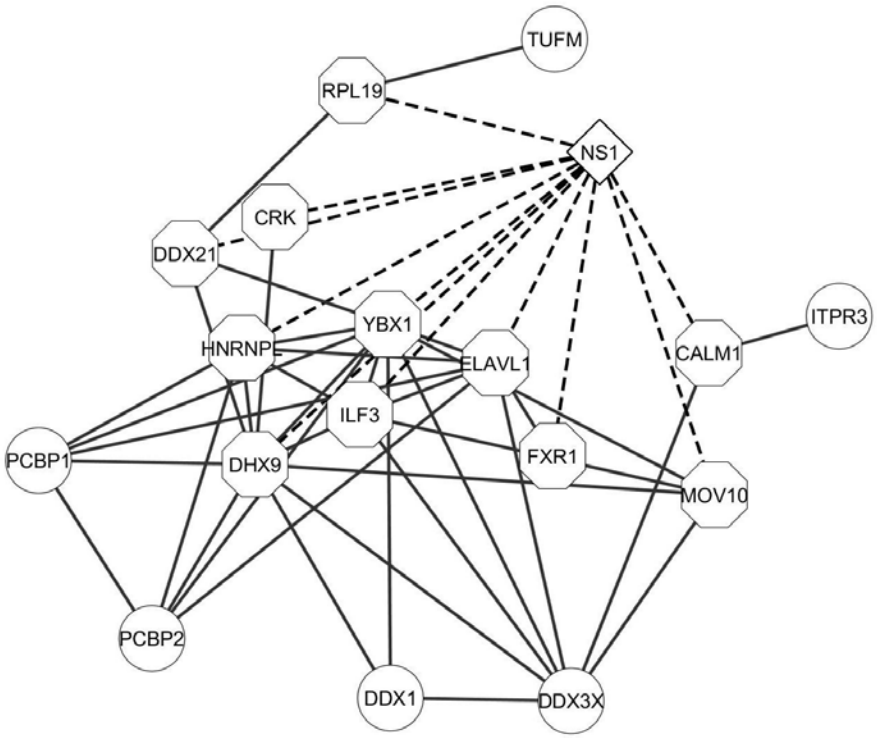


Figure 3.6 Interaction network of innate immunity related proteins. The proteins classified as belonging to the innate-immune response pathway (Table 3.2) were analyzed by STRING database and an interaction network was generated using Cytoscape. The edges connecting NS1 and known NS1-interacting host proteins are represented by dashed lines and the NS1-interacting host protein nodes are highlighted by an octagon shaped border. Nodes represent the proteins in the network. Edges represent the interaction connecting the two nodes.

3.4.8 Validation of NS1 interaction with RNA helicases DHX9 and DDX3

Influenza being an RNA virus, RNA helicases could have a critical role in the virus life cycle. Several RNA helicases such as DDX1, DHX9, DDX3, DDX21, DDX17 and DHX15 were identified in the NS1-interacting complex. Hence, I was interested in understanding whether any of these helicases interact with NS1 and the mechanism by which they affect the virus life cycle. A previously published study in our lab, confirmed DHX9 as an NS1 interaction partner and its pro-viral role in enhancing viral transcription and replication during infection was also established (326). In chapter 4 of this thesis, DDX3 has been studied and confirmed to be a NS1 interaction partner. Additionally, knockdown of DDX3 enhanced virus replication and was found to play an antiviral role mediated through stress granule formation (see chapter 4). These two studies confirmed DHX9 and DDX3 as authentic NS1 interaction partners, serving as a validation for the approach used in this work to study the cellular proteins interacting with NS1 protein.

3.4.9 Conclusion

Through bioinformatics analysis of the NS1-interacting complex, we grouped the identified cellular partners into diverse functional categories. This shows that the large protein complex associating with NS1 form part of smaller complexes with distinct cellular functions. Even though our data cannot characterize the cellular partners as direct or indirect NS1 interacting partners, our analysis provides an insight into the potential NS1 regulated cellular pathways important for influenza infection. Validation and characterization of these associations will provide a deeper understanding of the virus-host interplay, the co-evolution mechanisms that moulded the host-pathogen relationship and will help in the identification of new therapeutic

targets to control influenza infection and disease pathogenesis.

CHAPTER 4: DDX3 INTERACTS WITH INFLUENZA A NS1 AND NP PROTEINS AND EXERTS ANTIVIRAL FUNCTION THROUGH THE REGULATION OF STRESS GRANULE FORMATION

Relationship of this study to the dissertation.

In the previous study, DDX3 was identified to be associated with NS1 as part of a larger NS1 interacting protein complex. Therefore, in this study we tested and characterized the interaction of DDX3 with NS1 and other viral proteins. Existing literature about DDX3 function in SGs as an essential nucleating factor (486), prompted us to explore the function of DDX3 in influenza virus-induced SGs.

4.1 Abstract

DDX3 belongs to the DEAD box RNA helicase family and is a multifunctional protein affecting the life cycle of a variety of viruses. DDX3 was identified as a NS1 associating partner in chapter 3. However, its role in influenza virus infection is unknown. In this study, the potential role of DDX3 in influenza A virus life cycle was explored. The interaction of DDX3 and NS1 was first validated. In the search of other potential viral proteins that interact with DDX3, NP was also observed to interact with DDX3 in virus-infected cells. The contribution of the different domains in DDX3 to its NS1- and NP- interaction was examined. Stress granules (SGs) are known to be antiviral and do form in influenza A virus-infected cells expressing defective NS1 protein. Additionally, a recent study showed that DDX3 is an important SG-nucleating factor. Thus, the role of DDX3 in affecting influenza A virus infection through regulation of SGs was further explored. Results from this work showed that SGs were formed in infected cells upon infection with a mutant influenza A virus lacking a functional NS1 (del NS1) protein and DDX3

co-localized with NP in SGs. Furthermore, the DDX3 helicase domain was identified not to interact with NS1 and NP; however, it was essential for DDX3 localization in virus induced SGs. Knockdown of DDX3 resulted in impaired SG formation and led to increased virus titers. DDX3-NP interaction may be important for NP recruitment into SGs and DDX3-NS1 interaction may play a role in NS1 mediated SG inhibition in an eIF2α-independent manner. Taken together, these results identified DDX3 as an antiviral protein with a role in virus-induced SG formation.

4.2 Introduction

DDX3 belongs to the DEAD box RNA helicase family and harbors ATPase and RNA helicase activities (145). Like most other DEAD box helicases, DDX3 is a multifunctional protein with functions related to RNA metabolism, RNA export, ribosome biogenesis, cellular signalling and apoptosis (19, 480). DDX3 is known to enhance antiviral innate immunity by interacting with specific proteins of the type I IFN pathway (547). However, many viruses employ viral proteins such as Vaccinia virus (VACV) K7, Hepatitis B virus (HBV) pol and Hepatitis C virus (HCV) core protein to counteract DDX3 function and in turn use DDX3 to enhance their own replication (20, 76, 459, 472, 563). Contrary to its antiviral function, DDX3 is required for the replication of several viruses such as Human Immunodeficiency virus (HIV), West Nile virus (WNV), Japanese encephalitis virus (JEV), HCV and Norovirus (547). Therefore, existing literature portrays DDX3 both as a host factor required for viral replication as well as a component of the antiviral innate immune response.

Stress granules (SGs) are discrete cytoplasmic foci containing untranslated mRNA in nucleoprotein aggregates. They form in eukaryotic cells in response to a variety of environmental stress conditions including viral infections (409). The first step in the signalling cascade leading to SG assembly is the phosphorylation of eukaryotic translation initiation factor-2α (eIF2α), which can be regulated by any of the four serine/threonine kinases, namely double stranded RNA-dependent protein kinase R (PKR), Heme-regulated translation inhibitor kinase (HRI), PKR-like endoplasmic reticulum kinase (PERK) and General Control Nonderepressible 2 (GCN2) (17, 402, 434). PKR is activated by heat, UV irradiation and viral infections (580), HRI is activated in erythroid cells subject to oxidative stress and when levels of free heme are limiting during hemoglobin assembly (167, 191), PERK is activated in response to unfolded protein

accumulation in endoplasmic reticulum (195, 422) and GCN2 is activated during amino acid deprivation (281). The phosphorylation of eIF2α reduces the availability of ternary complex eIF2-GTP-$tRNA_iMet$, which is required to load the initiator $tRNA_iMet$ onto the small ribosomal subunit to initiate translation (267). This results in the accumulation of stalled translation preinitiation complexes containing the translationally inactive messenger ribonucleoproteins, which recruit the RNA-binding proteins such as T-cell intracellular antigen-1 (TIA-1) and TIA-1 related protein (TIAR). These RNA-binding proteins in turn mediate the formation of SGs (266).

Several RNA helicases including DDX3 have been shown to localize in SGs (69). A recent study reported that DDX3 localized in the SGs, induced by a variety of cellular stresses including sorbitol, arsenite, DTT, heat shock treatment and UV irradiation. Further, DDX3 was found to be a SG-nucleating factor and DDX3-eIF4E interaction is essential for SG formation (486). Many viruses induce SGs through the activation of PKR kinase and in some cases GCN2 by the detection of viral RNA in the cytoplasm (46, 350). Most viruses including influenza A virus have mechanisms to inhibit SG formation, implicating the antiviral role of SGs in the virus life cycle (409). In case of influenza A viruses, NS1 protein is known to inactivate PKR, thereby preventing eIF2α phosphorylation and SG formation (272). Besides NS1, the nucleoprotein (NP) and polymerase-acidic protein-X (PA-X) have also been shown to aid influenza virus in overcoming stress induced translation arrest (271). Besides inducing translation arrest, SGs have also been shown to play a role in interferon (IFN) synthesis by sequestering retinoic acid inducible gene I (RIG-I) and influenza viral RNA (vRNA), thereby serving as a platform for the sensing of viral RNA by RIG-I (408). In addition, other antiviral proteins such as MDA5, LGP2, RNaseL, OAS and PKR also localize in influenza virus-induced SGs (408). These studies underscore the antiviral role that SGs play in influenza infection and highlight the involvement

of DDX3 in virus life cycle and SG formation. These studies prompted the exploration of the role of DDX3 in influenza A virus induced SG formation.

Several host factors involved in RNA metabolism including DDX3 have been shown to associate with the viral polymerase complex and co-localize with NP (244). However, detailed studies on the viral interaction partners and the function of DDX3 during influenza A virus replication were not conducted. Another study, attempting to assess the effect of DDX3 downregulation on influenza A virus polymerase activity, could not determine conclusively whether DDX3 regulates this function (58).Thus, in this study the role of DDX3 during influenza virus infection and the mechanism of DDX3-mediated regulation on influenza virus replication was investigated. DDX3 was established as an interaction partner with the viral NS1 and NP proteins and it was observed to localize in virus induced SGs. NS1 was able to counteract virus-induced SG formation and DDX3 localization into these SGs. The domains in DDX3 that are critical for interaction with NS1, NP and SG formation was also identified. Moreover, knockdown of DDX3 impaired SG formation and increased virus titers upon infection with PR8 NS1 deletion virus. Thus, DDX3 was established as an antiviral protein for influenza A virus infection with a prominent role in regulating SG formation, which warrants further study and understanding.

4.3 Materials and Methods

4.3.1 Cells and viruses. Madin-Darby canine kidney (MDCK) cells and new born porcine tracheal epithelial (NPTr) cells were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) (Life Technologies). 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS.

Influenza A/Puerto Rico/8/34 (H1N1) (PR8-WT) and A/Sw/SK/18789/02 (H1N1) (SIV/SK-WT) viruses were propagated in 11-day-old embryonated chicken eggs as described previously (487). PR8 virus lacking NS1 protein (del NS1) was kindly provided by Dr. Garcia-Sastre and was propagated in Vero cells maintained in MEM with 10% FBS. PR8-WT and SIV/SK-WT virus were titrated by plaque assay on MDCK cells, while del NS1 was titrated on Vero cells. PR8 virus carrying the mutations R38A and K41A in NS1 was rescued by reverse genetics (214). The virus was propagated using a MDCK cell line stably expressing the NS1 protein (MDCK-NS1) and titrated by plaque assay on the MDCK-NS1 cell line.

4.3.2 Antibodies and reagents. Rabbit polyclonal NS1 and NP antibodies were generated in our laboratory as previously described (488). The other antibodies were purchased from different sources as follows: Mouse anti-HA antibody and Mouse anti-Flag M2 antibody (Sigma-Aldrich), Rabbit anti-flag DYKDDDDK tag antibody (Cell signaling technology), Mouse anti-Influenza A NP AA5H antibody (AbD Serotec), Rabbit polyclonal antibody to DDX3 (Abcam), Rabbit polyclonal antibody to PABP1 (Abcam), Rabbit polyclonal to HA tag – ChIP grade (Abcam), Mouse monoclonal antibody to β-actin (Cell Signaling Technology), Goat anti-TIA-1 antibody (Santa Cruz), Donkey anti-Rabbit IgG secondary antibody, Alexa Fluor 405 (Abcam), Donkey anti-Mouse IgG secondary antibody, Alexa Fluor 488 (Life Technologies), Donkey anti-Goat secondary antibody, Alexa Fluor 633 (Life Technologies), IRDye 680RD anti-

Rabbit antibody (LI-COR), IRDye 800CW anti-mouse antibody (LI-COR). Horse serum used in immunofluorescent staining was purchased from Life Technologies. Stellaris FISH probes specific to PR8 M vRNA for Fluorescence *in situ* hybridization (FISH) assay were purchased from Biosearch technologies.

4.3.3 Transfection and immunoprecipitation (IP). To examine DDX3 interaction with the viral proteins, 293T cells were seeded at a density of 1×10^6 cells/well in six-well plates. One μg of each plasmid expressing the protein of interest was transfected using TransIT-LT1 as per the manufacturer's recommendation. For transfection in NPTr cells, Lipofectamine LTX with PLUS reagent (Life Technologies) was used as per the manufacturer's recommendation. The plasmids used for transfection include, pcDNA-HA-DDX3, pcDNA-SK-NP, pcDNA-PR8-NP, pcDNA-PR8-NS1 and pCMV-3×Flag-PR8-NS1, pCMV-3×Flag-DDX3 (DDX3), pCMV-3×Flag-core helicase DDX3 (DDX3-CH), pCMV-3×Flag-C-term deletion DDX3 (DDX3-del CTD) and pCMV-3×Flag-N-term deletion DDX3 (DDX3-del NTD). The protein of interest was cloned into pCMV-3×Flag and pcDNA-HA plasmids such that the Flag- or HA-tag is fused to the N-terminal sequence of the expressed fusion protein. For examining protein interactions by IP, the cell lysate was collected in 1 ml Flag lysis buffer (FLB) (50 mM Tris HCl, pH 7.4, with 150 mM NaCl, 1 mM EDTA, and 1% TRITON X-100). The cell lysate was then sonicated and cleared of cellular debris by centrifugation. For RNase A treatment, RNase A (Life Technologies) was added to the lysate at a concentration of 10 μg/ml and incubated on ice for 30 mins before proceeding with the immunoprecipitation assay.

For IP, 1.5 μg of mouse anti-HA (Sigma-Aldrich) or mouse monoclonal anti-Flag M2 (Sigma-Aldrich) antibody or mouse anti-Influenza A NP (AbD Serotec) antibody was added to the cell lysate and incubated with gentle rocking at 4°C for 1 hr and 15 mins. Then, 35 μl of

Dynabeads Protein G (Life Technologies) was added to the lysate and incubated for another 1 hr and 15 mins with gentle rocking at 4°C. The beads were then washed extensively with FLB and the precipitated proteins were subjected to Western blotting with appropriate antibodies. The IP data presented are representative of multiple experiments.

4.3.4 Western blotting. Samples were resolved by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto nitrocellulose membranes (Bio-Rad). Membranes were blocked with 10% skim milk for 30 minutes and then incubated with a primary antibody diluted in Tris Buffered Sodium chloride solution with 0.1% Tween-20 (TBST) at 4°C overnight. The following primary antibodies were used for Western blotting: Rabbit polyclonal to NS1 and Rabbit polyclonal to NP (in-house generated), Rabbit anti-Flag DYKDDDDK tag antibody (Cell signaling technology), Rabbit polyclonal antibody to DDX3 (Abcam), Rabbit polyclonal to HA tag–ChIP grade (Abcam), Mouse monoclonal antibody to β-actin (Cell Signaling Technology). After washing in TBST, the membranes were incubated in TBST containing IRDye 680RD Goat anti-Rabbit IgG antibody or IRDye 800CW Goat anti-Mouse IgG antibody. Membranes were washed again in TBST and scanned using an Odyssey imager (Li-Cor Biosciences).

4.3.5 Immunofluorescent staining. NPTr cells were grown on glass chamber slides. After the experimental treatment and/or infection, the cells were washed with PBS, fixed with 4% Paraformaldehyde (PFA) in PBS for 15 mins at room temperature and then permeabilized with ice-cold methanol for 15 mins at room temperature. The cells were washed in PBS, blocked in 5% horse serum in PBS for 45 mins and then incubated with the primary antibody in the blocking buffer for 2 hrs at room temperature or overnight at 4ºC (266). The following primary antibodies were used for immunostaining: Mouse anti-Flag M2 antibody (Sigma), Rabbit anti-

Flag DYKDDDDK tag antibody (Cell signaling technology), Mouse anti-Influenza A NP AA5H antibody (AbD Serotec), Rabbit polyclonal antibody to DDX3 (Abcam), Rabbit polyclonal antibody to PABP1 (Abcam), Goat anti-TIA-1 antibody (Santa Cruz). The cells were again washed in PBS and incubated with the Alexa Fluor conjugated secondary antibodies in the blocking buffer for 1 hr at room temperature. After washing in PBS, the cells were mounted using Prolong Gold Antifade reagent (Life Technologies). Multiple images of different fields of view were captured using Leica TCS SP8 confocal laser microscope. A representative of the multiple images is presented in the results.

For the quantification of the number of SG forming cells, approximately 50 cells showing positive immunostaining for NP were considered from two random microscopy panels. The number of cells showing punctuate TIA-1 staining among the NP positive cells were counted visually.

4.3.6 Knockdown of DDX3. NPTr cells were plated at a density of 4×10^4 cells/well in 24-well plate, 3.8×10^4 cells/well in 4-well chamber slide and 8×10^4 cells/well in 12-well plate. Next day, medium was replaced with OptiMEM and the siRNA containing transfection mix was made with OptiMEM and Lipofectamine 2000 as per manufacturer's protocol. The siRNA mixture containing four independent siRNAs targeting DDX3 (GS1654) and the Negative siRNA (SI03650318) were obtained from Qiagen. The transfection mix was added to the cells in OptiMEM for 5-6 hrs. Then, it was replaced with complete media and incubated for 48 hrs before proceeding with the experimental treatment.

4.3.7 FISH Assay. For the FISH assay, cells were washed with PBS and fixed with 4% Paraformaldehyde (PFA) for 10 min at room temperature after virus infection. After washing with PBS, the cells were permeabilized with 70% ethanol overnight at 4°C. The cells were then

incubated in a hybridization solution [10% dextran sulfate, 2mM vanadyl-ribonucleoside complex, 0.02% RNA-free BSA, 1mg/ml E.coli tRNA, 2× saline-sodium citrate (SSC) and 10% formamide] containing 125 nM vRNA probe (Stellaris FISH probes, Biosearch Technologies) at 28°C overnight in the dark. The cells were then washed again and imaged with a Leica TCS SP8 confocal laser microscope after adding the mounting solution. The Stellaris FISH probes against the M vRNA segment were conjugated with the Quasar 670 Dye. The probes were custom designed using the Stellaris probe designer (version 4.1) with the sequence of the M segment as the target. A total of 39 probes were generated and this mixture of vRNA probes were used in the FISH assay to visualize the M vRNA in the infected cells.

To visualize DDX3 and NP, primary antibodies against the two proteins were added to the hybridization solution along with the vRNA probe during the overnight incubation period described above. Next day, the cells were washed and incubated with the respective Alexa Fluor secondary antibodies in the hybridization solution for 1 hr in the dark at room temperature. Cells were washed again and observed with the confocal microscope. Multiple images of different fields of view were captured using Leica TCS SP8 confocal laser microscope. A representative of the multiple images is presented in the results.

4.4 Results

4.4.1 DDX3 interacts with viral NS1 and NP proteins

DDX3 is known to affect the life cycle of a number of viruses by interacting with their respective viral proteins such as HIV Tat, HCV core, HBV pol and VACV K7 (480). Therefore, I speculated that DDX3 could affect the influenza A virus life cycle by interacting with one or several of the influenza A virus proteins during infection. To identify the DDX3 interaction partners, I tested the interaction between HA-tagged DDX3 and the viral proteins expressed during infection by IP. 293T cells were first transfected with the HA-tagged DDX3 plasmid and then were infected with the influenza virus for 11-12 hrs. The cell lysate was subjected to IP with an antibody against the HA tag. The precipitated proteins were then subjected to Western blotting with antibodies against the HA-tag, viral NS1 protein and NP protein, respectively. As shown in Fig. 4.1A, expression of the viral proteins is similar in the input of the infected cell lysates (lane 1 and 3); both the NS1 and NP proteins were co-precipitated readily with HA-DDX3 (lane 2) but not with HA-vector (lane 4). Neither HA-DDX3 nor the viral proteins were detected in the input and pull-down samples, when HA-vector was transfected in uninfected cells (lane 5 and 6). These data demonstrated that the viral proteins NS1 and NP interact with the DDX3 protein during infection.

To examine whether or not NS1 and NP interaction is dependent upon the expression of other viral proteins, 293T cells were co-transfected with Flag or HA-tagged DDX3 and NS1 or NP expressing plasmids and their interaction was tested by precipitating DDX3 from the lysate. To verify NS1 and DDX3 interaction, the Flag-DDX3 or Flag-vector plasmid was transfected along with or without NS1 expressing plasmid and the cell lysates were subjected to IP with Flag antibody. As shown in Fig. 4.1B, the input NS1 protein expression was similar in lane 1 and 3,

but NS1 was detected in the precipitated complex only when Flag-DDX3 was expressed and not when Flag-vector was expressed (lane 2 vs. 4). To verify NP and DDX3 interaction, HA-DDX3 or HA-vector plasmid was transfected along with or without the NP expressing plasmid (note, Flag-beads bind to NP protein non-specifically, thus I used HA-tag) and the cell lysates were subjected to IP with the HA antibody. As shown in Fig. 4.1C, the input NP protein expression was similar in lane 1 and 3, but NP was detected in the precipitated complex only when HA-DDX3 was expressed and not when HA-vector was expressed (lane 2 vs. 4). These results showed that the NS1 and NP proteins can interact with DDX3 independent of infection and other viral components.

To further confirm this interaction, reciprocal pull-down assay was conducted. Flag-NS1 or Flag-vector plasmid was co-transfected with the HA-DDX3 or HA-vector plasmid in 293T cells and the cell lysates were then subjected to IP using the Flag antibody. As shown in Fig. 4.1D, Flag-NS1 co-precipitated HA-tagged DDX3 (lane 2), while HA-DDX3 was not detected in the precipitated complex expressing Flag-vector and HA-DDX3 (lane 4). For DDX3 and NP interaction, 293T cells were transfected with HA-DDX3 or HA-vector and then were infected with the wild-type (WT) virus. The cells were subjected to IP with NP antibody. As shown in Fig. 4.1E, HA-DDX3 co-precipitated with NP (lane 2) in infected cell lysate. No HA-DDX3 was detected in the precipitated complex of the mock-infected cell lysate, which does not express NP (lane 4). When HA-vector was expressed in infected cells, although NP was precipitated successfully, no HA-DDX3 was detected (lane 6). These results demonstrated that NS1 and NP interact with DDX3 during infection and the interaction is specific.

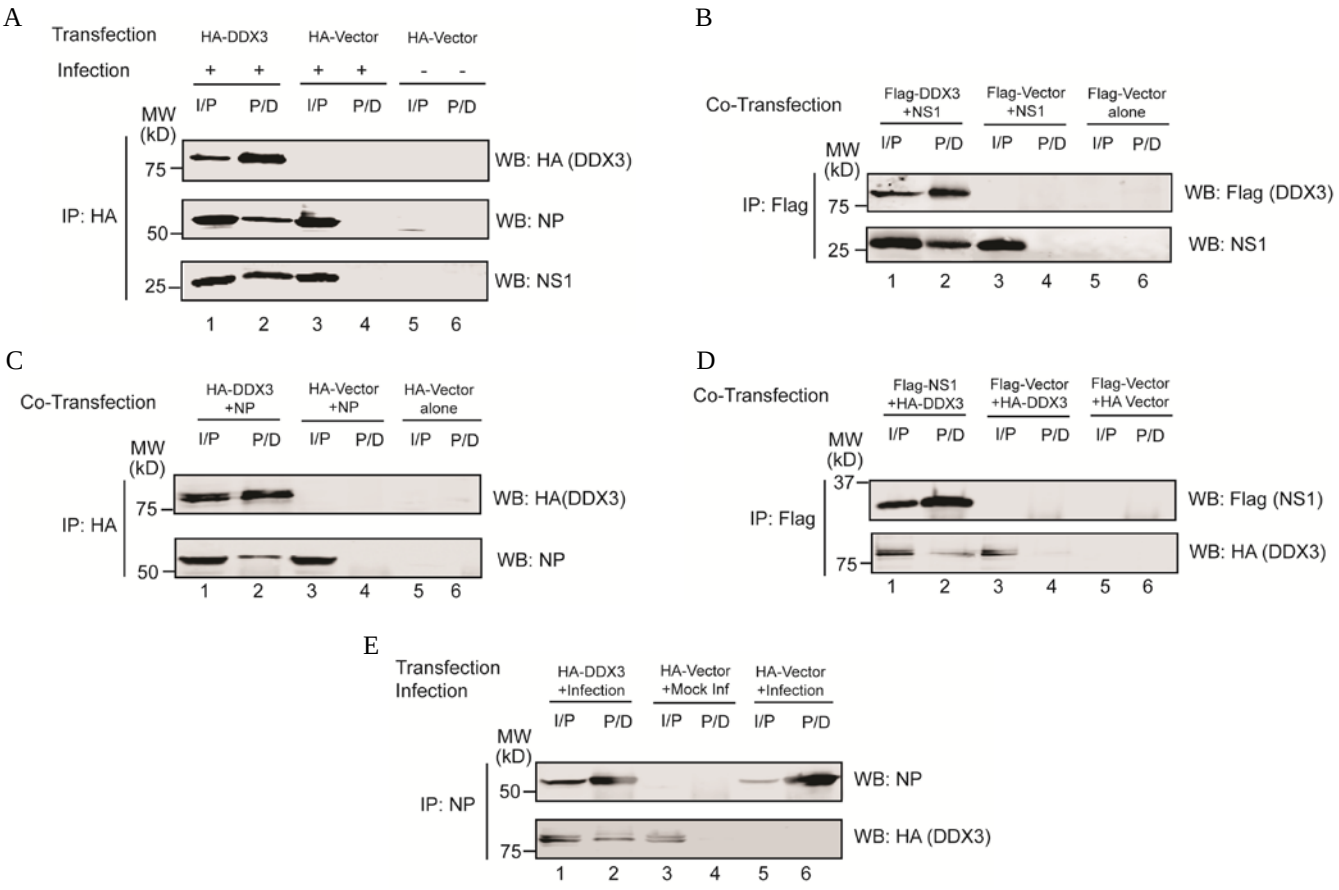


Figure 4.1 Identification and characterization of viral proteins interacting with DDX3. A) 293T cells were transfected with HA-DDX3 or HA-vector plasmid. At 36 h.p.t., cells were infected with SK-WT at an MOI of 1 or left uninfected. Cell lysates were prepared at 11-12 h.p.i and subjected to IP with HA-antibody. Precipitated proteins were subjected to Western blotting using antibodies against HA-tag, NS1 and NP proteins. B) Flag-DDX3 or flag vector plasmid was co-transfected with or without PR8-NS1 expressing plasmid in 293T cells. At 48 h.p.t., cell lysates were collected and subjected to IP with Flag antibody. Precipitated proteins were subjected to Western blotting using antibodies against Flag-tag and NS1 protein. C) HA-DDX3 or HA vector plasmid was transfected with or without SIV/SK-NP expressing plasmid in 293T cells. At 48 h.p.t., cell lysates were collected and subjected to IP with HA antibody. Precipitated proteins were subjected to Western blotting using antibodies against HA-tag and NP protein. D) Flag-PR8 NS1 or flag vector plasmid was co-transfected with HA-DDX3 or HA-vector in 293T cells. At 48 h.p.t., cell lysates were collected and subjected to IP with Flag antibody and Protein-G dynabeads. Precipitated proteins were subjected to western blotting using antibodies against Flag-tag and HA-tag. E) HA-DDX3 or HA vector plasmid was transfected into 293T cells and were either infected with SIV/SK-WT at an MOI of 1 or left uninfected. At 11-12 h.p.i., cell lysates were collected and subjected to IP with NP antibody. Precipitated proteins were subjected to Western blotting using antibodies against NP protein and HA-tag. I/P refers to input and P/D refers to pull-down. The data presented is representative of multiple independent experiments.

4.4.2 DDX3-NS1 and DDX3-NP interaction is RNA independent

NS1, NP and DDX3 exhibit RNA binding activity (436, 439, 496). Therefore, I was interested in investigating whether DDX3-NS1 and DDX3-NP interactions were mediated by RNA. To this end, plasmids expressing the NS1 protein and HA-DDX3 were co-transfected in 293T cells and the cell lysates were pre-treated with RNase A before subjecting to IP with HA antibody. Efficient degradation of RNA in the sample by the RNase A treatment was confirmed by measuring rRNA ratio (28S/18S) and RNA integrity number (RIN) (291, 473) using Agilent 2100 Bio analyzer (data not shown). As shown in Fig. 4.2A, while NS1 was not co-precipitated with HA-vector (lane 4), NS1 was co-precipitated with HA-DDX3 irrespective of the RNase A treatment (lane 2 vs. 5). It is well established that the substitution of R38 and K41 amino acids to alanine in NS1 disrupts its RNA binding activity (566). Therefore, NS1 with the R38A/K41A mutation (MT NS1) and HA-DDX3 were co-expressed in 293T cells and HA-DDX3-MT NS1 interaction was tested by IP using an antibody against HA tag. Surprisingly, MT NS1 was not co-precipitated with HA-DDX3 (Fig. 4.2B, lane 2). Additionally, the interaction of HA-DDX3 and MT NS1 expressed during infection with the PR8 virus expressing the NS1 protein containing R38A/K41A mutations (MT virus) was also tested (Fig. 4.2C). Similar to the results in co-transfection with plasmids, the MT NS1 expressed during infection did not co-precipitate with HA-DDX3 (Fig. 4.2C, lane 2). These data suggest that even though DDX3-NS1 interaction is RNA independent, the R38/K41 site in NS1 that is important for RNA binding activity by itself is essential for interaction with DDX3.

To investigate the RNA dependency of DDX3-NP interaction, cells were co-transfected with the plasmids expressing HA-DDX3 and NP respectively. Cell lysates were treated with RNase A before being subjected to IP with HA antibody. The RNase A treatment efficiency was

tested as described before. As shown in Fig. 4.2D, Western blotting detected the NP protein in the precipitated complex in HA-DDX3 expressing cell lysate irrespective of the RNase A treatment (lane 2 vs. 5), whereas NP was not co-precipitated with HA-vector (lane 4). This suggested that the interaction between DDX3 and NP is independent of RNA.

4.4.3 C-terminal domain of DDX3 has a predominant role in mediating DDX3 interaction with NS1 and NP

DDX3 belongs to the DEAD box family of proteins and like all the helicases in the family contains a core-helicase domain flanked by a highly variable N- and C-terminal region (451, 496). The core-helicase domain in human DDX3 comprises residues 168-582, while the flanking N- and C-terminal regions comprise residues 1-167 and 583-662 respectively (215). Based on the sequence alignment with other DEAD box helicases, the core-helicase domain contains the nine conserved motifs involved in ATPase, helicase and RNA binding activity (153). The N-terminal region contains a conserved leucine rich nuclear export signal and is critical for CRM1-mediated nuclear export and eIF4E binding (480, 486, 496), while an SR-rich region in the C-terminus is associated in an interaction with the nuclear export receptor NXF1/TAP (298). Therefore, Flag-tagged plasmids expressing different truncated DDX3 mutants were constructed and their ability to interact with NS1 and NP was examined. As shown in Fig. 4.3A, plasmids expressing Flag-tagged full length DDX3 and three truncation mutants expressing the core helicase domain of DDX3 alone (AA 168-582, DDX3-CH), DDX3 lacking the C-terminal domain (AA 1-582, DDX3- del CTD) or DDX3 lacking the N-terminal domain (AA 168-662, DDX3- del NTD) were constructed. To identify the domains critical for NS1 and NP interaction, 293T cells were co-transfected with one of the Flag-tagged DDX3 truncation mutant plasmids and a plasmid expressing either NS1 or NP. Cell lysates were subjected to IP

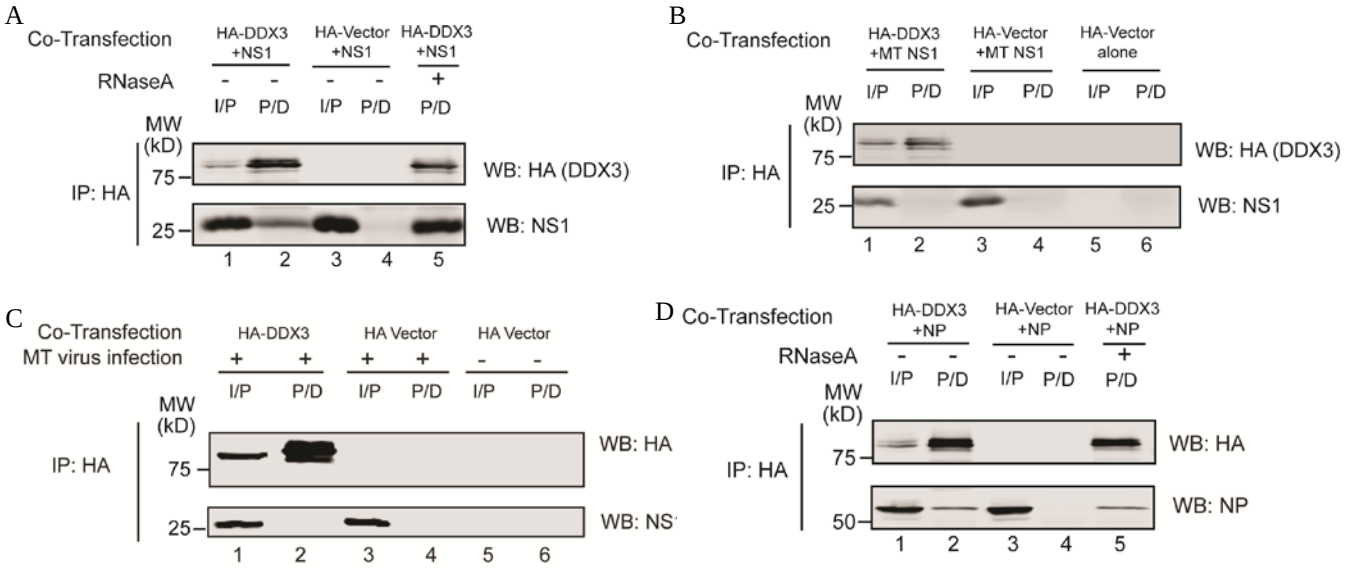


Figure 4.2 RNA dependency of DDX3 interaction with NS1 and NP. A) PR8-NS1 expressing plasmid was co-transfected with HA-DDX3 or HA-vector plasmid in 293T cells. At 48 h.p.t., cell lysate was collected, pre-treated with RNaseA at 10 μg/ml or left untreated and subjected to IP with HA antibody. B) HA-DDX3 or HA-vector plasmid was co-transfected with or without plasmid expressing PR8-NS1 R38A/K41A (MT NS1) in 293T cells. At 48 h.p.t., cell lysate was collected and subjected to IP with HA antibody. C) 293T cells were transfected with HA-DDX3 or HA-vector plasmid. At 24 h.p.t., cells were either infected with PR8 virus carrying R38A/K41A mutation in NS1 (MT virus) at an MOI of 10 or left uninfected. At 24 h.p.i., cell lysate was collected and subjected to IP with HA antibody. (A, B, C) Precipitated proteins were subjected to Western blotting using antibodies against HA-tag and NS1 protein. D) SIV/SK-NP expressing plasmid was co-transfected with HA-DDX3 or HA-vector plasmid in 293T cells. At 48 h.p.t., cell lysates were collected, pre-treated with RNase A at 10 μg/ml or left untreated and subjected to IP with HA antibody. Precipitated proteins were subjected to Western blotting using antibodies against HA-tag and NP protein. I/P refers to input and P/D refers to pull-down. The data presented is representative of multiple independent experiments.

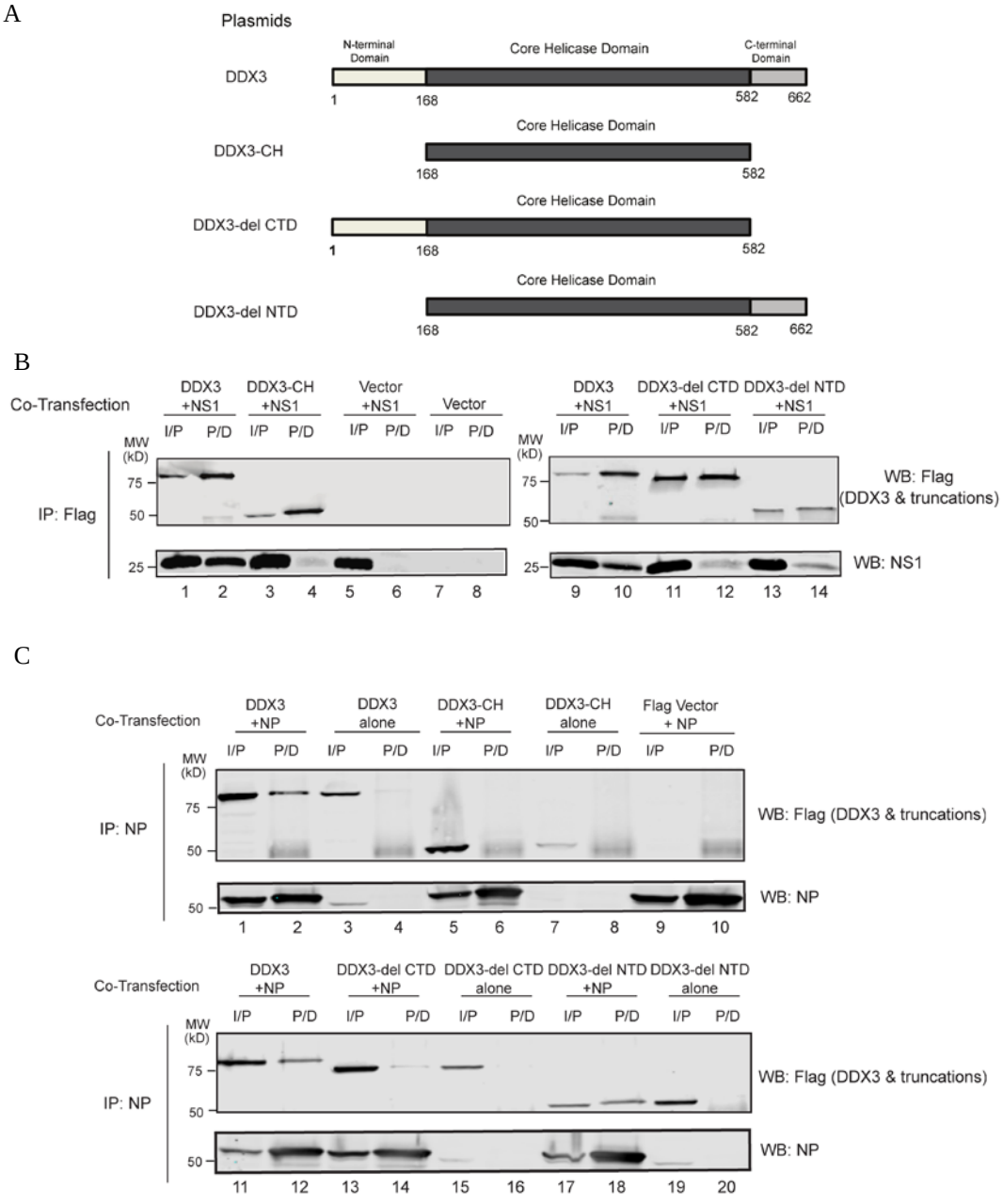


Figure 4.3 Identification of DDX3 protein domains critical for viral protein interaction. A) Flag-tagged plasmid expressing different DDX3 truncations were constructed to identify the domains important for NS1 and NP interaction and for SG formation. A schematic of the domains cloned into each plasmid is shown in the figure. (B, C) PR8-NP or PR8-NS1 expressing plasmid was co-transfected with one of the Flag-tagged plasmids expressing different DDX3 truncations in 293T cells. At 48 h.p.t., cell lysate was collected and subjected to IP with Flag antibody (B) or NP antibody (C). Precipitated proteins were subjected to Western blotting using antibodies against NS1 protein and Flag-tag (B), or NP protein and Flag-tag (C). I/P refers to input and P/D refers to pull-down. The data presented is representative of multiple independent experiments.

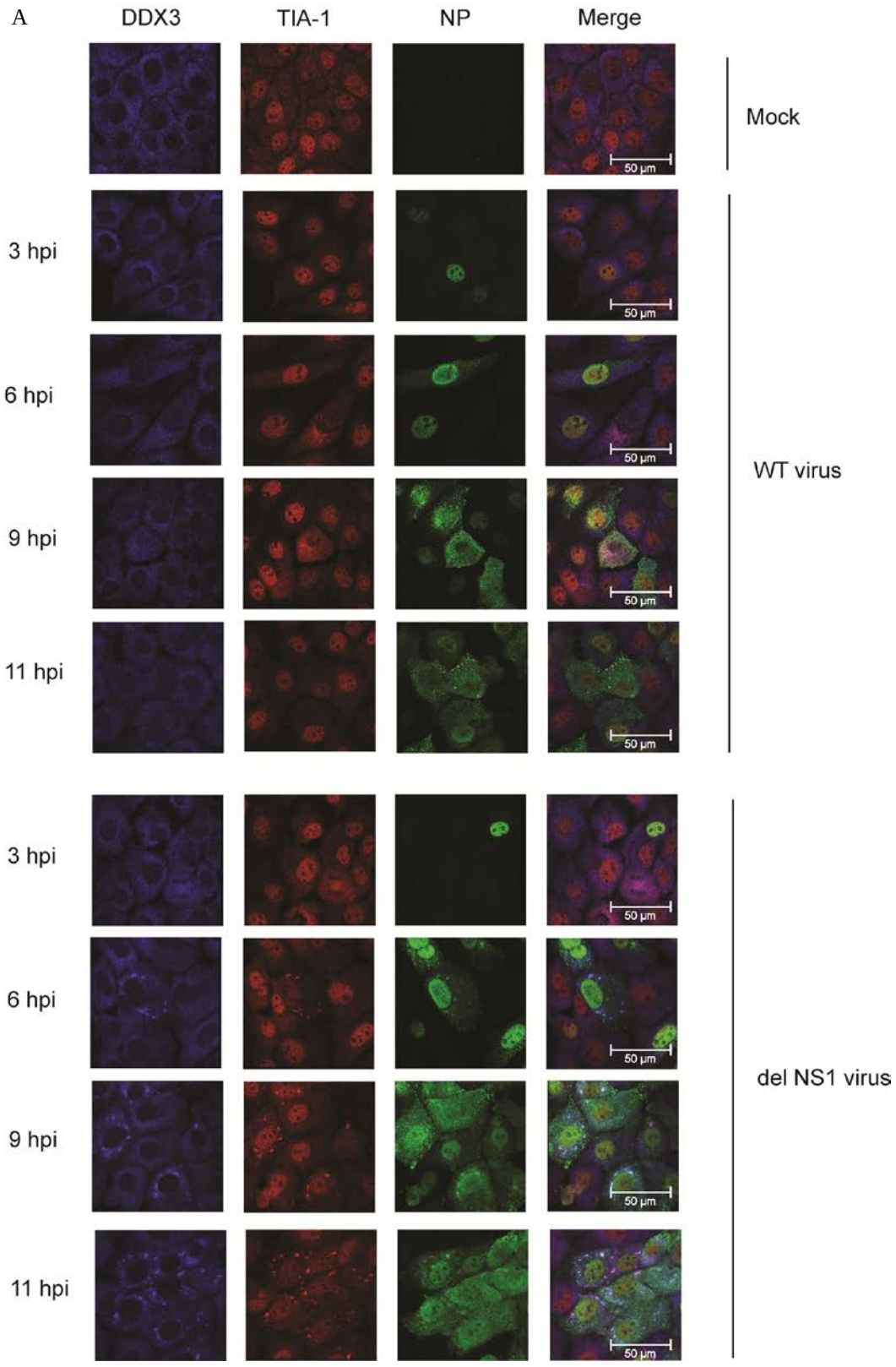
with the Flag antibody (for DDX3-NS1 interaction) or with the NP antibody (for DDX3-NP interaction). As shown in Fig. 4.3B, the full-length DDX3 protein demonstrated strong interaction with NS1 (lanes 2 and 10), whereas the core-helicase domain of DDX3 lost the ability to interact with NS1 (lane 4). Addition of the N-terminal domain slightly restored the interaction with NS1 (lane 12), whereas the addition of the C-terminal region had a stronger influence on restoring the interaction with NS1 (lane 14). Considering the amount of pulled down truncated DDX3 in this sample, the effect is more profound. IP of DDX3 truncation and NP showed similar results to those observed in Fig. 4.3B. As shown in Fig. 4.3C, while the full-length DDX3 protein was co-precipitated with NP (lanes 2 and 12), the core-helicase domain of DDX3 was not (lane 6). While addition of the N-terminal domain of DDX3 restored interaction slightly (lane 14), C-terminal domain addition had a stronger impact on restoring the interaction with NP (lane 18). Additionally, none of the DDX3 truncations or full-length DDX3 bound to the beads when expressed alone, demonstrating the specificity of the interaction of DDX3 and its truncated proteins with NP (lanes 4, 8, 16 and 20). These results suggested that the C-terminal domain (AA 583-662) and the N-terminal domain (AA 1-167) are essential for DDX3 interaction with its viral partners, although the C-terminal domain has a more prominent role than the N-terminal domain.

4.4.4 DDX3 localizes in the SGs in response to del NS1 virus infection

DDX3 has been documented to localize to cytoplasmic SGs and has been reported to be an essential SG-nucleating factor (179, 298, 486). Several other host proteins such as NF90, RAP55 and FMRP known to localize to SGs and involved in SG formation have also been shown to associate with influenza NP and NS1 (381, 565, 576, 610). Influenza A virus lacking a functional NS1 and those that are impaired in NS1 RNA binding activity have been shown to induce robust SG formation, while the WT virus expressing a fully functional NS1 does not

induce SGs throughout the virus life cycle (272, 408). A recent study revealed that viral NP is recruited into SGs and that the expression of NS1 is able to inhibit the formation of RAP55 and NP associated SGs (381). These studies led to the speculation that DDX3 could localize in influenza virus-induced SGs and may affect the virus life cycle by interacting with NP in the SGs. In order to test this hypothesis, I first examined SG formation and DDX3 localization upon virus infection in NPTr cells. Unlike many human cell-lines such as A549 and HeLa cells derived from carcinomatous tissues, NPTr cell-line was established following serial culture of primary cells (136). Additionally, the cell-line is derived from trachea, which is the primary site of influenza virus replication during infection (353). Thus, infection of NPTr cells would resemble conditions similar to natural infection and hence we used NPTr cells to study SG formation.

I infected NPTr cells with either a recombinant PR8 virus completely lacking the functional NS1 protein (del NS1) (156) or the WT PR8 virus and observed the formation of SGs at different time points by staining the SG specific marker T-cell restricted intracellular antigen-1 (TIA-1, red) (266). In addition, I also stained the viral NP (green) protein and cellular DDX3 (blue) to identify the infected cells and the localization of DDX3. As shown in Fig. 4.4A, no cytoplasmic punctate staining with TIA-1 was observed in the mock-infected cells and at any of the time points tested in the WT virus-infected cells, indicating SGs did not form in these conditions. However, in the del NS1 virus-infected cells, SGs started to form at 6 h.p.i. and were sustained throughout the time points tested. More strikingly, DDX3 co-localization with TIA-1 in the virus-induced SGs at all the time points when SG formation was observed. In order to confirm the formation of SGs in del NS1 virus-infected cells, the cells were stained for another SG marker PABP1 (266). The cells were also stained for NP (green) to identify the infected cells


A
DDX3
TIA-1
NP
Merge
Mock
3 hpi
6 hpi
9 hpi
11 hpi
WT virus
3 hpi
6 hpi
9 hpi
11 hpi
del NS1 virus
50 µm

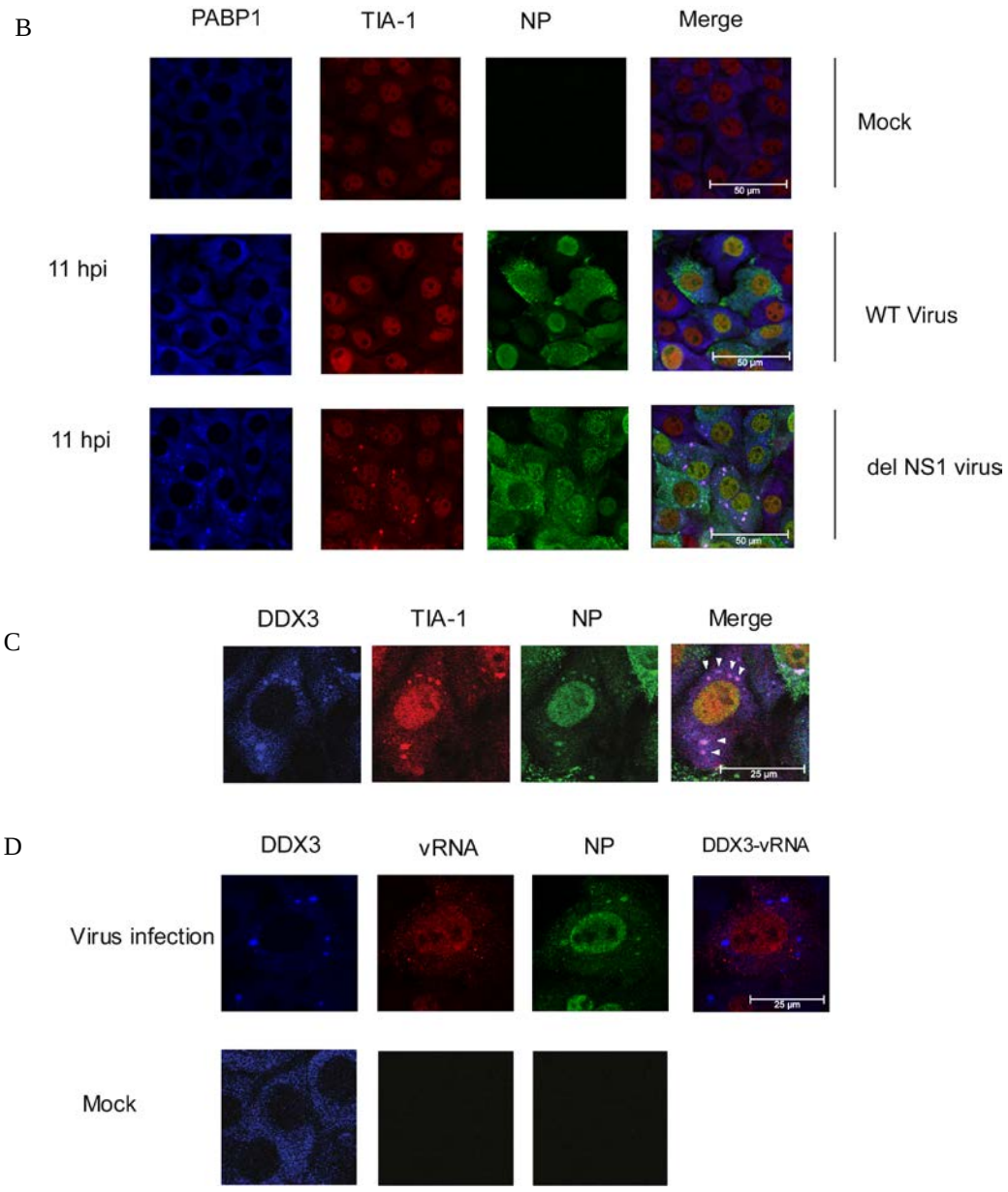


Figure 4.4 Kinetics of SG formation in PR8-WT and del NS1virus-infected cells and vRNA and NP localization in SGs. A) SG formation was analyzed by immunofluorescent staining of NPTr cells either mock-infected or infected with PR8-WT or del NS1 virus at an MOI of 0.5. The cells were stained at predetermined time points. Virus-infected cells were identified by antibody staining for NP (green), SGs were indicated by staining for TIA-1 (red) and DDX3 localization was observed by staining for DDX3 (blue). B) SG formation was further confirmed by immunofluorescent staining of NPTr cells either mock-infected or infected with PR8-WT or del NS1 at an MOI of 0.5. The cells were stained at 11 h.p.i. Virus-infected cells were identified by antibody staining for NP (green), SGs were indicated by staining for TIA-1 (red) and PABP1 (blue), both of which are SG markers. C) Zoomed in image of a del NS1 virus-infected cell stained at 11 h.p.i. for DDX3 (blue), TIA-1 (SG marker) (red) and NP (green). The white arrowheads show NP co-localization with TIA-1 and DDX3, all of which form punctate structures characteristic of SG formation and localization. D) NPTr cells were infected with del NS1 virus at an MOI of 0.5 or mock uninfected. At 11 h.p.i., the cells were subjected to FISH (red) using probes specific for vRNA of M segment and counter stained with antibodies against DDX3 (blue) and NP (green). A representative image from multiple fields of view is shown in the figure.

and TIA-1 (red). As shown in Fig. 4.4B, no punctate staining with PABP1 (blue) or TIA-1 (red) was observed in the mock-infected and WT virus-infected cells. However, TIA-1 and PABP1 co-localized and formed cytoplasmic punctate staining in the del NS1 virus-infected cells. Thus, these results confirm the formation of SGs and present TIA-1 as an authentic marker for observing SG formation in del NS1 infected cells.

4.4.5 DDX3 and NP co-localize in SGs but vRNA is not sequestered in virus induced SGs

The above described IP experiments clearly demonstrate the interaction between the viral NP protein and cellular DDX3 during infection (Fig.4.1A, 4.1C, 4.1E and 4.2D). Therefore, I was interested in studying where the NP and DDX3 interaction could occur in virus-infected cells. In the immunofluorescent staining experiment, I noticed that NP also formed some granules in the cytoplasm, which co-localized with DDX3 and TIA-1 staining (Fig. 4.4C), suggesting that DDX3 interacts with NP in the virus-induced SGs. Since the NP protein encapsidates influenza viral RNA (vRNA) to form the viral ribonucleoprotein (vRNP) complex, which is essential for viral transcription and replication (436), I wanted to understand whether the NP staining in the SGs is a result of vRNP being recruited to virus-induced SGs. FISH assay was conducted using a probe against the M vRNA segment. As shown in Fig 4.4D, FISH analysis clearly showed that vRNA (red) did not co-localize with the granular NP (green) in SGs. Note that, granular DDX3 was always observed to co-localize with TIA-1 in virus-infected cells (Fig. 4.4A and 4.4C). Therefore, in this particular experiment, DDX3 (blue) granule formation was used as a marker for the SGs. These results demonstrated that NP and DDX3 co-localized in virus-induced SGs, while vRNA/vRNP is not sequestered in these SGs.

4.4.6 Influenza virus NS1 inhibits virus-induced SG formation and DDX3 localization in SGs

SGs did not form at any of the time points tested with the WT virus infection, but SG formation was readily observed in the cells infected with the del NS1 virus (Fig 4.4A) starting at 6 h.p.i. Therefore, it was speculated that expression of the NS1 protein might be inhibiting the virus-induced SG formation and DDX3 localization in SGs in the WT virus-infected cells. To test this, SG induction upon del NS1 virus infection was studied in cells expressing the WT NS1 protein. NPTr cells were transfected with plasmids expressing Flag-tagged NS1 protein (Flag-NS1) or Flag-vector and then the cells were infected with the del NS1 virus. To study DDX3 recruitment into the SGs, cells were stained with antibodies against DDX3 (blue), TIA-1 (red) and Flag (green) (Fig. 4.5A). To monitor del NS1 virus-infected cells, the cells were also stained with antibodies against Flag (blue), NP (green) and TIA-1(red) (Fig. 4.5B). As expected, SGs and granular DDX3 were not found in the NS1 expressing cells upon del NS1 virus infection formation, such as oxidative stress induced SG formation via treatment with sodium arsenite (Fig 4.5A and 4.5B). Additionally, the ability of NS1 to inhibit other forms of stress induced SG formation, such as oxidative stress induced SG formation via treatment with sodium arsenite (NaAs) was also tested (369). For this, NPTr cells were transfected with the plasmid expressing Flag-tagged NS1 protein (Flag-NS1) or Flag-vector and then treated with NaAs for 1 hr. Cells were then stained with antibodies against DDX3 (blue), TIA-1 (red) and Flag (green) (Fig. 4.5C). Contrary to the results observed with del NS1 virus induced SGs, NS1 expression did not interfere with NaAs induced SG formation and was even recruited to these SGs in a few cells (Fig. 4.5C).

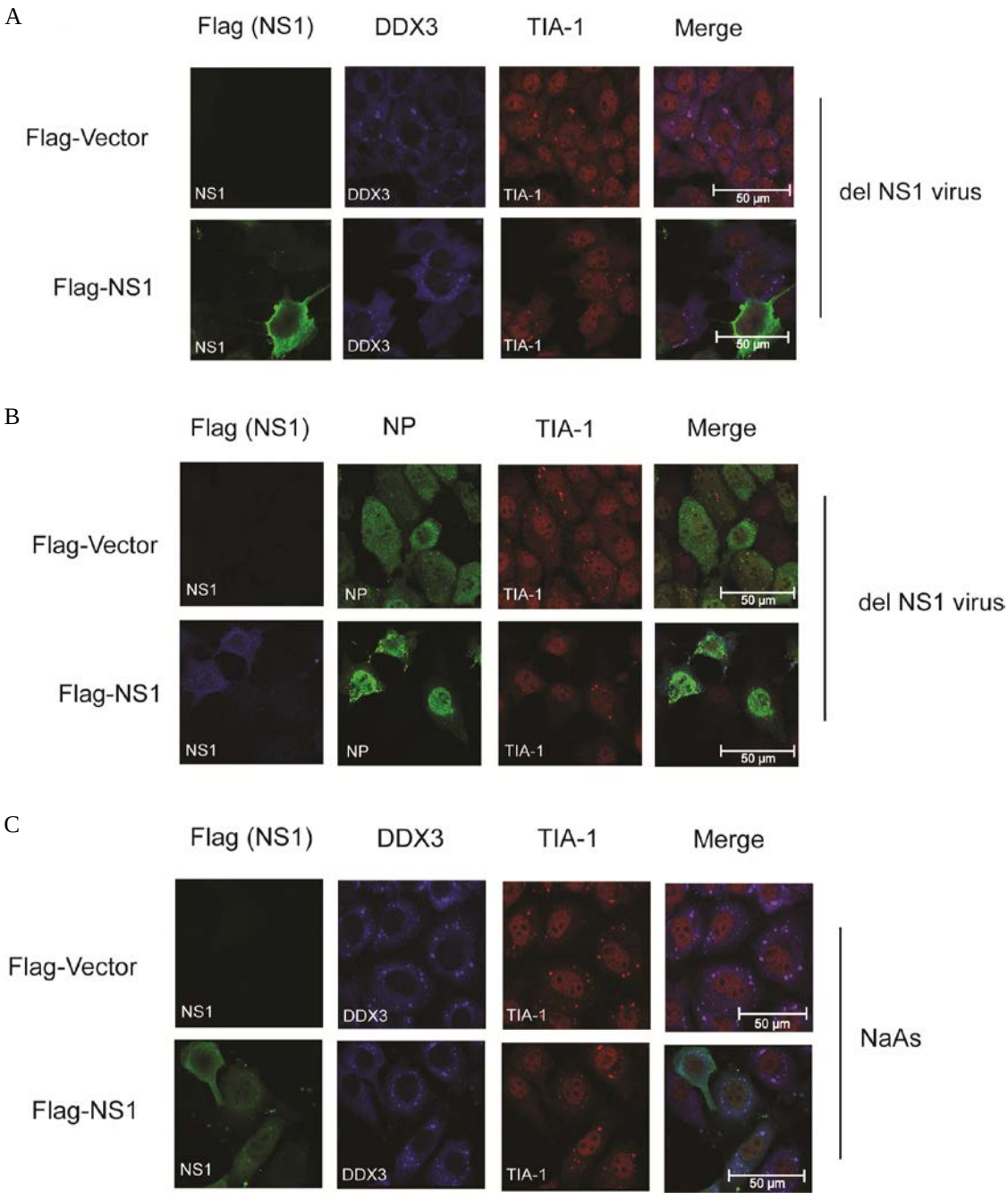


Figure 4.5 Effect of NS1 on virus-induced and NaAs-induced SG formation. NPTr cells were transfected with 500 ng of Flag-NS1 or Flag-vector plasmid (A, B). At 36 h.p.t., the cells were infected with del NS1 virus at an MOI of 0.5. At 11 h.p.i., one set of cells were stained with antibodies against Flag-tag (green), DDX3 (blue) and TIA-1(SG marker) (red) (A) and the other set of cells were stained with antibodies against Flag-tag (blue), NP (green) and TIA-1 (SG marker) (red) (B). C) At 46-48 h.p.t., the cells were treated with 0.75 mM NaAs for 1 hr and were then stained with antibodies against Flag-tag (green), DDX3 (blue) and TIA-1(SG marker) (red). A representative image from multiple fields of view is shown in the figure.

4.4.7 The core-helicase domain of DDX3 alone is sufficient for its localization into del NS1 virus induced SGs

To further the understanding of DDX3 function in virus-induced SGs, studying the relative importance of the DDX3 domains in SG localization was considered. NPTr cells were transfected with plasmids encoding either the Flag-tagged full-length or the truncated versions of DDX3 (described in Fig. 4.3A) and then infected with the del NS1 virus. At 11 h.p.i., cells were stained with antibodies against Flag (blue), TIA-1 (red) and NP (green). As shown in Fig. 4.6, full-length DDX3 co-localized with the virus induced SGs and so did the core-helicase domain (DDX3-CH) and the C-terminal deleted DDX3 (DDX3-del CTD). However, of the many microscopic fields examined, none of the N-terminal deleted DDX3 (DDX3-del NTD) expressing cells exhibited SG formation upon virus infection.

4.4.8 DDX3 down-regulation interferes with SG formation and enhances virus replication

The dominant negative effect of the N-terminal deleted DDX3 on SG formation (Fig 4.6) led to the hypothesis that DDX3 is not just recruited passively into SGs and might have a critical function in the formation of SGs. To confirm this speculation, NPTr cells were treated with siRNA specific to DDX3 (siRNA-DDX3) and SG formation in del NS1 virus-infected cells was observed (Fig. 4.7A). The infected cells were stained with antibodies against DDX3 (blue), TIA-1 (red) and NP (green). Treatment with the DDX3 siRNA resulted in a significant reduction of endogenous DDX3 expression when compared to the off-target siRNA treated cells (siRNA-OT) (Fig 4.7A, DDX3 panels) and interestingly, virus-induced SG formation was also suppressed in the cells with DDX3 downregulation (Fig. 4.7A, TIA-1 and NP panels). Notably, the quantitation of infected cells exhibiting virus-induced SGs showed that the number of SG forming cells diminished by 60% in the DDX3 siRNA treated cells when compared to the off-target siRNA

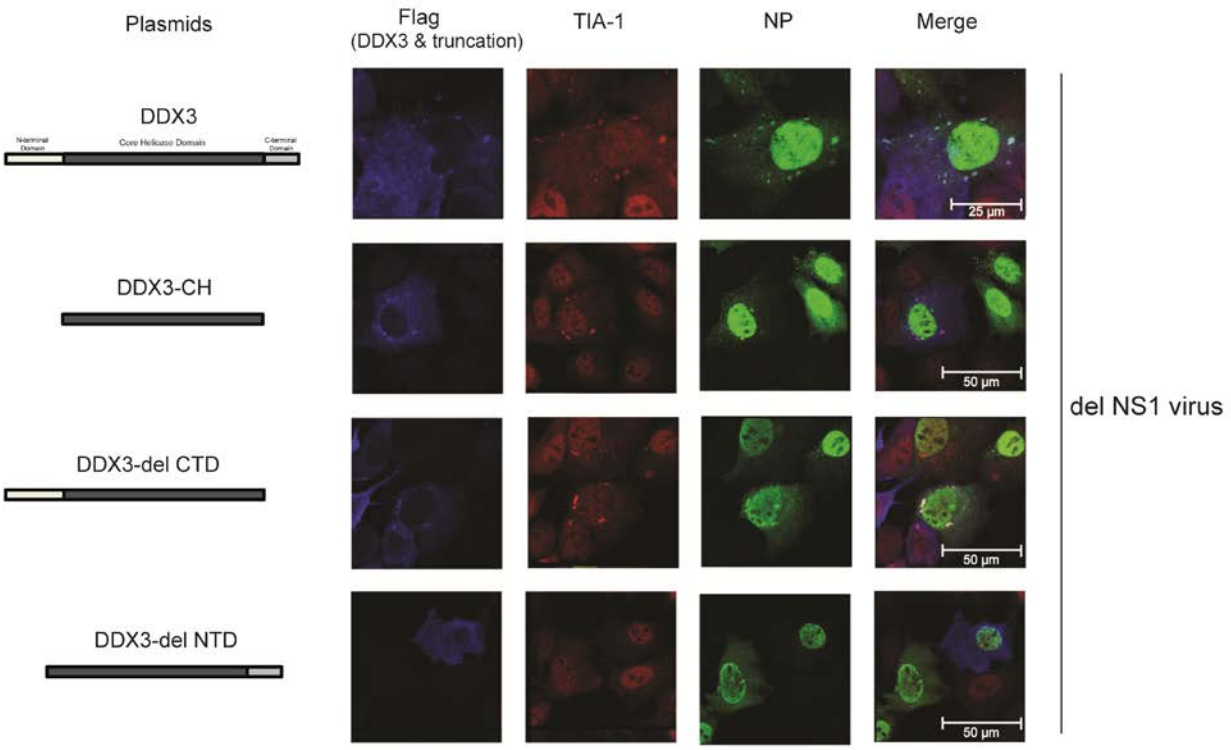
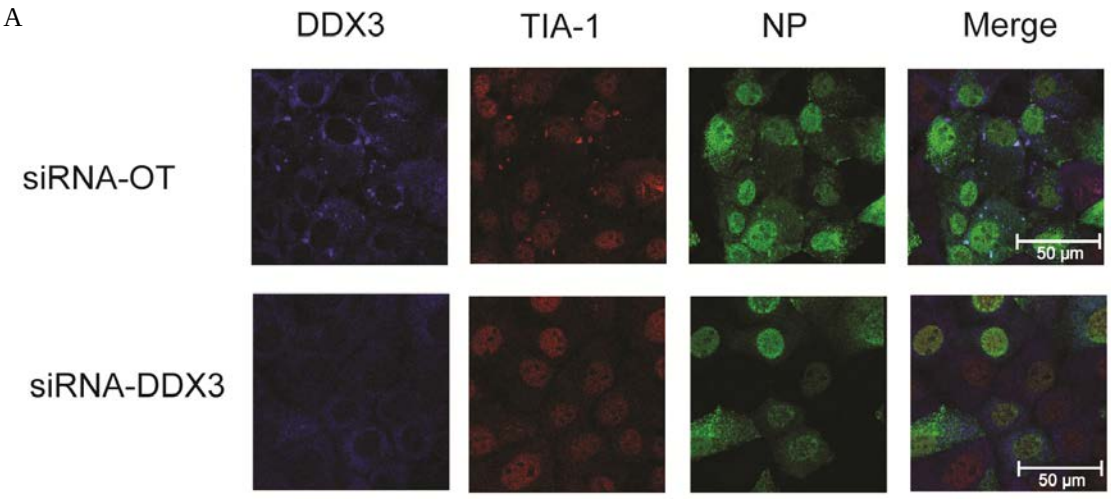


Figure 4.6 Characterization of DDX3 domains in virus -induced SG formation. NPTr cells were transfected with 500 ng of plasmids expressing flag-tagged DDX3 truncations. At 36 h.p.t., the cells were infected with del NS1 virus at an MOI of 0.5 and at 11 h.p.i., cells were stained with antibodies against Flag-tag (blue), TIA-1(SG marker) (red) and NP (green). A representative image from multiple fields of view is shown in the figure.

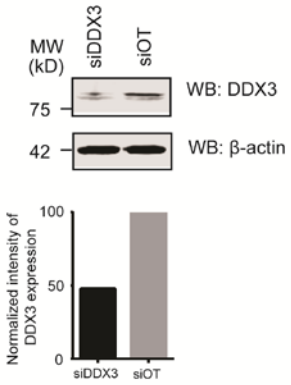
treated cells. This observation suggested that DDX3 is not just passively recruited to the SGs but has a critical function in the formation of virus induced SGs.

From the above data, it is clear that DDX3 has a critical function in virus-induced SG formation, but the effect of DDX3 downregulation and SG formation on virus replication is not known. Thus, DDX3 siRNA and off-target siRNA treated cells were infected with either PR8 WT, del NS1 or SIV/SK-WT virus (a field isolated strain) (250) and the virus titer was examined every 12 hrs. Downregulation of endogenous DDX3 expression was confirmed by subjecting the DDX3 siRNA and off-target siRNA treated cells to Western blotting with the antibodies against DDX3 and β-actin (Fig 4.7B). The normalization of DDX3 level to the β-actin level in the same sample showed that the siRNA-DDX3 treated cells demonstrated a 50% reduction in endogenous DDX3 expression compared to the off-target siRNA treated cells (Fig. 4.7B). Moreover, DDX3 siRNA treatment resulted in an increased virus titer with the del NS1 virus infection at all the time points tested. The peak difference with 10-fold increase in virus titre was observed at 36 h.p.i. However, DDX3 downregulation did not cause any significant difference in the virus titers upon PR8-WT virus infection and SIV/SK-WT virus infection (Fig. 4.7C). The degree of cytopathic effect on the infected cells was also more severe in the del NS1 virus-infected and DDX3 knocked-down cells, while DDX3 knockdown alone did not result in the cytotoxicity (Fig. 4.7D).

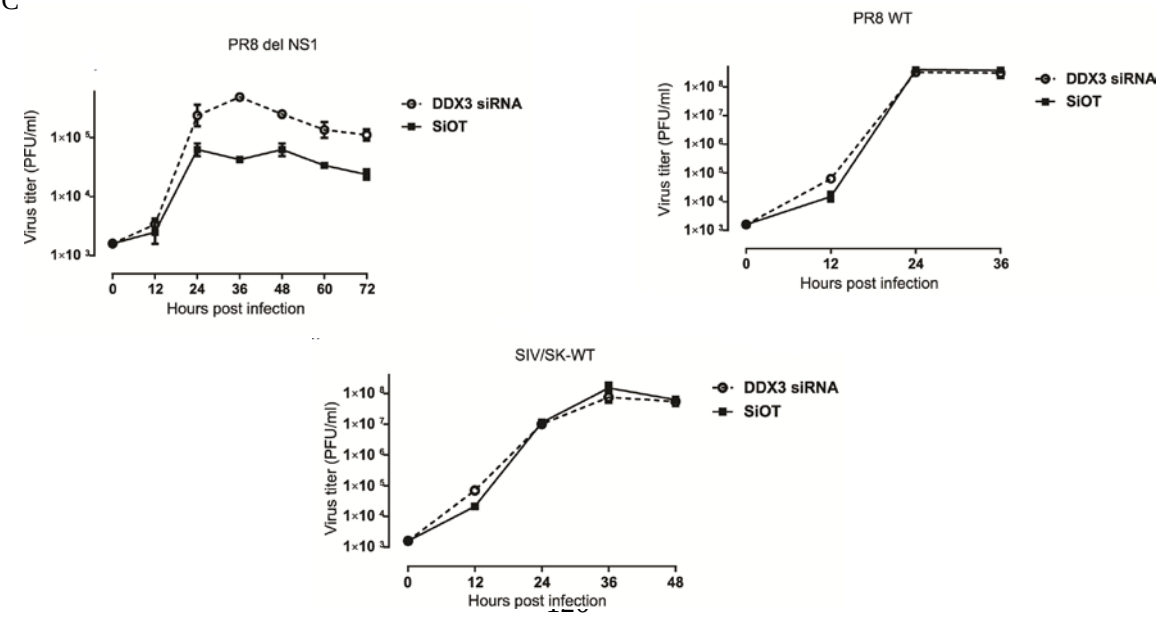
A


DDX3
TIA-1
NP
Merge
siRNA-OT
siRNA-DDX3
50 μm
50 μm

B


MW (kD)
siDDX3
siOT
75
42
WB: DDX3
WB: β-actin
Normalized intensity of DDX3 expression
100
50
0
siDDX3
siOT

C


PR8 del NS1
Virus titer (PFU/ml)
Hours post infection
DDX3 siRNA
SiOT
PR8 WT
SIV/SK-WT

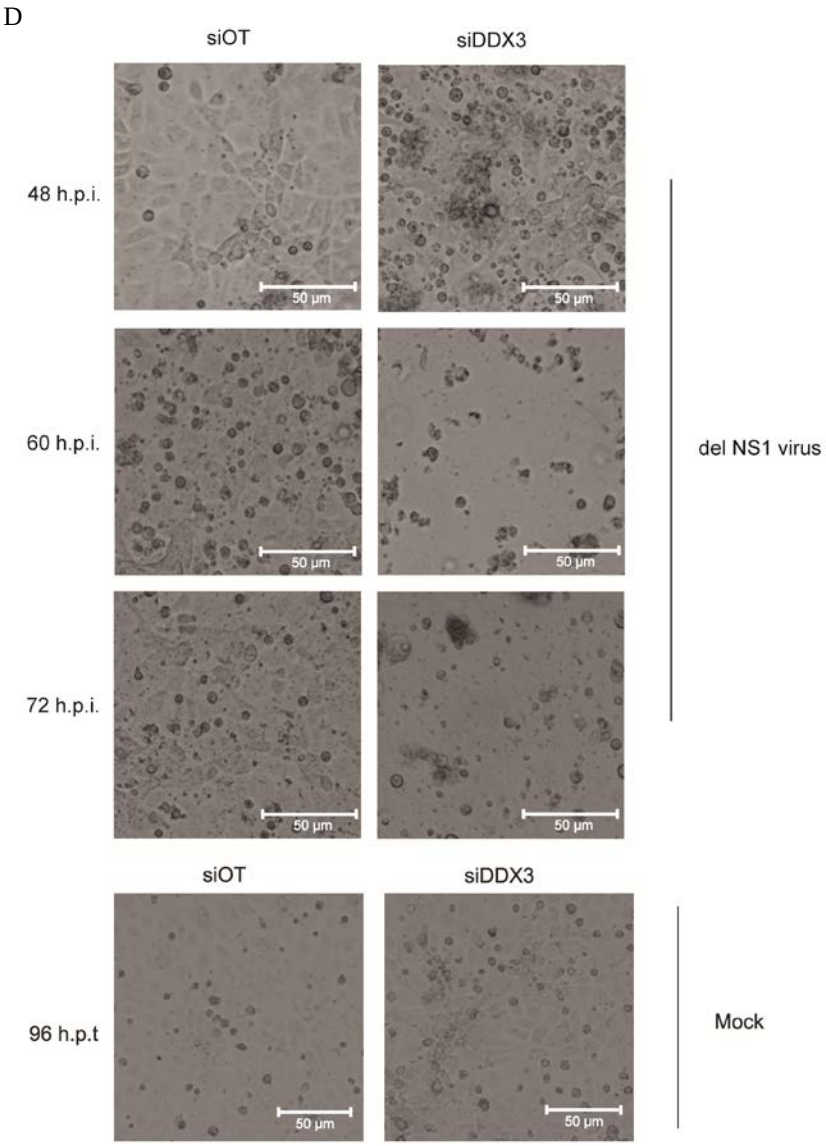


Figure 4.7 Effect of DDX3 downregulation on virus-induced SG formation and virus titer. NPTr cells were transfected with siRNA specific to DDX3 or off-target siRNA. A) At 48 h.p. siRNA treatment, the cells were infected with del NS1 virus at an MOI of 0.5. At 11 h.p.i., the cells were stained for DDX3 (blue), TIA-1 (SG marker) (red) and NP (green). A representative of multiple fields of view is shown in the figure. B) At 48 h.p. siRNA treatment, the cell lysates were subjected to Western blotting with antibodies against DDX3 and β-actin. The level of DDX3 expression was determined by normalizing the intensity of the DDX3 bands with the corresponding β-actin bands for each siRNA treatment and is represented as a graph below the western blot images. At 48 h.p. siRNA treatment, the cells were infected in triplicates with either PR8-WT, del NS1 or SIV/SK-WT virus at an MOI of 0.01. The supernatant was collected every 12 hrs and the virus titer at each time point was determined by plaque assay. A growth curve was plotted using the mean titer values at each time point and the associated standard deviation is displayed as error bar (C). The cytopathic effect of the cells infected by del NS1 virus and mock-infected cells were documented under the microscope (D).

4.5 Discussion

DEAD-box proteins are the largest helicase family in eukaryotes and are multifunctional proteins involved in various aspects of RNA metabolism, cell-cycle regulation, tumorigenesis and virus life cycle (19). Several RNA helicases including DDX1, DDX6, RHAU, eIF4A and DDX3 localize in SGs (69). Among these, DDX3 has been reported to function as an essential component for SG assembly and interacts with other SG proteins such as eIF4E and PABP1 (486). DDX3 interaction with eIF4E traps eIF4E and the associated mRNA in a translationally inactive complex, thus inhibiting cap-dependent translation (485). The formation of this inactive complex and the inhibition of translation in turn triggers SG assembly (486). This study shows that infection with the del NS1 virus triggers SG formation and that DDX3 and NP localize in these SGs (Figures 4.4A and 4.4C). The impairment of virus-induced SG formation and increased replication of del NS1 virus upon DDX3 knockdown was also observed (Figures 4.7A, 4.7B and 4.7C). These results suggested that DDX3 functions as an antiviral protein and DDX3 co-localization in virus-induced SGs may have a major contribution to this antiviral function. This is in agreement with the previous studies, which have shown SG formation to negatively affect influenza virus replication (272, 381). The antiviral function of DDX3 could be exerted in several ways. In one study, it was reported that the vRNA along with RIG-I is sequestered into virus-induced SGs and that these SGs serve as a platform for RNA detection by RIG-I (408). Because DDX3 interacts with NP, it is possible that DDX3 sequesters the NP bound vRNA into the SGs. Even though staining for RIG-I in the del NS1virus-infected cells was not performed, the results clearly showed that the vRNA does not localize in SG-containing NP (Fig. 4.4D). Therefore, it seems less likely that DDX3 exerts its antiviral role through facilitating vRNA sensing by RLR in the SGs. Another possible mechanism of antiviral function exists by

sequestering the viral mRNA into SGs through DDX3 interaction with the translation initiation factor eIF4E. During influenza virus infection, eIF4E binds the viral mRNA in the cytoplasm after nuclear export and triggers the recruitment of other factors such as eIF4G and PABP1 to initiate translation (48). As mentioned above DDX3 inhibits translation by binding eIF4E and locking it in a translationally inactive state in SGs. Therefore, it is conceivable that DDX3 inhibits viral mRNA translation by binding to eIF4E-viral mRNP complex, trapping it in a translationally inactive state and thereby sequestering the eIF4E-viral mRNP into the SGs. However, as the FISH assay requires a mixture of probes for the target RNA molecules, it would be difficult to differentiate cRNA from viral mRNA. Thus, additional studies are needed to confirm the viral mRNA localization in SGs and to explore the contribution of DDX3-eIF4E interaction to SG nucleation. The third possible mechanism is through sequestration of NP into the SGs via DDX3-NP direct interaction, as observed from the IP (Figures 4.1A, 4.1C, 4.1E) and immunofluorescent staining experiments (Figures 4.4A, 4.4C). NP is a highly conserved viral protein with a primary function of encapsidating the viral genome (436). However, NP is a multifunctional protein with additional functions in the nuclear and cytoplasmic trafficking of vRNA, the viral polymerase activity and in the regulation of transcription and replication during the virus life cycle (84, 318). Therefore, sequestration of NP protein from the appropriate cellular compartments into SGs as observed in this study (Fig 4.4C), could have a serious negative effect on viral replication. Taken together, these data suggest that DDX3-NP interaction could sequester NP into the virus-induced SGs, isolating it away from the proper cellular compartments. This reduces the availability of NP to wield its normal cellular functions and has an unfavourable effect on virus replication. Additionally, ectopic NP expression at high levels inhibits SG formation in an eIF2α-independent manner through an unknown mechanism (271).

Thus, NP sequestration into the SGs could serve as a mechanism to counteract its SG antagonistic function.

It has been reported that NaAs treatment induces oxidative stress and SG assembly via activating the eIF2α kinase HRI (369), while del NS1 virus infection activates the PKR kinase. NS1 is able to inhibit PKR activation but not the HRI kinase activation (272, 381), which might explain the ability of NS1 to supress virus-induced SG formation (Fig. 4.5A, 4.5B) and the inability of NS1 to suppress NaAs-induced SG formation (Fig. 4.5C). Failure of NS1 to suppress SG assembly in NaAs-treated cells was also observed by Khaperskyy et al., in his study (271). These data provide a glimpse into the distinct pathways activated by NaAs treatment and influenza virus for SG assembly.

Studies with DDX3 truncations in inducing SG formation revealed that the core helicase domain of DDX3 alone is sufficient for SG localization during virus infection, while addition of the N-terminal domain does not affect its localization (Fig. 4.6). Interestingly, we observed that addition of the C-terminal domain though has a negative effect on the formation of SGs itself (Fig. 4.6). This prompts the speculation that the N-terminal deleted DDX3 has a dominant negative effect on SG formation. Moreover, downregulation of DDX3 also weakens virus-induced SG formation (Fig. 4.7A). These results portray DDX3 as an essential factor for virus-induced SG formation, which is supportive of another study which identified DDX3 as an essential nucleating factor for oxidative stress-induced SG formation (486).

In this study, it was observed that the WT virus-infected cells do not form SGs throughout the infection at all the time points tested (Fig. 4.4A). The NS1 protein was identified to be responsible for the SG inhibitory function, since SGs did not form in the del NS1 virus-infected cells upon NS1 expression (Figures 4.5A, 4.5B). Previous studies have demonstrated the

ability of NS1 to inhibit SG formation (271, 381). The NS1 protein is believed to suppress SG formation through its ability to inhibit the activation of eIF2α kinase PKR. However, formation of SGs can occur independent of eIF2α phosphorylation (eIF2α-P), upon treatment with drugs that interfere with translation initiation and when certain translation initiation factors are depleted with siRNA treatment (100, 279, 366, 382). More specifically, SGs are induced by depletion of PABP1, eIF4E and by preventing eIF4E association with eIF4G (382). Many viruses such as Poliovirus, Herpes Simplex Virus (HSV) and Mammalian Orthoreovirus are known to modulate SG formation independent of eIF2α-P (137, 440, 577). HSV ICP8 protein binds to G3BP, while Poliovirus viral 3C proteinase cleaves G3BP to counteract eIF2α-independent SG formation (418, 577). Hence, an alternative NS1-mediated mechanism to inhibit the SG formation downstream of eIF2α may exist. The NS1 protein of influenza is reported to enhance viral mRNA translation through facilitating ribosomal recruitment by interacting with the viral mRNA, eIF4G and PABP1 (70). In this study, the existence of DDX3 and NS1 interaction is clearly demonstrated (Figures 4.1A, 4.1B, 4.1D). DDX3 interaction with eIF4E prevents eIF4E-eIF4G association thereby trapping the associated mRNA in a translationally inactive complex inducing the formation of SGs (486). DDX3 also interacts with PABP1, which is a key component of SGs (486). During WT influenza virus infection, it is reasonable to speculate that NS1 besides interacting with DDX3, also associates with the viral mRNA, PABP1 and eIF4F (comprises eIF4E, eIF4G and eIF4A). This might prevent DDX3 binding to eIF4E and PABP1. Inability of DDX3 to interact with eIF4E and PABP1 could in turn suppress SG formation, which might be an additional mechanism to explain the resistance of WT virus-infected cells to SG formation.

In this study, the role of DDX3 as an antiviral protein during influenza virus infection is reported for the first time. DDX3 localizes in SGs upon virus infection and is critical for virus-induced SG formation. DDX3 is able to interact with the viral proteins NS1 and NP in infected cells, NP but not vRNA is sequestered in the SGs. As reflected in the discussion, NP recruitment into the SGs mediated through DDX3-NP interaction could contribute to DDX3's antiviral function; and NS1 interaction with DDX3 might suppress SG formation in WT virus-infected cells. Supportive of SG mediated DDX3 antiviral function, DDX3 downregulation suppresses SG formation and increases virus titer in the del NS1 virus-infected cells. Overall, a novel antiviral function for DDX3 was discovered, which is mediated through SG formation during influenza virus infection.

CHAPTER 5: THE ROLE OF DDX3 IN REGULATING INTERFERON BETA EXPRESSION DURING INFLUENZA VIRUS INFECTION

Relationship of this study to the dissertation.

In Chapter 3, DDX3 was identified associated with NS1 as part of a larger NS1-interacting complex. Additionally, DDX3 was also identified as an innate-immunity related protein. Many studies with various viruses portray DDX3 as a regulator of IFNβ induction, which is mediated through DDX3 interaction with different components of the RIG-I mediated IFNβ induction pathway (19, 547). Therefore, we investigated the functional role of DDX3 in influenza virus-induced IFNβ induction pathway.

5.1 Abstract

DDX3 is a multifunctional protein and has been reported to affect the life cycle of various viruses. More importantly, DDX3 regulates antiviral innate immunity by affecting the various steps of interferon beta (IFNβ) signalling pathway during infection by several viruses. However, its function in influenza A virus-induced IFNβ induction is unknown. In this work, the role of DDX3 in influenza A virus-induced IFNβ expression is investigated. Through siRNA gene knockdown, the RIG-I was identified as the dominant signalling pathway responsible for IFNβ induction during influenza A virus infection. DDX3 downregulation and overexpression studies showed that DDX3 inhibits influenza A virus-induced IFNβ expression. Additionally, DDX3 inhibition of IFNβ expression using Low Molecular Weight (LMW) poly I:C, which is another RIG-I ligand, was also confirmed. Then, attempts were made to identify the mechanism by which DDX3 exerts its IFNβ inhibitory effect. RNA competition assay showed that RIG-I affinity to LMW poly I:C and influenza viral RNA (vRNA) is much higher than that of DDX3. Furthermore, DDX3 downregulation enhanced titers of an influenza virus lacking the NS1 gene

(PR8 del NS1), while it did not affect the titers of a WT virus (SIV/SK), which possess a fully functional NS1 gene. In the previous chapter the increase in PR8 del NS1 virus titers was observed to correlate with decreased stress granule (SG) formation in cells with DDX3 downregulation. Overall, the results suggest that, even though DDX3 has a function in IFNβ inhibition, its role in SG formation is dominant during PR8 del NS1 virus life cycle, while additional mechanisms might be in place to counteract the increased IFNβ induction during WT virus infection. The mechanism by which DDX3 exerts its IFNβ inhibitory function still needs further study.

5.2 Introduction

Influenza A virus infection initiates in the respiratory tract and in most cases is restricted within this organ (533). Upon entering the host through the upper respiratory tract, the virus has to counter a number of host defence mechanisms to facilitate its spread and replication in the host. One of the most important defence mechanisms employed by the host during virus infections is the secretion of type I interferons (IFNs) (257). The type I IFN family is primarily comprised of cytokines IFNα and IFNβ and induces an antiviral state in both the infected and neighbouring bystander cells via the induction of transcription of a set of genes called IFN stimulated genes (ISGs) (230). The ISGs interfere with multiple stages of the virus life cycle through various mechanisms and are responsible for maintaining the antiviral state in the infected and neighbouring uninfected cells (230). While plasmacytoid dendritic cells are the predominant producers of IFNα, most cell-types and more importantly epithelial cells produce IFNβ in response to influenza infection (140, 248).

IFNα/β secretion usually occurs in response to stimulation of receptors known as pattern recognition receptors (PRRs) by ligands of microbial origin (294, 390). These receptors can be located on the cell surface, in the endosomal compartments or in the cytosol and are divided into three main classes, namely: Toll-like receptors (TLRs), Retinoic acid inducible gene I (RIG-I) like receptors (RLRs) and Nucleotide oligomerization and binding domain (NOD) like receptors (294). The two major sensors involved in type I IFN induction during influenza infection are the TLRs and RLRs (154). Among the TLRs, TLR3 and TLR7 are important for influenza virus detection and they recognize double-stranded RNA (dsRNA) and single-stranded RNA (ssRNA) respectively in the endosomal compartment (53, 344). Binding of the RNA ligand to TLR3 and

TLR7 results in the recruitment of adaptor molecules TRIF and Myd88 respectively. Additionally, the influenza A virus RNA is also recognized in the cytoplasm by RIG-I, which belongs to the RLR family (600). RIG-I recognizes 5'-triphosphate dsRNA with a minimum length of around 10 bp, which resembles the "panhandle" structure of the vRNA generated during influenza virus infection (14, 285, 328). Binding of the RIG-I ligand results in a conformational change that exposes the caspase activation and recruitment domain (CARD), leading to RIG-I ubiqutination by TRIM25 and RIPLET, which promotes the interaction of RIG-I with the adaptor molecule MAVS (151, 412). Interaction of all three receptors with their respective adaptors results in the subsequent activation of transcription factors such as IFN regulatory factor-3 (IRF3), IFN regulatory factor-7 (IRF7) and Nuclear factor kappa beta (NFκB), leading to type I IFN production (429).

DDX3 is an ATPase-dependent RNA helicase, which belongs to the DEAD – box RNA helicase family (95, 145, 235). DDX3 has been implicated as a target of several viral proteins and is known to regulate different stages of the life cycle of a variety of viruses such as Hepatitis C virus (HCV), Hepatitis B virus (HBV), West Nile virus (WNV), Human immunodeficiency syndrome virus-1 (HIV), Japanese Encephalitis virus (JEV) and Vaccinia virus (VACV) (19). More importantly, DDX3 has been shown to have a role in antiviral innate immunity through enhancing IFNβ induction by targeting different host factors in the interferon signalling pathway (19). During virus infection, several transcription factors including IRF-3 are activated which translocates to the nucleus from the cytoplasm and triggers type-I IFN production (429). Following virus infection, IRF-3 activation occurs via phosphorylation by the kinases TBK1 and IKKε (429). DDX3 has been reported to upregulate IFNβ production through its interaction with IKKε or TBK1 (472, 497). DDX3 also acts as a transcriptional regulator by binding to the IFNβ

promoter (497). Furthermore, DDX3 has been shown to bind dsRNA and interact with RIG-I and its adaptor MAVS, acting as a scaffold to enhance MAVS-mediated IFN signalling (413). In order to offset the augmented IFN signalling, several viruses have been shown to target DDX3 as well. HCV core protein can disrupt DDX3-MAVS interaction (411), while HBV core protein disrupts DDX3-TBK1/IKKε interaction (563, 603) and suppress IFNβ induction. Similarly, VACV K7 protein targets DDX3 and inhibits IFNβ induction (472).

Even though many studies explored the function of DDX3 in regulating IFNβ induction during virus infection, there is no such study investigating DDX3 and its role in regulating influenza induced IFNβ induction. Therefore, this work utilized gene knockdown and overexpression studies to understand whether DDX3 has any regulatory role in influenza A virus-induced IFNβ induction. DDX3 exerts an inhibitory effect on influenza A virus-induced IFNβ induction and the competition assays with RIG-I ligands, poly I:C and influenza vRNA, demonstrated that DDX3 does not exert its inhibitory effect by competing with RIG-I for its ligand. In addition, this study on influenza A virus replication in DDX3 downregulated cells showed that the increase in IFNβ transcription does not affect influenza A virus replication.

5.3 Materials and Methods

5.3.1 Cells and viruses. Madin-Darby canine kidney (MDCK) cells and new born porcine tracheal epithelial (NPTr) cells were maintained in minimum essential medium (MEM) supplemented with 10% fetal bovine serum (FBS) (Life Technologies). 293T cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS.

A/Sw/SK/18789/02 (H1N1) (SIV/SK) viruses were propagated in 11-day-old embryonated chicken eggs as described previously (487). PR8 virus lacking the NS1 protein (PR8 del NS1) was kindly provided by Dr. Garcia-Sastre and was propagated in Vero cells maintained in MEM with 10% FBS. PR8 virus was titrated by plaque assay on MDCK cells and PR8 del NS1 virus was titrated on Vero cells.

5.3.2 Antibodies and reagents. Rabbit polyclonal NS1 antibody was generated in our laboratory as previously described (488). The other antibodies were purchased from different sources as follows: Rabbit anti-Flag DYKDDDDK tag antibody (Cell signaling technology), Rabbit polyclonal antibody to DDX3 (Abcam), Rabbit polyclonal to HA tag – ChIP grade (Abcam), Mouse monoclonal antibody to β-actin (Cell Signaling Technology), Rabbit monoclonal antibody to Phospho-IRF3 (Ser396) (Cell signaling technology), Rabbit polyclonal to IRF3 (Abcam), IRDye 680RD anti-Rabbit antibody (LI-COR), IRDye 800CW anti-mouse antibody (LI-COR). Other reagents were purchased from the following sources as follows: RNeasy mini kit (Qiagen), SuperScript III Reverse Transcriptase (Life Technologies), Platinum SYBR Green super mix (Bio-Rad), Pierce Magnetic RNA-Protein pull-down kit (Thermo Fisher Scientific), Biotin (Long Arm) Hydrazide (Vector laboratories), BrightStar BioDetect kit (Ambion), IRDye 800CW streptavidin (LI-COR), TransIT-LT1 (Mirus Bio LLC), Lipofectamine

LTX with PLUS (Life Technologies), Lipofectamine 2000 (Life Technologies), Low Molecular Weight (LMW) poly I:C (Invivogen), BX795 (Invivogen), MAXIscript T7 *in vitro* transcription kit (Ambion).

5.3.3 Plasmid construction. pcDNA-HA-DDX3 was kindly provided by Dr. Suresh Tikoo (VIDO-InterVac, University of Saskatchewan, Canada). pCMV-3×Flag-DDX3 and pCMV-3xFlag-RIG-I plasmids were used for expression of Flag-DDX3 and Flag-RIG-I in 293T cells. Full-length DDX3 was amplified from pcDNA-HA-DDX3 plasmid and cloned into pCMV-3×Flag plasmid at EcoRI/KpnI sites generating the pCMV-3×Flag-DDX3 plasmid. For the IFNβ luciferase activity assay p125Luc plasmid, which encodes the luciferase gene under the control of the IFN-β promoter and pCMV-rLuc, which encodes renilla luciferase under the control of a CMV promoter were used.

5.3.4 Transfection and Western Blotting. For transfection in 293T cells, 2 μl of TransIT-LT1 was used for every 1 μg of plasmid DNA. TransIT-LT1 and DNA were diluted in Opti-MEM media and the mixture was incubated for 20 mins at room temperature, it was added to the cells overlaid in Opti-MEM media. The media was then replaced with complete media 5-6 hrs post transfection (h.p.t.). For NPTr transfection, 3 μl of Lipofectamine LTX and 0.5 μl of PLUS reagent was used for every 500 ng of plasmid DNA to prepare the transfection mix in Opti-MEM. After incubating the transfection mix at room temperature for 20 mins the mixture was added to the cells overlaid with Opti-MEM media. The media was changed to complete media 5-6 h.p.t. 500 ng of LMW poly I:C was transfected into 293T and NPTr cells complexed with 2 μl Lipofectamine 2000 to test for IFNβ expression and IFNβ promoter induction.

Cell lysate and purified proteins were resolved by sodium dodecyl sulfate-10% polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting was performed as described elsewhere (488).

5.3.5 siRNA transfection. NPTr cells were plated at a density of 4×10^4 cells/well in 24-well plate. Next day, medium was replaced with OptiMEM. The siRNA (final concentration of 100 nM) containing transfection mix was prepared with OptiMEM and Lipofectamine 2000 as per manufacturer's protocol. The transfection mix was added to the cells in OptiMEM. After 5-6 hrs, the media was replaced with complete media and incubated for 48 hrs before proceeding with the experimental treatment.

DDX3 siRNA (GS1654) and Negative siRNA (SI03650318) were obtained from Qiagen. Custom siRNA specific for RIG-I and TLR3 were also obtained from Qiagen. Three siRNAs targeted to different regions of porcine RIG-I gene are of the following sequences: 5'-CAUAACUCUUGGAGGCUUAdTdT-3', 5'-GGCAAAGAGCAUCUUUGAAdTdT-3', 5'-GGACCACUGACAGAUUUAAdTdT-3'. Three siRNAs targeted to different regions of porcine TLR3 genes are of the following sequences: 5'-AAUUGUUAAUAGCAUCAAAdTdT-3', 5'-GAGAAACUUUGCUUAGAAUdTdT-3', 5'-GGAAGAUAACAACUUUCCAdTdT-3'.

5.3.6 Real-Time PCR. RNA was extracted from cells using the RNeasy mini kit. 500 ng of extracted RNA was used for reverse transcription using oligo$(dT)_{20}$ primer and SuperScript III reverse transcriptase to generate cDNA. The cDNA was then combined with the primer set and SYBR green super mix and Quantitative real-time PCR (qPCR) reaction was performed in an iCycler IQ5 multicolor real-time PCR detection system (Bio-Rad, Hercules, CA). The following primers were used for Quantitative real-time PCR (qPCR): porcine IFNβ forward, 5'-

CCGAATTCGCTAACAAGTGCATCCTCC-3'; porcine IFNβ reverse, 5'-GCGAAGCTTTCAGTTCCGGAGGTAATC-3'; porcine DDX3 forward, 5'-GCCGCAAACAATACCCAATCTC-3'; porcine DDX3 reverse, 5'-CATAAACCACGCAAGGACGAAC-3'; porcine RPL19 forward, 5'-AACTCCCGTCAGCAGATCC-3'; porcine RPL19 reverse, 5'-AGTACCCTTCCGCTTACCG-3'.

The qPCR conditions were 95°C for 120 s, followed by 45 cycles with denaturation at 95°C for 15 s, annealing at 60°C for 30 s and elongation at 72°C for 30 s. The Cycle threshold (C_t) values of the internal control gene RPL19 was used for normalization of IFNβ and DDX3 expression. Data are presented as relative gene expression to that of untreated cells using the formula $2^{-(\Delta Ct \text{ of test sample} - \Delta Ct \text{ of untreated control sample})}$. Real-time PCR was conducted in triplicate for each sample and the mean value was calculated. Final figures represent the results from at least two independent experiments.

5.3.7 Luciferase reporter assay. 293T cells or NPTr cells were transfected with a plasmid expressing a firefly luciferase gene under the regulation of an IFNβ promoter (p125Luc), a plasmid expressing renilla luciferase under the regulation of a CMV promoter (pCMV-rLuc) for normalization and increasing concentrations of HA-DDX3 or Flag-DDX3 plasmid or the vector plasmids. Forty eight h.p.t., 293T cells were transfected with 500 ng LMW poly I:C using Lipofectamine 2000. Thirty h.p.t., NPTr cells were infected with PR8 del NS1 virus at an MOI of 1. The cell lysate was collected 16 h.p. poly I:C transfection or infection. The relative luciferase activity was then determined using the Dual-luciferase reporter assay system (Promega) as per the manufacturer's protocol.

5.3.8 *In vitro* transcription. For *in vitro transcription* of the NS vRNA, a DNA template for *in vitro* transcription was first generated by PCR using pHW-SK-NS plasmid (361) as template and the primers 5'-TAATACGACTCACTATAGGTAGAAACAAGGGTGTTTTTTAG-3' (forward primer) and 5'-GGCAAAAGCAGGGTGACAAAAA-3' (reverse primer). The forward primer contains T7 promoter sequence (TAATACGACTCACTATAGG) followed by the sequence corresponding to the 5' non coding region (NCR) of NS vRNA (TAGAAACAAGGGTGTTTTTTAG) while the reverse primer contains sequence complementary to the 3' NCR of NS vRNA. The DNA segment thus generated, containing the T7 promoter sequence and the SIV/SK NS gene segment, was then used as template in the *in vitro* reaction using the MAXIscript T7 *in vitro* transcription kit. The *in vitro* transcribed NS vRNA was resolved on a 4% urea polyacrylamide gel and visualized using 1× SYBR Gold Nucleic Acid Gel Stain (Life Technologies).

5.3.9 Biotinylation and RNA-protein pull down. LMW poly I:C was biotinylated using Pierce RNA 3' end desthiobiotinylation kit as per the manufacturer's recommended protocol. Briefly, 6.6 μg of LMW poly I:C was incubated with the RNA ligase reaction buffer, RNase inhibitor, Biotinylated Cytidine Bisphosphate, T4 RNA ligase and 30% Polyethylene glycol at 16°C overnight. Next day, the poly I:C in the reaction was separated into the aqueous phase using chloroform:isoamyl alcohol mixture and precipitated with ethanol. The precipitated pellet was then re-dissolved in water and contains the purified biotinylated LMW poly I:C (BPIC). PR8 vRNA and NS vRNA were biotinylated using Biotin (Long arm) hydrazide as described elsewhere (73). Briefly, the T7 transcribed and purified vRNA was diluted in 100 mM sodium acetate, pH 4.5 to a final volume of 90 μl. Then 10 μl of $NaIO_4$ solution (21.4 mg/ml) was added, mixed and incubated for 1 hr at 4°C in the dark. The RNA from this reaction was then purified

by RNeasy mini kit and the RNA was resuspended in 70 µl RNase-free water. To this, 10 µl of 1M sodium acetate pH 6.0 and 20 µl of Biotin (Long arm) Hydrazide dissolved in dimethyl formamide (18.5 mg/ml) were added and incubated overnight at room temperature in the dark. Next day, biotinylated RNA was recovered by purification using the RNeasy mini kit.

For detection, BPIC and vRNA were dot blotted onto Amersham Hybond-N membrane (GE Healthcare) and cross-linked using a commercial UV-light cross-linking instrument at 120 mJ/cm^2 for a 60 second exposure. After cross-linking, BrightStar biodetect kit or IRdye 800CW streptavidin were used for detection of the biotinylated ligand. For detection using the BrightStar biodetect kit, the membrane containing the UV-cross-linked RNA was washed in wash buffer several times and then blocked in blocking buffer for 30 mins at room temperature. After blocking, the membrane was incubated with Strep-Alkaline Phosphatase diluted in the blocking buffer for 30 mins at room temperature. The membrane was again washed and a substrate for alkaline-phosphatase, CDP-Star was added to the membrane and exposed to an autoradiographic film for detection. For detection using the IRdye 800CW streptavidin, the UV-cross-linked membrane was blocked in Odyssey blocking buffer (Li-Cor) containing 1% SDS and then incubated with IRDye 800CW streptavidin (1:10000) in blocking buffer for 1 hr at room temperature. The membrane was then washed in PBS containing 0.1% Tween 20 and then scanned using an Odyssey imager (Li-Cor).

For studying the binding efficiency of RIG-I and DDX3 with LMW poly I:C and vRNA, Flag-RIG-I and Flag-DDX3 were first purified from 293T cells. 293T cells were transfected with 1 µg of pCMV-3×Flag-RIG-I or pCMV-3×Flag-DDX3 plasmids. At 48 h.p.t, the cell lysate was collected and subjected to immunoprecipitation using Anti-Flag M2 affinity gel (Sigma-Aldrich).

The immunoprecipitated proteins were eluted under native conditions by competition with Flag peptide (Sigma-Aldrich). The eluent thus contains the purified protein, which is then used in the RNA-protein pull down experiment. Interaction between purified Flag-DDX3, Flag-RIG-I and poly I:C or vRNA were studied using the Pierce RNA-protein pull down kit. Biotinylated LMW poly I:C (BPIC) or biotinylated vRNA were first bound to the Streptavidin magnetic beads supplied in the kit. The eluent containing either purified Flag-RIG-I or Flag-DDX3 (described above) were then incubated with the Streptavidin magnetic beads bound to either of the RNA species (BPIC or biotinylated vRNA) in protein-RNA binding buffer for 1 hr at 4°C with gentle rotation. The magnetic beads were then washed several times and the protein complex bound to either poly I:C or vRNA were eluted using the elution buffer supplied in the kit.

5.3.10 Statistical analysis. The statistical significance of differences was calculated using GraphPad Prism 6 (GraphPad Software, Inc., USA) with parametric t-tests to obtain the P-value. Data are shown as mean ± SD (standard deviation) of at least two independent experiments. Significant differences between treatments and controls are represented by an asterisk ($P < 0.05$) or two asterisks ($P < 0.01$).

5.4 Results

5.4.1 IFNβ mRNA expression is mediated through RIG-I during influenza virus infection

Influenza A virus recognition and IFN secretion can occur by a variety of PRRs such as TLR3, TLR7, TLR8, RIG-I and NLRP3 during virus infection (233). Among these, a subset of PRRs including the TLR3 and RIG-I receptors activate a distinct signalling pathway requiring the IKK related kinases TBK1 and IKKε and are also known to be the major receptors for IFNβ production (154, 260, 520). Therefore, NPTr cells were treated with BX795, an inhibitor of TBK1/IKKε (92), and then infected with the SIV/SK virus to confirm that the TLR3/RIG-I-mediated pathway does have a major contribution in influenza A virus-induced IFNβ expression. As shown in Fig. 5.1A, the IFNβ mRNA expression was significantly reduced in the BX795 treated cells at both 1μM and 10μM concentrations, when compared to the DMSO treated cells. This showed that TBK1/IKKε activation is indeed required for influenza A virus-induced IFNβ mRNA expression. Since both the TLR3 and RIG-I signalling utilize the TBK1/IKKε kinases for IFNβ induction, the relative contribution of the two PRRs in IFNβ signalling was tested. For this, the cells were treated with siRNA specific to TLR3 or RIG-I and the IFNβ mRNA expression after infection with SIV/SK virus was analysed. Both the TLR3 and RIG-I siRNA treatment resulted in a significant downregulation of the respective genes as quantified by qPCR (Fig. 5.1E and 5.1F). However, RIG-I downregulation and not TLR3 downregulation resulted in almost a 90% decrease in virus-induced IFNβ mRNA expression (Fig. 5.1C vs Fig. 5.1B). Since, TLR3 knockdown (KD) did not affect the IFNβ mRNA expression, I wanted to determine whether TLR3 is still functional in the NPTr cells. It has been reported that poly I:C present in the extracellular space (naked poly I:C) is internalized through endocytosis and is recognized by

TLR3, whereas poly I:C delivered intracellularly by complexing with a transfection reagent is recognized by cytoplasmic receptors such as RIG-I and MDA5 (8, 108). Therefore, if TLR3 is functional, IFNβ induction upon treatment with naked poly I:C should be inhibited in TLR3 KD cells. As shown in Fig. 5.1D, upon treatment with naked poly I:C, TLR3 downregulation resulted in a 65% decrease in the IFNβ induction, when compared to the cells treated with off-target siRNA (SiOT), while there was no significant decrease in the IFNβ expression in RIG-I downregulated cells, showing that TLR3 is indeed functional in NPTr cells.

5.4.2 DDX3 downregulation results in increased IRF-3 phosphorylation and IFNβ mRNA expression upon influenza virus infection

Next, the role of DDX3 in virus-induced IFNβ expression was examined. IRF3 phosphorylation occurs upstream of IFNβ transcription in the RIG-I signalling pathway (429). Therefore, the phosphorylation status of IRF3 in virus-infected cells during downregulated DDX3 expression was verified. NPTr cells were treated with either siRNA specific to DDX3 or SiOT and then infected with SIV/SK virus. IRF3 phosphorylation status in the DDX3 siRNA treated cells and SiOT treated cells were compared at 10 hrs post infection (h.p.i.) and at 12 h.p.i. by Western blotting. As shown in Fig. 5.2A, DDX3 siRNA treatment resulted in efficient knockdown of the gene (WB: DDX3 panel – lanes 1 vs 2 and lanes 3 vs 4). While total IRF3 protein expression remains the same irrespective of the siRNA treatment (WB: IRF3 panel), the level of phosphorylation of IRF3 is higher in the DDX3 siRNA treated cells when compared to the SiOT treated cells (WB: p-IRF3 panel - lanes 1 vs 2 and lanes 3 vs 4). Phosphorylated IRF3 dimerizes, translocates to the nucleus, associates with the IFNβ promoter region and acts as a transcription factor inducing IFNβ mRNA transcription (429). Hence, any increase in IRF3

phosphorylation should also result in increased IFNβ mRNA expression. Consequently, IFNβ mRNA expression in DDX3 KD cells upon SIV/SK infection was studied. As shown in Fig. 5.2D, DDX3 expression was efficiently downregulated upon treatment with the siRNA specific to DDX3. While, a 38-fold increase (when compared to untreated and uninfected cells) in IFNβ mRNA expression was observed in the SiOT treated/virus-infected cells, DDX3 downregulation resulted in a significant increase in IFNβ mRNA expression (92- fold increase when compared to untreated and uninfected cells) in response to virus infection, when compared to the SiOT treated/virus-infected cells (Fig. 5.2B). NS1 protein of influenza is a well-known IFN antagonist and influenza viruses with non-functional NS1 protein are robust inducers of IFNβ (23, 355). Therefore, I examined whether a similar IFNβ enhancement is also observed upon infection with the PR8 del NS1 virus, which lacks the NS1 gene (156). As shown in Fig. 5.2C, PR8 del NS1 virus infection resulted in a robust IFNβ mRNA expression (72-fold increase relative to uninfected and untreated cells) in the SiOT treated cells and DDX3 downregulation in PR8 del NS1 infected cells resulted in a significant enhancement in IFNβ mRNA expression (178-fold increase relative to uninfected and untreated cells), when compared to the SiOT treated and PR8 del NS1 virus-infected cells.

5.4.3 DDX3 regulates IFNβ mRNA expression triggered by the RIG-I agonist LMW poly I:C

From previous experiments, virus-induced IFNβ mRNA expression was observed to be mediated through RIG-I and DDX3 downregulation was established to affect the virus-induced IFNβ mRNA expression. Therefore, the effect of DDX3 in regulating IFNβ mRNA expression

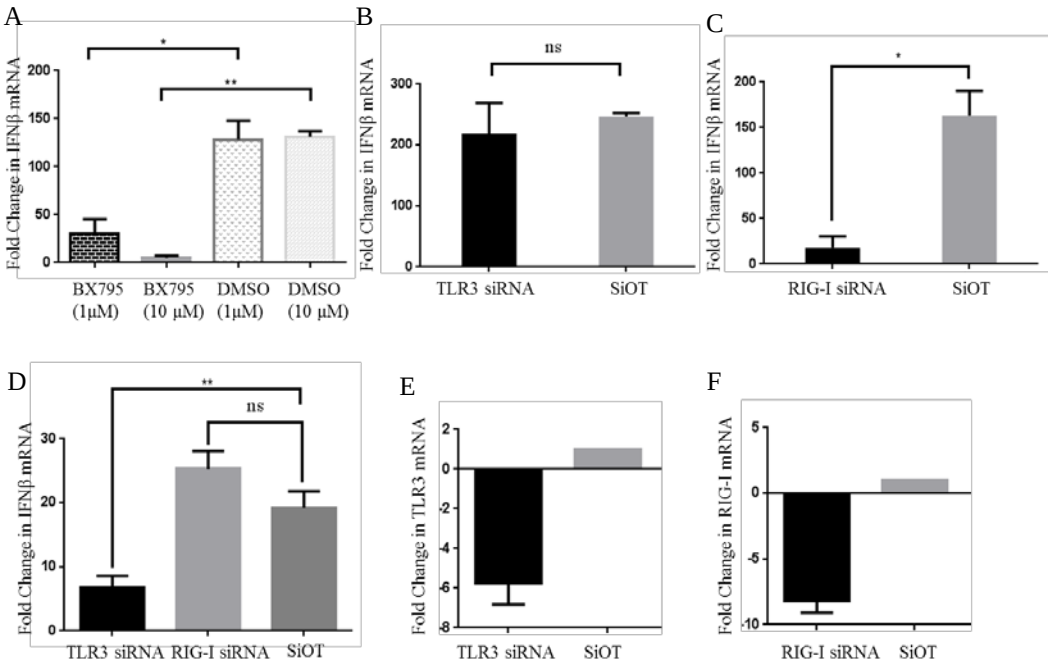


Figure 5.1 Influenza virus-induced IFNβ expression is mediated through the RIG-I pathway. A) NPTr cells were pre-treated with the TBK1/IKKε inhibitor at a concentration of 1 μM or 10 μM or mock-treated with the corresponding volume of DMSO for 1 hr. After 1 hr, the cells were infected with SIV/SK virus at an MOI of 1 and the inhibitor was present in the media throughout infection. B) NPTr cells were transfected with either siRNA specific to TLR3 or with off-target siRNA (SiOT) for 48-52 hrs. Then, the cells were infected with SIV/SK virus at an MOI of 1. C) NPTr cells were treated with either siRNA specific to RIG-I or with SiOT for 48-52 hrs. Then, the cells were infected with SIV/SK virus at an MOI of 1. (A, B, C) At 16 h.p.i., the cell lysate was collected and RNA extraction and reverse transcription performed to generate the cDNA. The cDNA was then used in qPCR experiments to determine IFNβ mRNA expression. D) NPTr cells were transfected with either TLR3 siRNA or RIG-I siRNA, SiOT, or left untreated and 48-52 h.p. siRNA treatment, the cells were treated with 250 ng of naked poly I:C. At 6 h.p. treatment, the cell lysate was collected and RNA extraction and reverse transcription performed. The level of IFNβ expression was determined using qPCR with the cDNA from each treatment as template. (E, F) NPTr cells transfected with TLR3 siRNA or RIG-I siRNA or SiOT were collected 48-52 hrs post treatment. (E) TLR3 expression and (F) RIG-I expression was determined using qPCR. Real-time PCR was conducted in triplicate for each sample and the mean value was calculated. Final figures represent the results from at least two independent experiments and the associated standard deviation is displayed as error bar. Significant differences between treatments and controls are represented by an asterisk ($P < 0.05$) or two asterisks ($P < 0.01$). Samples were considered statistically different with a significance of $p < 0.05\%$.

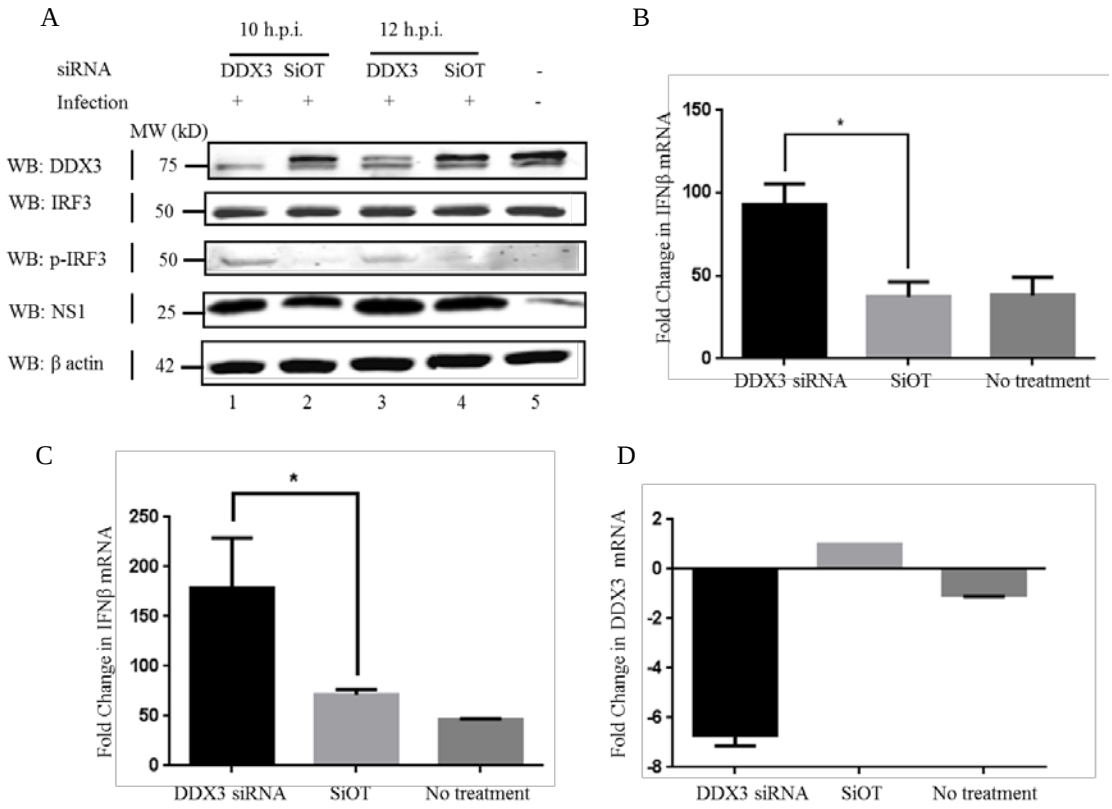


Figure 5.2 IRF3 phosphorylation and IFNβ mRNA expression is enhanced in DDX3 KD cells. NPTr cells were transfected with siRNA specific to DDX3 or SiOT for 48-52 hrs. A) Then, the cells were infected with SIV/SK virus at an MOI of 1. At 10 and 12 h.p.i., the cell lysate was collected and subjected to Western blotting using antibodies against DDX3, IRF3, Phospho-IRF3, NS1 and β-actin. B) Then, the cells were infected with SIV/SK virus at an MOI of 1. At 10 h.p.i., the cell lysate was collected and RNA extraction and reverse transcription performed to generate the cDNA. The cDNA was then used in qPCR experiments to determine IFNβ mRNA expression. C) Then, the cells were infected with PR8 del NS1 virus at an MOI of 1. At 8 h.p.i., the cell lysate was collected and RNA extraction and reverse transcription performed to generate the cDNA. The cDNA was then used in qPCR experiments to determine IFNβ mRNA expression. D) Then, the cell lysate was collected and RNA extraction and reverse transcription performed to generate the cDNA. The cDNA was then used in qPCR experiments to determine DDX3 mRNA expression. Real-time PCR was conducted in triplicate for each sample and the mean value was calculated. Final figures represent the results from three independent experiments and the associated standard deviation is displayed as error bar. Significant differences between treatments and controls are represented by an asterisk ($P < 0.05$) or two asterisks ($P < 0.01$). Samples were considered statistically different with a significance of $p < 0.05\%$.

induced by an alternative RIG-I agonist was examined. Short or LMW poly I:C ranging in size from 0.2 – 1 kb, when complexed with a transfection reagent has been reported to trigger IFNβ expression preferentially through the RIG-I pathway (108, 255). Therefore, first LMW poly I:C (complexed with lipofectamine 2000) was transfected in NPTr cells and the effect of TLR3 and RIG-I siRNA knockdown on IFNβ mRNA expression levels was studied. As shown in Fig. 5.3B, the TLR3 siRNA and RIG-I siRNA treatment resulted in the efficient knockdown of the respective genes. Even though TLR3 downregulation reduced the IFNβ mRNA expression levels, the level of decline was much more pronounced in the RIG-I KD cells when compared to the TLR3 KD cells (Fig. 5.3A). TLR3 KD resulted in a 30% decrease in the IFNβ mRNA expression levels, while RIG-I KD resulted in a 62% decrease in the IFNβ mRNA expression levels when compared to the SiOT treated cells. This showed that, the complexed LMW poly I:C triggers IFNβ mRNA expression preferentially through the RIG-I pathway. As a result, the effect of DDX3 KD on IFNβ mRNA expression induced by complexed LMW poly I:C was examined. As shown in Fig. 5.3D, DDX3 siRNA treatment resulted in efficient DDX3 KD. In addition, DDX3 downregulation resulted in a significant increase in the IFNβ mRNA expression induced by the complexed LMW poly I:C, when compared to the SiOT treated cells (Fig. 5.3C).

5.4.4 DDX3 overexpression decreases IFNβ promoter activity

For additional confirmation of the inhibitory effect of DDX3 on IFNβ expression, DDX3 was over expressed and IFNβ promoter activity was studied using luciferase reporter assay. IFNβ promoter activation during complexed LMW poly I:C treatment was first tested. Expression of HA-DDX3 or HA-Vector alone did not cause any significant induction of the IFNβ promoter, while treatment with the complexed LMW poly I:C (complexed and transfected with

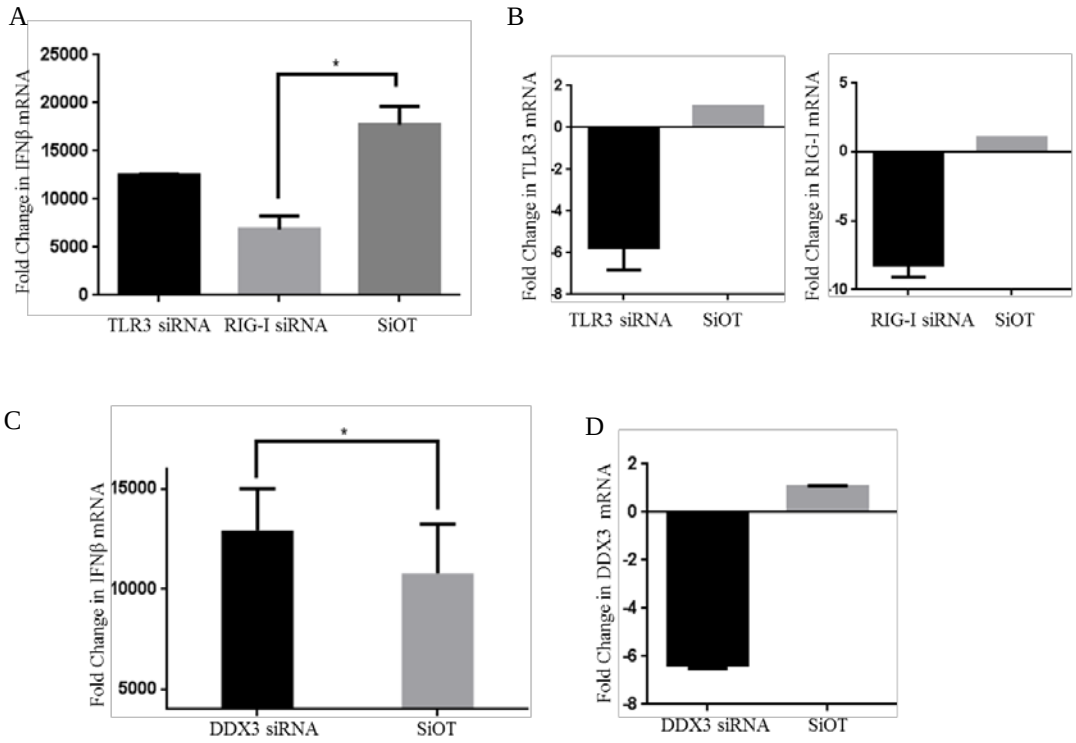


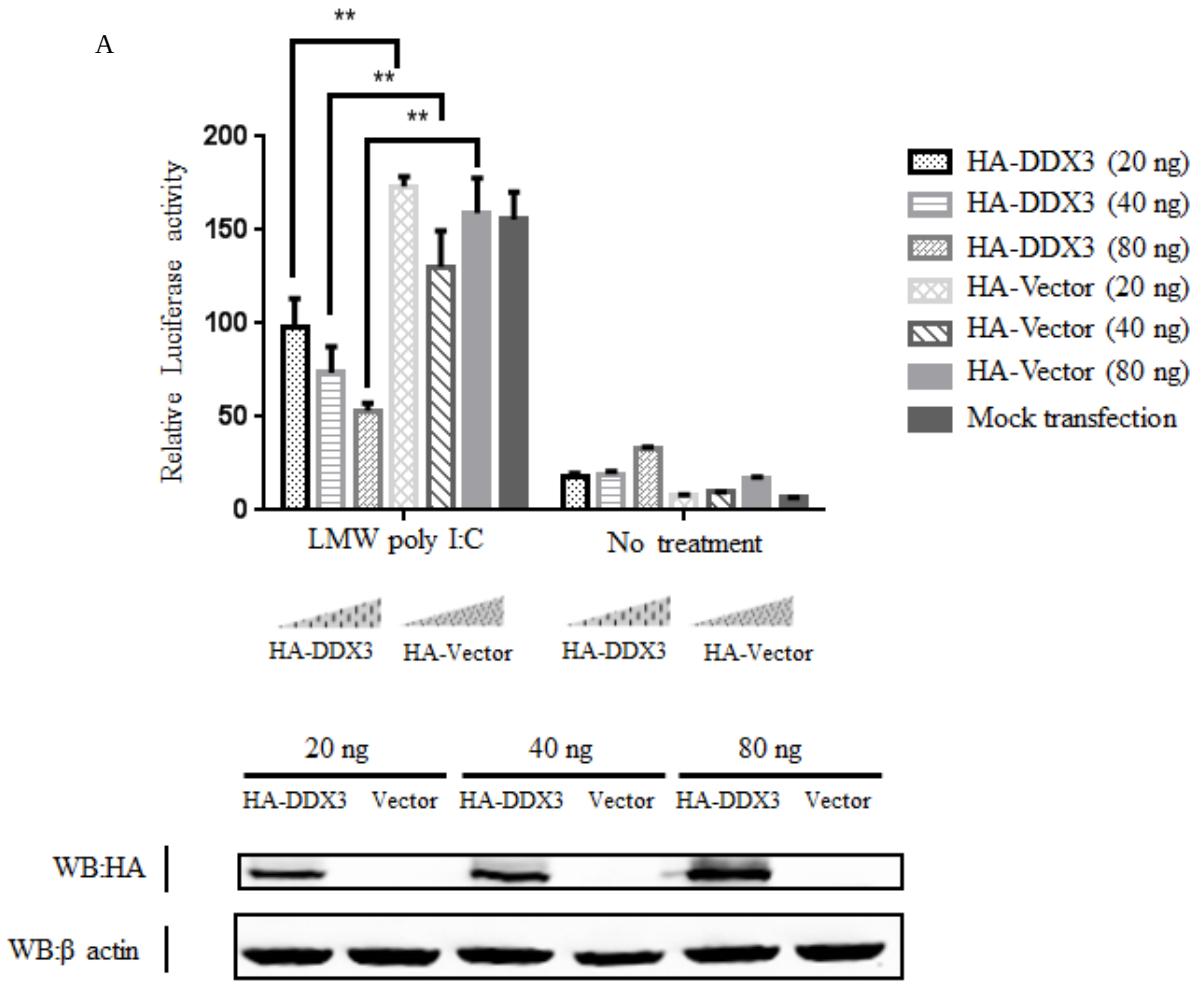
Figure 5.3 IFNβ mRNA expression mediated through complexed poly I:C. (A,B) NPTr cells were treated with TLR3 siRNA, RIG-I siRNA or SiOT for 48-52 hrs. A) The cells were then treated with LMW poly I:C complexed with lipofectamine 2000. At 6 h.p. treatment, the cell lysate was collected and RNA extraction and reverse transcription performed to generate the cDNA. The cDNA was then used in qPCR experiments to determine IFNβ mRNA expression. B) TLR3 siRNA, RIG-I siRNA and SiOT treated cells were collected and RNA extraction and reverse transcription performed to generate the cDNA. The cDNA was then used in qPCR experiments to determine TLR3 mRNA and RIG-I mRNA expression. (C, D) NPTr cells were transfected with siRNA specific to DDX3 or SiOT for 48-52 hrs. C) Then, the cells were treated with LMW poly I:C complexed with lipofectamine 2000. At 6 h.p. treatment, the cell lysate was collected and RNA extraction and reverse transcription performed to generate the cDNA. The cDNA was then used in qPCR experiments to determine IFNβ mRNA expression. D) Then, the cell lysate was collected and RNA extraction and reverse transcription performed to generate the cDNA. The cDNA was then used in qPCR experiments to determine DDX3 mRNA expression. Real-time PCR was conducted in triplicate for each sample and the mean value was calculated. Final figures represent the results from three independent experiments and the associated standard deviation is displayed as error bar. Significant differences between treatments and controls are represented by an asterisk ($P < 0.05$) or two asterisks ($P < 0.01$). Samples were considered statistically different with a significance of $p < 0.05\%$.

lipofectamine 2000) did result in a robust induction of the IFNβ promoter (Fig. 5.4A). Increasing the amount of plasmid expressing DDX3 suppressed the LMW poly I:C-induced IFNβ promoter activity significantly in a dose-dependent manner, while the IFNβ promoter activity in the cells expressing HA-vector was similar to the mock plasmid transfected cells (Fig. 5.4A). DDX3 expression level was monitored in the transfected cells by Western blotting using an antibody against the HA-tag and as shown in the lower panel of Fig. 5.4A, the DDX3 expression levels were robust and they increased with increasing amount of transfected HA-DDX3 plasmid.

IFNβ promoter activity induced by PR8 del NS1 virus infection was then tested. A dose-dependent decline in the IFNβ response was observed upon Flag-DDX3 expression in the virus-infected cells, while Flag-vector did not affect the IFNβ promoter activity (Fig. 5.4B). DDX3 expression levels were monitored by Western blotting using a Flag-tag antibody and increasing concentrations of the Flag-DDX3 plasmid correlated with increasing expression levels of the Flag-DDX3 protein (Fig. 5.4B – lower panel).

5.4.5 RIG-I and DDX3 bind to LMW poly I:C, but DDX3 is unable to compete with RIG-I for binding to poly I:C

Both RIG-I and DDX3 are known to bind to poly I:C and dsRNA (145, 240, 413). Therefore, I wanted to investigate whether DDX3 could compete with RIG-I for the RNA ligands (poly I:C and dsRNA), thereby limiting the recognition of ligand by RIG-I and in turn inhibiting IFNβ production. To test this, experiments were set up to confirm the ability of RIG-I and DDX3 to bind to poly I:C. Thus, Flag-RIG-I or Flag-DDX3 were expressed in 293T cells and were then purified under native conditions by immunoprecipitation (IP) using Flag antibody conjugated to agarose. The lysate and the eluent were subjected to Western blotting using Flag-


A
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200
150
100
50
0
Relative Luciferase activity
HA-DDX3 (20 ng)
HA-DDX3 (40 ng)
HA-DDX3 (80 ng)
HA-Vector (20 ng)
HA-Vector (40 ng)
HA-Vector (80 ng)
Mock transfection
LMW poly I:C
No treatment
HA-DDX3
HA-Vector
HA-DDX3
HA-Vector
20 ng
40 ng
80 ng
HA-DDX3
Vector
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WB:HA
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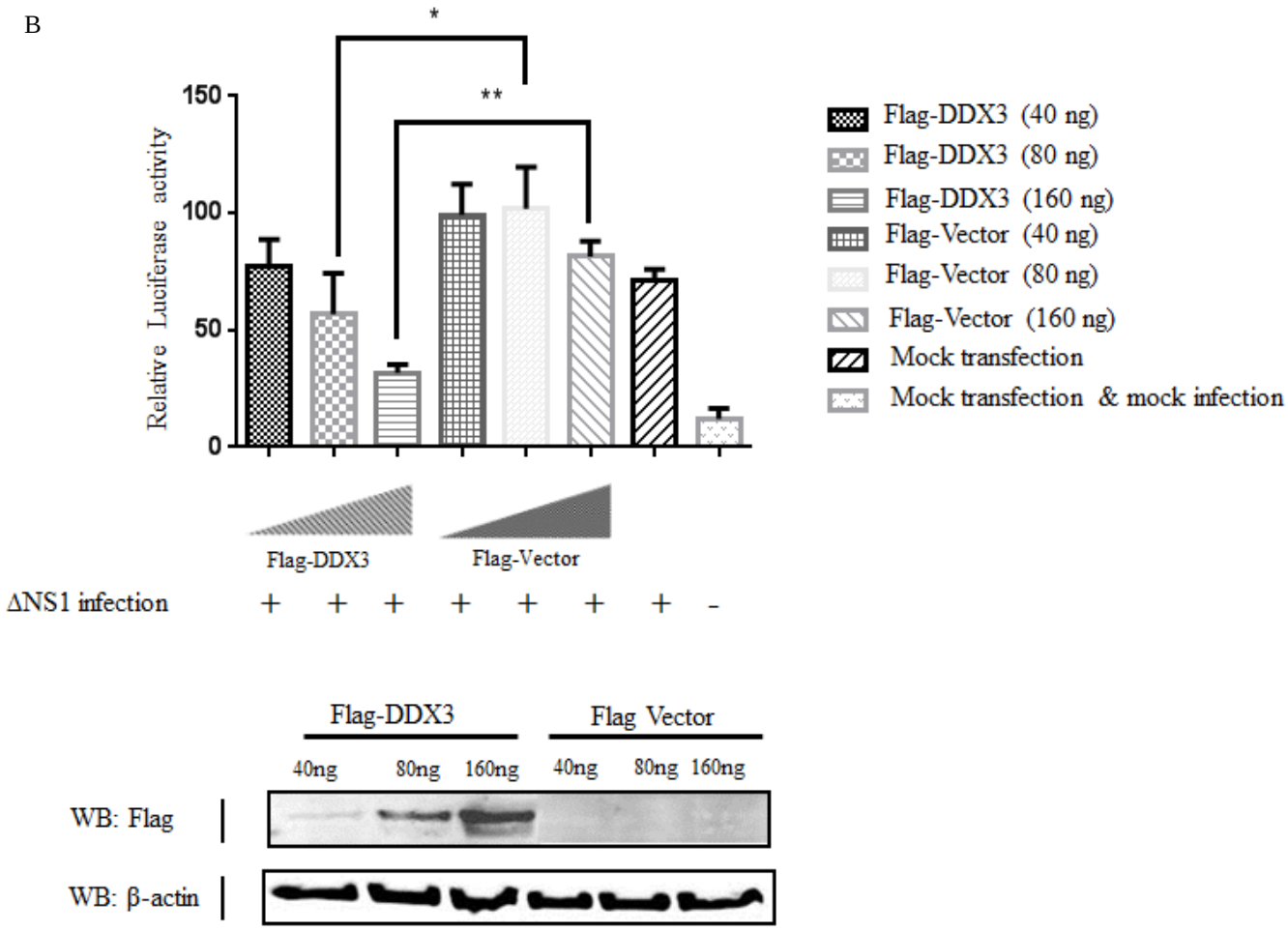

Figure 5.4 Effect of DDX3 overexpression on influenza virus and complexed poly I:C-mediated IFNβ mRNA expression. 293T cells (A) or NPTr cells (B) were transfected with different plasmids, one containing the firefly luciferase gene under the control of an IFNβ promoter and another one containing the renilla luciferase gene under the control of a CMV promoter (as a transfection control). A) Besides these plasmids, 293T cells were also transfected with plasmids expressing HA-DDX3 or the HA vector with increasing concentrations of the plasmid from 0 ng to 80 ng. At 48 hrs post transfection (h.p.t.), the cells were either treated with LMW polyI:C complexed with lipofectamine 2000 or left untreated. At 16 h.p. treatment, the IFNβ promoter activity was determined by measuring the luminescence using a luminometer. In order to confirm HA-DDX3 expression, one set of cells were collected 48 hrs post transfection and subjected to Western blotting using antibodies against the HA-tag and β-actin. β-actin expression was used as loading control. B) Besides these plasmids, NPTr cells were also transfected with plasmids expressing Flag-DDX3 or the Flag-vector with increasing concentrations of the plasmid from 0 ng to 160 ng. At 30 hrs post transfection, the cells were infected with PR8 del NS1 virus at an MOI of 1or left uninfected. At 16 h.p.i., the IFNβ promoter activity was determined by measuring the luminescence using a luminometer. In order to confirm Flag-DDX3 expression, one set of cells were collected 30 hrs post transfection and subjected to Western blotting using antibodies against the Flag-tag and β-actin. β-actin expression was used as loading control. Luciferase assay was conducted in triplicate for each sample and the mean value was calculated. Final figures represent the results from three independent experiments and the associated standard deviation is displayed as error bar. Significant differences between treatments and controls are represented by an asterisk ($P < 0.05$) or two asterisks ($P < 0.01$). Samples were considered statistically different with a significance of $p < 0.05\%$.

tag antibody. As shown in Fig. 5.5A, Flag-RIG-I and Flag-DDX3 were successfully expressed in the 293T cell-lysate input (lanes 1 and 5), while expression of the proteins were not observed in the vector transfected cell lysates (lanes 2 and 6). In addition, immunoprecipitation and elution (via competition with Flag peptide) of the two proteins was also successful, as observed from the significant enrichment of the two proteins in the eluent (lanes 3 and 7). Either of the purified proteins were then incubated with biotin-labelled LMW poly I:C (BPIC) bound to streptavidin magnetic beads and subjected to RNA-protein pull down. The poly I:C bound protein complex was purified using the streptavidin magnetic beads after extensive washing. BPIC was detected in the eluent, showing that BPIC was not washed away and was bound to the streptavidin beads throughout the procedure (Fig 5.5B - bottom panels). The proteins in the eluent were then subjected to Western blotting with Flag antibody. Both RIG-I and DDX3 were detected in the eluent containing BPIC, while neither of the two proteins were present in the eluent when unlabelled LMW poly I:C was used as a bait (Fig. 5.5B - top panels). Next, I tested whether DDX3 can compete with RIG-I for binding to the LMW poly I:C. Flag-RIG-I and Flag-DDX3 were purified from 293T cell lysate by IP. The lysate and the eluent were subjected to Western blotting using Flag-tag antibody. As shown in Fig. 5.5C, Flag-RIG-I and Flag-DDX3 were successfully expressed in the 293T cell-lysate input (lanes 1 and 2), while expression of the proteins were not observed in the vector transfected cell lysate (lane 3). In addition, immunoprecipitation and elution (via competition with Flag peptide) of the two proteins was also successful, as observed from the significant enrichment of the two proteins in the eluent (lanes 4 and 5). Purified RIG-I or DDX3 was incubated separately with BPIC or RIG-I and increasing amounts of DDX3 were incubated together with BPIC and the amount of RIG-I and DDX3 pulled down along with BPIC in the eluent was observed. If DDX3 competes with RIG-I for

poly I:C binding, one would observe an increase in the amount of DDX3 in the BPIC pull-down complex corresponding to a decline in the amount of RIG-I in the pull-down complex. However, no competition between DDX3 and RIG-I was observed. As shown in Fig 5.5D, DDX3 was barely detected in the eluents of biotin-labelled LMW poly I:C (lane 2 and 3), whereas intense RIG-I band was readily detected in the eluent (lane 1), indicating that DDX3 has a much lower affinity with poly I:C than RIG-I. Meanwhile, the addition of increasing amounts of DDX3 did not change RIG-I binding (lane 4 and 5).

5.4.6 RIG-I binds to influenza vRNA at a much higher affinity than DDX3

LMW poly I:C is not the authentic ligand during influenza A virus infection. Therefore, I wanted to confirm the above findings with influenza vRNA, which is the true RIG-I ligand during influenza A virus infection (444). For this purpose, the vRNA extracted from purified PR8 virions (PR8 vRNA) and *in vitro* transcribed vRNA of the NS gene segment (NS vRNA) were tested (Fig. 5.6A). Flag-RIG-I and Flag-DDX3 protein were purified from 293T cell lysates by IP with Flag-M2 Agarose (Fig. 5.6B). Biotinylated PR8 vRNA and NS vRNA were incubated with the purified Flag-RIG-I or Flag-DDX3 protein and the protein-vRNA complex was purified using streptavidin magnetic beads. Both of the biotin-labelled vRNA species bound efficiently to the streptavidin beads, since both the biotin-labelled PR8 vRNA and biotin-labelled NS vRNA were detected in the eluent of biotin-labelled RNA species (Fig. 5.6C – bottom panel). While RIG-I bound to both of the biotin-labelled vRNA species (lane 1 and 3), DDX3 did not bind to either of the biotin-labelled vRNA species (lane 5 and 7) (Fig 5.6C – top panel).

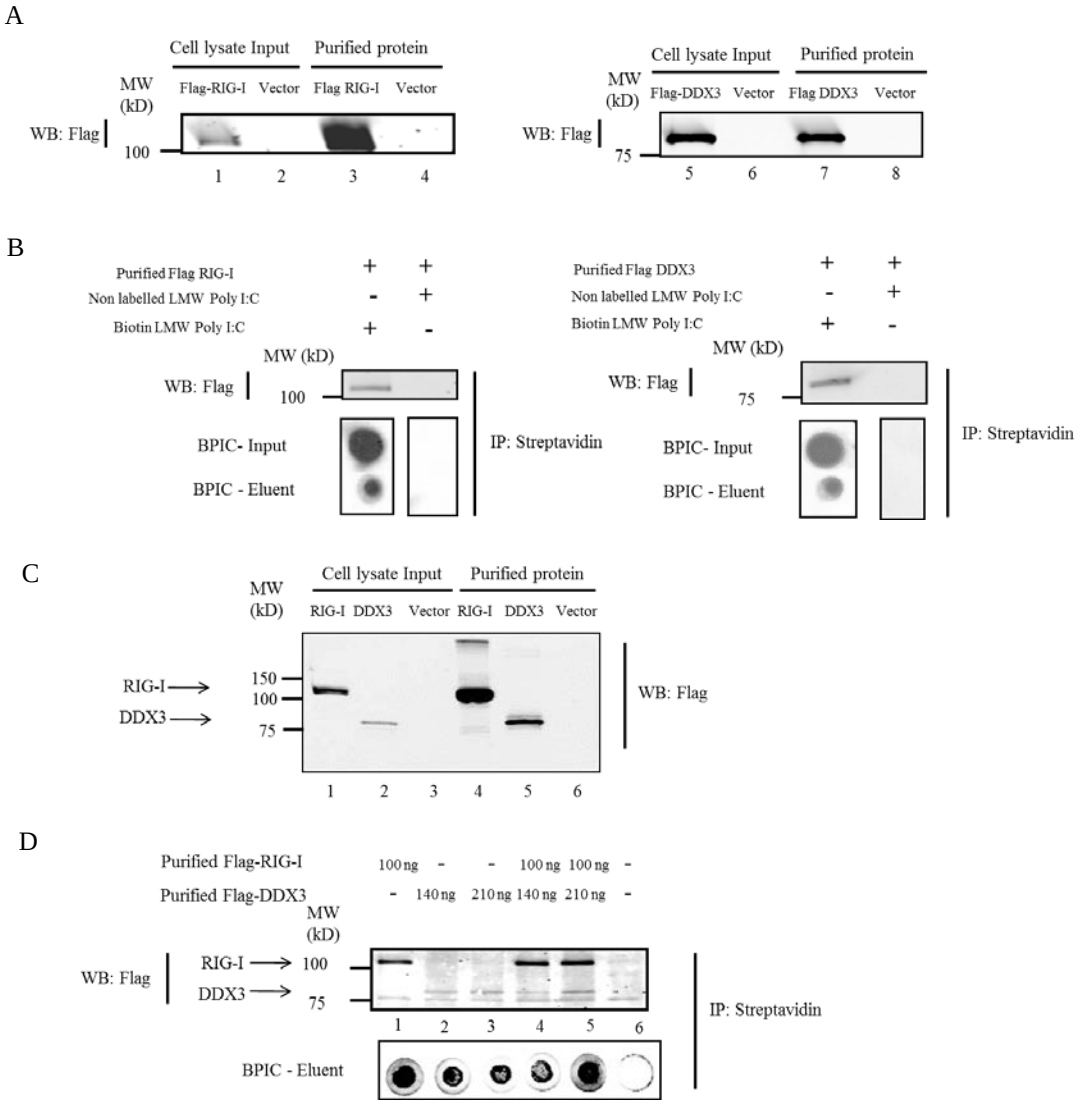

Figure 5.5 DDX3 and RIG-I competition assay with LMW poly I:C. (A,C) 293T cells were transfected with 1µg of plasmid expressing Flag-DDX3 or Flag-RIG-I or Flag-vector. At 48 h.p.t., cell lysate was collected, Flag-DDX3 and Flag-RIG-I were purified using Anti-Flag M2 Agarose. The input cell lysate and the purified protein were subjected to Western blotting using Flag-tag antibody. B) Biotinylated LMW poly I:C or non-labelled LMW poly I:C was first incubated with magnetic streptavidin beads and then washed to remove unbound poly I:C. The beads were then incubated with purified Flag-DDX3 or Flag- RIG-I, then washed several times to remove the unbound protein and the streptavidin bound poly I:C-protein complex was then eluted with the elution buffer. The eluent was then run on a gel and the bound Flag-DDX3 and Flag-RIG-I was then detected by Western blotting using Flag-tag antibody. The input poly I:C and eluent were then blotted onto a Hybond membrane, crosslinked by UV and detected using Ambion BrightStar BioDetect kit. D) The purified proteins were then mixed together at a ratio of 1:2 (lane 4- 100 ng Flag-RIG-I and 140 ng Flag-DDX3) or at a ratio of 1:3 (lane 5 – 100 ng Flag-RIG-I and 210 ng Flag-DDX3), Flag-RIG-I alone (lane 1- 100 ng), Flag-DDX3 alone at 140 ng (lane 2) or at 210 ng (lane 3) and then incubated with Biotinylated LMW poly I:C. The LMW poly I:C along with any interacting protein was isolated using Streptavidin Magnetic Beads. The amount of Flag-tagged protein in the eluent was determined by Western blot using an anti-Flag antibody and the eluent was blotted on a HyBond membrane and the biotinylated poly I:C present in the eluent was detected using Streptavidin conjugated with IRDye 800CW from Licor.

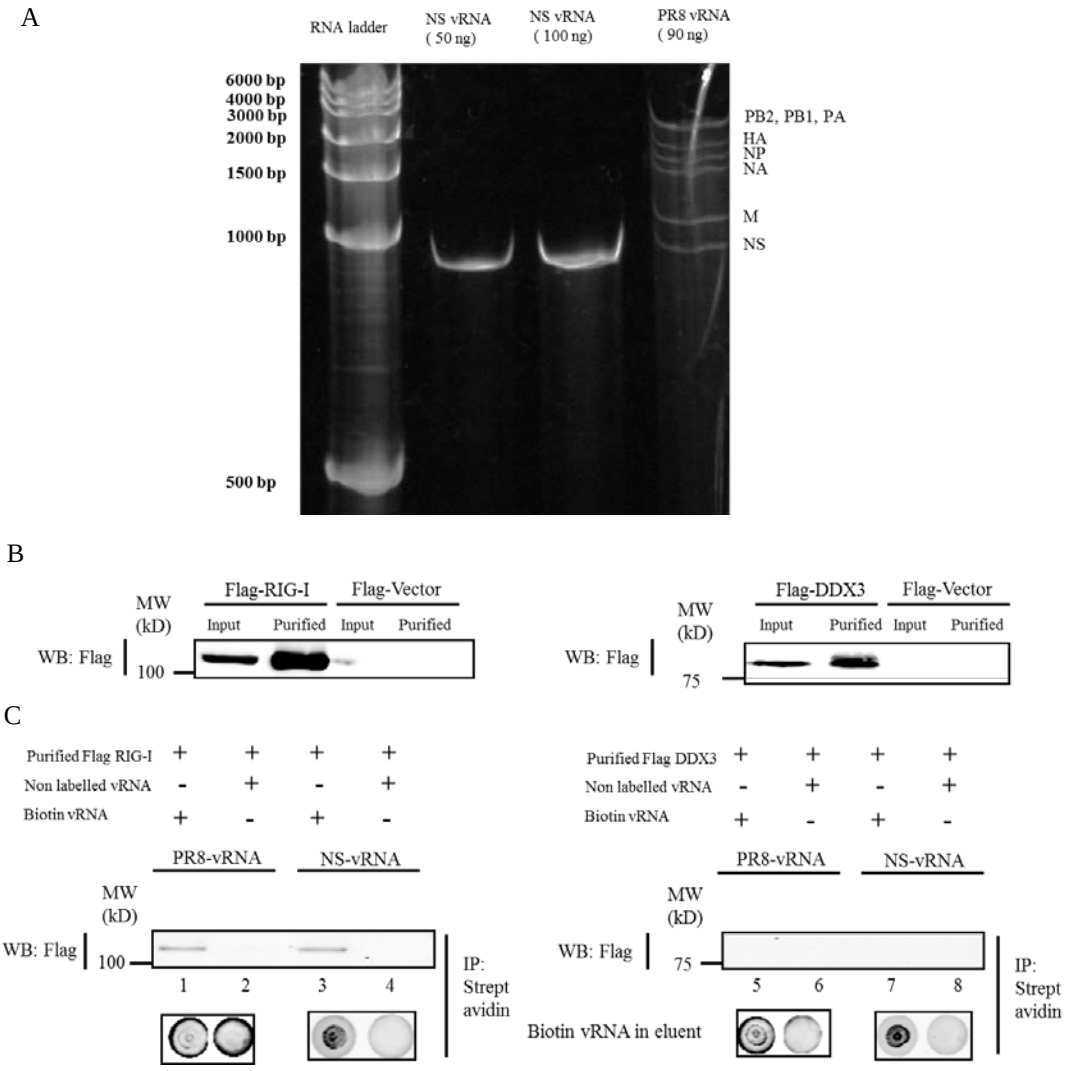

Figure 5.6 Viral RNA (vRNA) interaction with RIG-I and DDX3. A) Viral RNA was isolated from purified PR8 virion using the Qiagen RNeasy mini kit and NS vRNA of SIV/SK-WT virus was *in vitro* transcribed from a NS gene template containing the T7 promoter sequence using the Ambion MaxiScript T7 polymerase kit. The RNA was run on a urea-polyacrylamide gel along with a RNA ladder and visualized by staining with SYBR Gold. The identity of the bands corresponding to the different vRNA segments (for PR8 vRNA lane) is labelled on the right-hand side of the image. B) 293T cells were transfected with 1µg of plasmid expressing Flag-DDX3 or Flag-RIG-I or Flag-vector. At 48 h.p.t., cell lysate was collected and Flag-DDX3 and Flag-RIG-I were purified using Anti-Flag M2 Agarose. The input cell lysate and the purified protein were subjected to Western blotting using antibody against Flag-tag. C) Biotinylated or non-labelled PR8 vRNA, biotinylated or non-labelled NS vRNA was first incubated with magnetic streptavidin beads and then washed to remove RNA. The RNA bound beads were then incubated with purified Flag-DDX3 or Flag-RIG-I, then washed several times to remove the unbound protein and the streptavidin bound poly I:C-protein complex was then eluted with the supplied elution buffer. The eluent was then run on a gel and the bound Flag-DDX3 or Flag-RIG-I was then detected by Western blot using antibody against Flag-tag. The eluent was also blotted onto a Hybond membrane, crosslinked by UV and the vRNA present in the eluent were detected using Streptavidin conjugated with IRDye 800CW from Licor.

5.4.7 The overall effect of DDX3 regulation on IFNβ expression and SG formation affects PR8 del NS1 virus replication but not WT virus replication

Previously, in chapter 4, suppression of virus-induced SG formation was observed in DDX3 siRNA treated cells (Fig. 4.7A). Here, we observed an enhancement of virus-induced IFNβ expression in DDX3 siRNA treated cells. Even though DDX3-mediated IFNβ inhibition should be detrimental to virus replication, we observed an increase in the virus titer of PR8 del NS1 virus (chapter 4, Figures 4.7B and 4.7C). The increase in virus replication correlated with DDX3 inhibition on SG formation. These results suggest that while the SG regulatory function of DDX3 has a dominant role in influencing influenza virus replication, the IFNβ inhibitory function of DDX3 has only a minor role in the virus life cycle.

5.5 Discussion

The innate immune system detects viral infections through the recognition of unique molecules in the pathogen or through the recognition of unique molecules generated during infection called pathogen associated molecular patterns (PAMPS) (372, 373). Influenza A virus is recognized by members of at least three classes of PRRs: the toll-like receptors (TLR3, TLR7 and TLR8), RIG-I and the NOD-like receptor family member NLRP3 (233). TLR3 and RIG-I are the major sensors of influenza A virus infection in epithelial cells and distinct from other PRRs, they signal through the activation of TBK1/IKKε (260, 307, 520). Therefore, we tested the contribution of the two receptors in IFNβ signalling by using a TBK1/IKKε inhibitor BX795 (92), which showed that TBK1/IKKε activation does play a major role in virus-induced IFNβ induction (Fig. 5.1A). Then, the individual contribution of the two receptors was tested by siRNA-mediated knockdown and this showed that the RIG-I receptor and not the TLR3 receptor has a major role in virus-IFNβ induction (Figures 5.1B, 5.1C, 5.1E and 5.1F).

Many studies have shown that DDX3 enhances the activation of IRF3 and induction of IFNβ (183, 413, 472, 497). However, in this study I report that DDX3 inhibits IRF3 activation and IFNβ induction (Figures 5.2A, 5.2B, 5.2C, 5.3C, 5.4A and 5.4B). The reason for this discrepancy might be due to the multifunctional nature of DDX3 and differences in the cell-type and RIG-I ligand used in the studies. DDX3 is a multifunctional protein affecting every step of gene expression including transcription, RNA splicing, RNA export and translation (19, 471). Importantly, several studies have reported a role for DDX3 in translational regulation (19, 471). While initially, it was suggested that DDX3 has an inhibitory role on cap-dependent protein translation (485) another study reported that DDX3 does not have any significant effect on

general translation (310). In order to explain these contradictory results, it has been suggested that the level of DDX3 expression might influence whether it has a positive or negative effect on translation and that the overexpression of DDX3 might have a translation repressive effect (471). It is also interesting to note the diversity in the mechanisms proposed to explain the role of DDX3 in enhancing IFNβ induction. Even though existing studies agree that DDX3 upregulates IFNβ induction, there are differences in the mechanisms proposed by these studies to explain this enhancing effect. While many studies reported that DDX3 functions through TBK1/IKKε interaction to enhance IFNβ induction (183, 472, 497), another study showed that DDX3 does not affect TBK1/IKKε-mediated IFNβ induction and suggested a new mechanism where DDX3 acted as a viral sensor forming a complex with RIG-I/MDA5 and IPS-1 (413). DDX3 is also recruited to the IFNβ promoter region, suggesting that it can act as a transcriptional regulator (497). Hence, these studies show that DDX3 can influence multiple steps of the IFNβ gene expression and IFNβ induction pathway and that the observed phenotype can vary depending on experimental conditions such as level of DDX3 expression, cell-type and the ligand used. In line with this, the experimental conditions in this work differ considerably from the above described studies. The differences include the RIG-I ligand (LMW poly I:C vs poly I:C containing both low and high molecular weight species), differences in method of delivery and dosage of poly I:C, differences in plasmids and the amount transfected to express DDX3 and cell-type specific differences (porcine NPTr vs human cell line). In addition, it is also important to note that these studies were accomplished through infection with pathogens other than influenza. Observations from the previous study showed that DDX3 interacts with the viral proteins NP and NS1 and suggests a regulatory role for DDX3 in influenza induced stress granule (SG) formation (see chapter 4). Hence, DDX3 being a multifunctional protein could be utilized in functions and

pathways distinctly different from other pathogens through its interaction with viral proteins NS1 and NP during influenza infection. Therefore, another reason for the contradictory observation could be the differences in the life cycle of the pathogens. In this study, attempts were made to find out the mechanisms by which DDX3 inhibits IFNβ induction upon virus infection. DDX3 and RIG-I are both RNA helicases and are known to bind to dsRNA and poly I:C (145, 240, 413), but unlike RIG-I, DDX3 does not have a signalling domain corresponding to the RIG-I CARD domains (480, 496). RIG-I is a well-documented sensor of the viral RNA and poly I:C in the cytoplasm, which upon activation induces expression of the type I IFN (177). Therefore, the speculation that DDX3 might inhibit RIG-I activation by competing for the RIG-I ligands such as poly I:C and viral RNA were tested. However, *in vitro* experiments clearly demonstrate that RIG-I binds LMW poly I:C and influenza vRNA with an affinity much higher than that of DDX3 (Figures 5.5D and 5.6C). Therefore, it does not seem possible that DDX3 exerts its IFNβ inhibitory effect by competing with the RIG-I ligand. However, DDX3 being a multifunctional protein could contribute to DDX3-mediated IFNβ inhibition by several other mechanisms. DDX3 interacts with the influenza virus proteins NP, NS1 (see chapter 4) and has also been shown to form a complex with RIG-I and MAVS (413). In addition, NS1 interacts with RIG-I and is a major antagonist of IFNβ induction (378). Therefore, it may be interesting to study the interplay between the viral proteins, RIG-I and DDX3 in IFNβ induction. Also of interest would be understanding whether DDX3-mediated SG regulation (see chapter 4) has any role in IFNβ regulation, since one study has reported SGs as important platforms for viral RNA sensing (408).

Besides inhibiting type I IFN production at multiple steps of the IFN induction pathway, the influenza NS1 protein also inhibits the function of IFN-induced antiviral proteins (23, 290). Viral proteins other than NS1 have also been shown to inhibit IFN induction (180, 231, 551).

Therefore, even when IFN transcription is enhanced upon DDX3 downregulation (Figures 5.2B and 5.2C), mechanisms still exist to counteract IFNβ protein expression and IFNβ-mediated antiviral responses at the post-transcriptional level. This might explain the unresponsive effect of DDX3 downregulation and enhanced IFNβ transcription on SIV/SK virus replication (chapter 4, Figures 4.7B and 4.7C). However, PR8 del NS1 virus replication was affected by DDX3 downregulation. Even though DDX3 KD increased PR8 del NS1 virus-induced IFNβ expression (Fig. 5.2C), an anticipated reduction in the virus replication was not observed. In contrast, increased virus replication of the PR8 del NS1 virus in DDX3 KD cells was observed (chapter 4, Figures 4.7B and 4.7C). This could be explained by the multifunctional nature of the DDX3 protein (19). In this study, DDX3 was observed to be an essential factor of virus-induced stress granules (SGs) during PR8 del NS1 virus infection, as DDX3 KD suppressed virus-induced SG formation (see chapter 4, Fig. 4.7A). Additionally, DDX3 KD promoted PR8 del NS1 virus replication (see chapter 4, Figures 4.7B and 4.7C). Several studies show that SGs possess antiviral functions and SG formation is inhibitory to virus replication (272, 381, 409). Thus, suppression of SG formation correlated with the increase in PR8 del NS1 virus replication in DDX3 siRNA treated cells. DDX3 being a multifunctional protein, it could influence both the IFNβ induction and SG formation pathways during influenza virus infection. However, as these results suggest, the SG regulatory function of DDX3 might have a much profound effect on virus replication than the IFNβ regulatory function of DDX3.

In conclusion, the role of DDX3 in regulating virus-induced IFNβ expression has been established. DDX3 regulation on influenza induced IFNβ expression occurs upstream of IRF3 phosphorylation and the mechanism by which it inhibits IFNβ induction requires further study. Additionally, it will be interesting to study the phosphorylation status of IRF3, TBK1/IKKε upon

DDX3 downregulation and overexpression in LMW poly I:C transfected cells. This will give a better insight into the mechanism by which DDX3 inhibits IFNβ induction and to understand whether the repressive effect might be mediated at the translational level.

CHAPTER 6: GENERAL DISCUSSION AND CONCLUSIONS

Influenza A viruses cause frequent epidemics and annual deaths of about 500,000 people worldwide (535), an effective prophylactic and therapeutic measure against influenza infection is essential. The effectiveness of the vaccines developed each year varies depending on several factors and the currently available therapeutic arsenal against influenza virus infections is limited to two main classes of drugs, namely the inhibitors of viral neuraminidase (zanamivir and oseltamivir) and of the viral ion channel protein M2 (rimantadine and amantadine) (102, 205). The emergence of resistant virus strains to these drugs poses a serious problem for the public health and global economy (66, 398). Therefore, there is an urgent need for the discovery of new antiviral drugs, which are protected from the virus drug resistance mechanisms. There is a growing realisation among the scientific community that the best approach to achieving this aim, would be to target cellular host factors instead of targeting the traditional virus factors. While the virus-factors are under pressure to mutate when confronted with drugs affecting the virus life cycle, host-factors are not. Another significant advantage of this approach is that by targeting cellular factors, a broad-spectrum antiviral activity could be achieved, since several viruses may depend on the same cellular pathway. Additionally, by targeting cellular factors, there is a possibility that existing drugs approved for human disorders could be re-directed for use in antiviral therapy.

In spite of these advantages, there are also some disadvantages in targeting cellular factors. As cellular factors may be critical for proper functioning of the cell and in regulating homeostasis, interfering with these processes might lead to cytotoxic and undesirable effects. To minimize this undesirable effect, treatment time has to be limited, which makes this approach

more suitable for treating acute virus infections such as influenza virus infections. Some of the recent studies have been successful in employing approaches to validate drugs targeting host factors, in order to achieve antiviral activity against influenza (105, 570). One among them is the sialidase drug DAS181, which removes sialic acids on respiratory epithelial cells and prevents virus attachment to the receptor (42). Resistance to the drug was minimal and unstable upon extensive passaging of influenza virus in the presence of DAS181 (540). Other approaches using inhibitors to target cellular proteases and the Raf/MEK/ERK signalling pathway have also been demonstrated to be successful in inhibiting virus infection (60, 61, 120). Treatment with immunomodulatory drugs targeting the host immune system, such as COX-2 inhibitors and S1P agonists have been found to alleviate tissue damage caused by virus-induced cytokines and also suppress virus replication (312, 313, 359). Additionally, many other inhibitors that target cellular factors are known to affect various stages of the influenza virus life cycle leading to reduced virus replication (386). Thus, targeting host proteins could lead to the development of novel and broad-spectrum antivirals. Therefore, identification of cellular factors that affect virus replication is critical.

Many different strategies have been used to identify and study cellular interaction partners of viral proteins. One of the earliest and widely used approach is the yeast-two-hybrid (Y2H) assay, which was employed by Shapira et al. to study host protein interactions with 10 major influenza A virus proteins (478). Many of the earliest known cellular factors affecting influenza virus replication were discovered using this approach and it is still used in recent studies to identify novel virus-host interaction partners (184, 394, 405, 481). Recently, the Y2H assay system was used to identify host-factors interacting with NS1 and NEP proteins. Seventy-nine cellular proteins interacting with NS1 and NEP proteins from 9 different influenza virus

strains were identified (104). However, the disadvantage of using this approach is that only dual interactions can be studied and virus-host protein interactions in the context of virus infection or in specific protein complexes cannot be studied.

Genome wide siRNA screens have also been used to identify cellular factors required for viral replication (501, 571). Numerous studies have used the RNAi approach and have been critical for expanding the knowledge about the cellular pathways important for virus replication (64, 193, 252, 287, 478). Even though RNAi is a powerful method, there are some limitations to this approach. High-throughput RNAi screening is restricted by the number of genes covered in the siRNA library, the efficiency of knockdown and the inability to study genes that affect cell-growth and survival. Even though a large amount of data generated through these studies is available on the cellular factors involved in virus replication, there is very little overlap among the different studies (105, 501, 571). The lack of overlap suggests that these datasets are far from being comprehensive and hence, additional studies are needed for identification and validation of the viral protein interaction partners.

Another approach employed is to isolate protein complexes by affinity tag purification using tagged viral protein constructs. The proteins in the isolated complex can then be identified using mass spectrometry. Cellular proteins that interact with the influenza vRNP and the polymerase complex have been identified using this approach (244, 365). However, the viral proteins were expressed artificially and hence the observed interactions may not take place in virus-infected cells. It would be ideal to express the tagged viral protein in the context of infection, which would need integration of the tag sequence within the virus genome. The segmented nature of influenza A virus genome and specific packaging mechanisms of viral

RNA, make incorporation of the affinity tag at either the N- or C-terminus of the influenza A virus ORF challenging (118). However, advancements in the understanding of the architecture of influenza A virus genome have made possible the generation of recombinant viruses encoding affinity-tagged viral proteins for studying viral protein interactions from virus-infected cells (324, 326, 441).

In this study, a strategy was devised to insert a Strep-tag sequence in NS1 protein in order to analyze protein interactions of NS1 occurring during physiological virus replication. The flexible and variable nature of the linker region between the RNA-binding domain (RD) and effector domain (ED) of NS1 has been demonstrated in several studies. Large scale sequence analysis of the NS1 sequence from various influenza A virus strains showed that the amino acid residues D74-L77 and K79-R83 in the linker region between the RD and ED of NS1 is highly variable (101). Moreover, an X-ray structure study on NS1 observed the region corresponding to residues 75-79 as not well-defined, which shows that the linker region is highly flexible in nature (57). This region is amenable for modifications, which was demonstrated by previous studies in our lab involving tetracysteine (TC) tag and Strep-tag insertions in NS1 (57, 324, 326). Taking advantage of this knowledge, an eight amino acid Strep-tag sequence was inserted in the SK02 NS1 linker region and a recombinant SK02 virus encoding a Strep-tag NS1 (SIV/SK-544) with growth properties and protein expression kinetics similar to the WT virus was successfully rescued (Section 3.4.1). This resulted in the introduction of the Strep-tag sequence in NS1 without affecting the packaging signals or splicing of NS gene segment. Most of the existing studies to identify NS1-interacting host partners have been performed with well-established cell lines. However, studying NS1-host protein interactions in primary cell lines would yield novel interaction partners. The epithelial cells in the respiratory tract serve as the primary sites of viral

replication during influenza infection (353). Therefore, epithelial cells from the trachea of healthy pigs were isolated. The purity was then confirmed by FACS analysis using a specific marker for epithelial cells called cytokeratin (Section 3.4.2). The epithelial cells were then used for infection with the recombinant virus.

The Strep-tag sequence is engineered to bind with high affinity and reversibly to strep-tactin, enabling the isolation of intact protein complexes (464). Thus, SRECs were infected with SIV/SK-544 and the presence of Strep-tag sequence in the NS1 protein facilitated the purification of an intact NS1-interacting protein complex (Section 3.4.2). The host and viral proteins present in the purified NS1 – host protein complex were identified by LC-MS/MS. Since NS1 interaction with cellular proteins is critical for its multifunctional nature, the host proteins present in the purified complex could in turn provide an understanding of the pathways regulated by NS1 during infection. Therefore, DAVID bioinformatics resources was used to group proteins in my dataset and identified the pathways enriched by these proteins. Several host functions important for virus replication were identified by this analysis and protein-interaction networks derived using STRING database. Cytoscape helped visualize the interactions among the cellular proteins and NS1 in each group (Section 3.4.3-3.4.6). Thus, bioinformatics analysis of the protein dataset provided an insight into the different host functions that may be regulated through NS1 during virus infection. Many of the previously known NS1-interacting proteins such as PABP1, DHX9, DDX21 and CRKL were identified in this study. Additionally, 92 out of the 192 proteins analysed in my dataset were listed as known NS1 interactors in the VirHostNet 2.0 database, which increased the confidence in my dataset. Meanwhile, some of the well-known NS1 binding partners such as p85β and CPSF30 were not identified in the NS1-interacting protein complex. The absence of these well-known NS1-interacting partners could be due to the

polymorphisms in the protein sequence of the NS1 used in this study. Examples of these polymorphisms playing a role in binding to different cellular partners have been demonstrated in other studies (104, 478).

SGs are aggregates of inactive mRNPs enriched in translation initiation factors and 40S ribosome subunits (15). SG formation is triggered by a variety of environmental stress conditions including viral infection. The stress conditions induce the activation of a variety of different eIF2α kinases all of which result in the phosphorylation of eIF2α. This triggers SG formation (15). Virus infection commonly activates PKR kinase which in turn phosphorylates eIF2α, resulting in the inhibition of translation initiation (350). SGs are generally inhibitory to virus replication due to their role in inducing translational arrest. As a result, viruses typically suppress SG formation at some point in their life cycle. The mechanisms used by viruses to disrupt SG formation are varied. These include: i) cleavage of proteins essential for SG formation, ii) co-opting of SG proteins to other functions necessary for virus replication and iii) inhibition of PKR activation (336). Studies demonstrated that poliovirus protein 3C proteinase cleaves the SG-nucleating protein G3BP1 and prevents SG formation during the late stages of infection (577); HCV redistributes several P-body and SG proteins such as DDX6, DDX3, G3BP1, RCK/p54 and Xrn1 to virus induced cytoplasmic lipid droplets, thereby facilitating virus replication and restricting SG assembly at the same time (21). Influenza A virus on the other hand inhibits SG formation throughout the virus life-cycle by antagonizing PKR activation and thereby preventing eIF2α phosphorylation (272). SG inhibition is mediated through the PKR antagonistic function of NS1 and influenza viruses with functional defects in NS1 protein are unable to suppress SG formation (272, 408). Moreover, two other influenza virus proteins NP and PA-X have also been

shown to inhibit both eIF2α-dependent and independent SG formation through unknown mechanisms (271).

DDX3 was identified as a NS1-interacting partner during virus infection in this study (Figures 4.1A and 4.1B). This prompted the study of the function of DDX3 in influenza A virus life cycle. Given the fact that DDX3 has been reported to localize in SGs and function as an essential factor for SG formation in response to a variety of stresses such as sorbitol, arsenite, heat shock, DTT and UV irradiation (486), I set up my research direction to study DDX3's function in regulating influenza A virus infection through SG formation. The interaction of DDX3 with NS1 and NP proteins was confirmed by IP experiments either in the context of virus infection or with ectopically expressed proteins. Furthermore, the interactions were demonstrated to be RNA independent (Section 4.4.1-4.4.2). Upon immunostaining PR8 del NS1 virus-infected cells for DDX3 and the SG marker TIA-1, it was shown that DDX3 does localize in PR8 del NS1 virus induced SGs. SGs started to appear from 6 h.p.i. and DDX3 localized in SGs at all the time points tested until 11 h.p.i, where SG formation was observed (Section 4.4.4). Strikingly NP also formed granules and co-localized with DDX3 and TIA-1 in SGs, which suggested that DDX3 and NP interaction occurs in virus-induced SGs. While NP localization in SGs has been observed in other studies (271, 381, 408), Onomoto et al. reported that NP localized to the SGs is associated with the vRNA and the SGs serve as a platform for vRNA sensing by RIG-I (408). However, in this study vRNA was not sequestered in the SGs as demonstrated by FISH analysis, suggesting that the NP localized in SGs is not associated with vRNA (Section 4.4.5). Additionally, no SG formation was observed in WT virus-infected cells at any of the time points tested, which could be explained by the SG inhibitory activity of NS1, possibly through preventing PKR phosphorylation (Section 4.4.4 and 4.4.6). SG formation has been reported to

suppress influenza virus replication in other studies, based on the observed reduction in NP expression intensity and the number of NP expressing cells (272, 381). Whether SG formation results in a reduction in the actual virus titers was not tested. In my study, influenza virus titers upon DDX3 knockdown were tested. DDX3 downregulation resulted in SG suppression, which correlated with increased virus titers in PR8 del NS1 virus-infected cells (Section 4.4.8), suggesting that DDX3 is an antiviral protein with a critical role in virus-induced SG formation.

In addition to DDX3, another SG component RAP55 has also been reported to be involved in SG/P-body (PB) formation and has also been shown to interact with NS1 (381). It was shown that NS1 inhibits RAP55 associated SG/P-body formation. The study also reported that NS1 and NP colocalize in RAP55 associated P-bodies. Moreover, P-bodies formed during the early stages of WT virus infection and retreated as the infection progressed into the late stages (381). Similar to SGs, P-bodies contain translationally inactive mRNAs. But, unlike SGs they contain the proteins needed for mRNA degradation such as the decapping enzyme DCP1 and 5'-3' exonuclease XRN1 (127). While SGs are induced by different stress responses, P-bodies are present in resting cells as well as in stress induced cells and both granules can increase in size and number in response to stress (110, 127). Recent studies suggest that SGs and P-bodies physically interact with each other and many components including mRNA and proteins may be exchanged between the two compartments (127, 268). Based on the above findings it might be interesting to study the role of P-bodies and whether SG and P-body interact and exchange components during influenza A virus infection. Interestingly Ded1p, the yeast homologue of DDX3 is a component of P-bodies and is involved in the movement of mRNAs between the polysomes and P-bodies (40, 41). Moreover, DDX3 has been observed to localize in the interface of SGs and P-bodies and has been suggested to be involved in regulating the transfer of

molecules between the two granular compartments (486). Therefore, DDX3 could have additional roles if a P-body-SG linkage exists during influenza infection.

DDX3 has also been reported to interact with different components of the IFNβ induction pathway and enhance IFNβ expression (19). DDX3 achieves this through its interaction with IKKε, TBK1 and by directly binding to the IFNβ promoter (19). Several viruses have evolved mechanisms to counteract this function by employing viral proteins to interact with DDX3 and subvert its IFN modulatory activity. HCV core protein, HBV pol protein and VACV K7 protein interact with DDX3 and counteract its IFN modulatory function (480). However, interaction of these proteins with DDX3 affects virus replication by interfering with other stages of the virus life cycle. For example, HCV core protein interaction with DDX3 re-localizes DDX3 to HCV assembly sites called lipid droplets and facilitates HCV replication, while HBV pol interaction with DDX3 interferes with the initial step of reverse transcription and negatively affects virus replication (21, 562). DDX3 also interacts with the 3'UTR of HCV genomic RNA and induces a CBP/p300-mediated transcriptional program, which is beneficial for HCV replication (320). DDX3 is required for the replication of several other viruses. DDX3 promotes the translation of JEV viral RNA and many P-body components including DDX3 are recruited to WNV replication sites (76, 317). Many studies have also reported that DDX3 is required for HIV replication and this function is mediated by its interaction with viral proteins Rev and Tat (388, 593). These studies show that DDX3 can affect virus life cycle through multiple mechanisms and underscores the multifunctional nature of the protein. Additionally, it is interesting to note that several SG/PB components, such as PABP1, YBX1, STAU1, ILF3, DDX1, FXR1, G3BP1, hnRNP K, ATXN2L, MOV10 were identified in the NS1-interacting protein complex (16, 51, 211). PABP1, YBX1, STAU1, FXR1, hnRNP K have proviral roles in influenza replication (70,

129, 258, 542, 610), while DDX1, ILF3 and MOV10 have antiviral functions (565, 605, 607). Therefore, it would be interesting to investigate how the functions of these proteins affect SG formation and how SG formation affects the functions of these proteins in the context of virus replication.

Even though the role of DDX3 in regulating virus induced IFN was studied, it remains unknown for influenza A virus replication. Thus, the potential contribution of DDX3 to this critical host defence pathway was explored. Since TLR3 and RIG-I are the major receptors for sensing influenza virus infection (260, 307, 520), the relative contribution of the two pathways to influenza virus-induced IFNβ induction was first determined. Gene knockdown assay showed that IFNβ expression in influenza virus-infected cells is mediated primarily through the RIG-I pathway, while TLR3 contribution to IFNβ induction is minimal (Section 5.4.1). Downregulation of DDX3 resulted in increased IRF3 phosphorylation and increased IFNβ mRNA expression in WT influenza virus-infected cells. A similar increase in IFNβ expression upon DDX3 downregulation was also observed in PR8 del NS1 virus-infected cells and LMW poly I:C transfected cells, which is a RIG-I agonist (108, 255) (Sections 5.4.2-5.4.3). The ability of LMW poly I:C to trigger IFNβ expression preferentially through the RIG-I pathway was also confirmed (Section 5.4.3). In agreement with the results observed upon DDX3 downregulation, DDX3 over-expression suppressed influenza A virus and LMW poly I:C induced IFNβ induction (Section 5.4.4).

DDX3 and RIG-I are both capable of binding to RNA (145, 240, 413). Therefore, I investigated whether DDX3 could exert its IFNβ inhibitory effect by competing with RIG-I for its ligand. Competition assays with purified RIG-I, DDX3 and biotinylated LMW poly I:C

showed that DDX3 could not compete with RIG-I for binding to poly I:C due to its low affinity to the molecule when compared to RIG-I (Section 5.4.5). Studies with biotinylated vRNA also showed the binding affinity of RIG-I to vRNA to be much higher than DDX3 (Section 5.4.6). Even though, DDX3 downregulation increased influenza A virus induced IFNβ expression, it did not have any effect on SIV/SK virus replication, while PR8 del NS1 virus replication was enhanced (Sections 4.4.8 and 5.4.7). The ability of NS1 protein to inhibit the function of ISGs and inhibit the multiple steps of IFNβ expression pathway (23, 290) might explain the lack of any effect on SIV/SK virus replication in DDX3 knockdown cells. While DDX3 does increase IFNβ mRNA expression in PR8 del NS1 virus-infected cells, the SG inhibitory function has a much more profound effect resulting in the observed increase in virus replication.

Many known antiviral components such as OAS, RNaseL, RIG-I, MDA5 and several regulators of RIG-I activation including TRIM25, RIPLET and MEX3C have been shown to co-localize in SGs (409). A recent study also suggests that SGs might act as a platform for facilitating the RIG-I sensing of viral RNAs during influenza infection (408). More importantly, constitutively expressed DDX3 acts as a viral RNA sensor during the initial stages of infection by forming a complex with MAVS and thereby triggering IFNβ production (413). Considering the localization of DDX3 in SGs and its known interaction with the components of RIG-I signalling pathway, it would be interesting to investigate whether and how DDX3 localization in SGs affects RIG-I-mediated IFNβ induction during influenza A virus infection.

In order to gain a better understanding of the role of DDX3 in influenza replication, several key findings resulting from this study could be explored further as follows. Strong interaction between the viral proteins NS1 and NP and DDX3 was observed in this study.

Identification of domains in NS1 and NP that are important for DDX3 interaction could help in understanding the SG recruitment of NP and NS1 and the role of these proteins in SG formation. More importantly, the role of NS1-DDX3 interaction in SG formation and influenza A virus replication needs further analysis. In this study, the R38A/K41A mutation in NS1 disrupts interaction with DDX3, showing that these two amino acids are important for DDX3 interaction. Influenza A virus with this mutation is able to induce PKR phosphorylation and SG formation (272). The inability of this mutant NS1 to interact with DDX3 could be utilized to study the role of DDX3-NS1 interaction in SG formation, if PKR phosphorylation induced SG formation does not occur. Therefore, further studies on DDX3-interacting domains in NS1 protein, could help in developing a mutant NS1 capable of inducing PKR phosphorylation but still able to interact with DDX3 and vice versa. Such a mutant could help dissect the relative contribution of NS1-DDX3 interaction in SG recruitment and formation. Even though this study shows that DDX3 antiviral activity correlates with SG formation, the mechanism of antiviral activity still needs to be determined. Studies on determining the identity of viral RNA components (vRNA, cRNA or mRNA) recruited to SGs and the stage of virus life cycle that is negatively impacted by the SG formation could provide more information on the antiviral mechanism. As discussed previously, P-bodies have been observed during the early stages of WT virus infection. DDX3 could have a role in the formation of P-bodies, which possess mRNA silencing activity similar to SGs (127). Therefore, studies on the role of P-bodies and the function of DDX3 in P-body formation could provide a better understanding on the impact of mRNP granules on influenza A virus replication. Moreover, exploring the role of SGs in influenza A virus-induced IFNβ induction may also provide knowledge on the antiviral mechanisms of SGs.

In conclusion, successful isolation of an intact NS1 protein complex from primary SREC infected with a recombinant Strep-tag NS1 virus was demonstrated. Bioinformatics analysis of the protein dataset helped identify important host functions that may be regulated by NS1 during influenza A virus infection. DDX3 as an antiviral protein with a critical role in influenza A virus-induced SG formation was established. DDX3 also regulates IFNβ expression in influenza A virus-infected cells, which demonstrates the multifunctional nature of the protein. However, the NS1 protein of influenza A virus is able to suppress SG formation in WT virus-infected cells and might also counteract the effects of increased IFNβ transcription. This shows the evolutionary interplay between the host and virus in developing defense strategies for survival and replication of the virus. Additionally, results from this study also suggest that the function of DDX3 in SG formation has a strong effect on virus replication when compared to its IFNβ regulatory function.

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APPENDIX I: PUBLICATIONS, CONFERENCES, SCHOLARSHIPS AND AWARDS

Publications:

1. Lin L, Li Y, Pyo HM, Lu X, **Raman SN**, Liu Q, Brown EG, Zhou Y. 2012. *Identification of RNA Helicase A as a Cellular Factor That Interacts with Influenza A Virus NS1 Protein and Its Role in the Virus Life Cycle*. **J Virol**. 86(4):1942-54.

   My contribution – Determination of mutant virus titers by plaque assay for the growth curve experiment. Percentage contribution – 10%.

2. **Raman SN**, Liu G, Pyo HM, Cui YC, Xu F, Ayalew LE, Tikoo SK, Zhou Y. 2016. *DDX3 interacts with influenza A NS1 and NP proteins and exerts antiviral function through regulation of stress granule formation.* **J. Virol.** doi:10.1128/JVI.03010-15

   My contribution – Conception and design of experiments, execution of experiments, acquisition and analysis of data, and drafting of the manuscript. Percentage contribution – 90%.

Conferences:

1. **Raman SN**, Zhou Y. November 12-13, 2010. *Determination of Temperature sensitive loci for Swine Influenza SIV/SK/2001 (H1N1).* Canadian pandemic preparedness meeting, Montreal, QC, Canada. (poster presentation)
2. **Raman SN**, Zhou Y. March 11, 2011. *Determination of Temperature sensitive loci for Swine Influenza SIV/SK/2001 (H1N1).* The 18th annual Life and Health Sciences research day, University of Saskatchewan, Saskatoon, SK, Canada. (poster presentation)
3. **Raman SN**, Zhou Y. June 1, 2012. *Cytokine expression profile of influenza infected primary swine respiratory epithelial cells (SRECs).* Li Ka Shing Institute of Virology and Gairdner foundation symposium, Edmonton, AB, Canada. (poster presentation)

4. **Raman SN**, Zhou Y. March 9, 2012. *Rescue of a swine influenza virus with strep-tagged NS1 to study virus-host protein interactions in swine respiratory epithelial cells (SRECs).* The 19th annual Life and Health Sciences research day, University of Saskatchewan, Saskatoon, SK, Canada. (poster presentation)
5. **Raman SN**, Zhou Y. July 22, 2012. *Rescue of a swine influenza virus with strep-tagged NS1 to study virus-host protein interactions in swine respiratory epithelial cells (SRECs).* The 31st annual meeting of American Society of Virology, Madison, WI, USA on. (poster presentation)
6. **Raman SN**, Zhou Y. March 15, 2013. *Identification of host factors in swine respiratory epithelial cells that contribute to host anti-viral defense and influenza virus replication.* The 20th annual Life and Health Sciences research day, University of Saskatchewan, Saskatoon, SK, Canada. (poster presentation)
7. **Raman SN**, Zhou Y. June 16-21, 2013. *DDX1 and DDX3 as potential host factors in regulating influenza virus replication in Swine Respiratory Epithelial Cells (SRECs).* The 15th International Negative Strand Virus meeting in Granada, Spain. (poster presentation)
8. **Raman SN**, Zhou Y. May 28-30, 2014. *Role of DDX3 in IFNβ response during influenza infection.* The 2014 Prairie Infectious Immunology Network Conference, Hecla, MB, Canada. (poster presentation)

Scholarships and awards:

1. Sep 2010-Aug 2011, Vaccinology and Immunotherapeutics departmental scholarship, University of Saskatchewan, Value is CAD 8000.
2. Sep 2011-Aug 2012, Vaccinology and Immunotherapeutics departmental scholarship, University of Saskatchewan, Value is CAD 8500.

3. Sep 2012-Aug 2013, Vaccinology and Immunotherapeutics departmental scholarship, University of Saskatchewan, Value is CAD 9000.
4. 2012, Saskatchewan Innovation and Opportunity scholarship. Value is CAD 10000.
5. 2013, Saskatchewan Innovation and Opportunity scholarship. Value is CAD 10000.
6. 2012, Student travel award, University of Saskatchewan, to attend the Li Ka Shing Institute of Virology and Gairdner Foundation Symposium, Value is CAD 350.
7. 2012, Student travel award, American Society of Virology, to attend The American Society for Virology the 31st annual meeting. Value is USD 500.
8. 2013, Student travel award, University of Saskatchewan, to attend the 15th International Negative Strand Virus meeting. Value is CAD 550.

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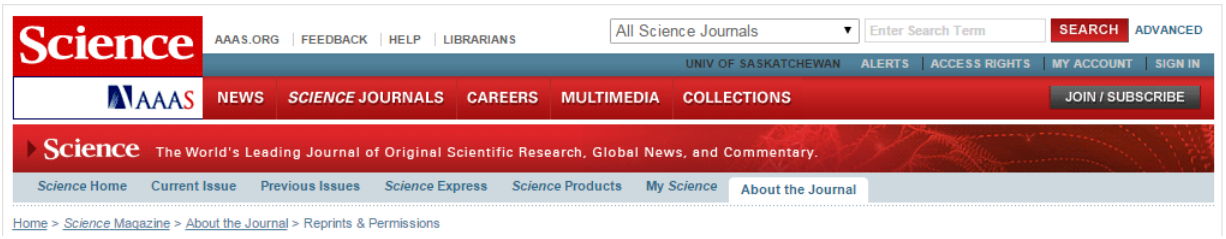

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