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Functional analysis of interactions between influenza A virus protein NS1 and cellular proteins TRBP and PACT

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Doctor of Philosophy,
The University of Edinburgh*

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**Division of Infection and Pathway Medicine
College of Medicine and Veterinary Medicine
The University of Edinburgh**

Declaration

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Abstract

Seasonal and pandemic Influenza virus infections cause about three to five million cases of severe illness and about 250,000 to 500,000 deaths world-wide annually according to the WHO. Although investigated intensively, Influenza virus pathogenesis is still not very well understood and hard to predict. Influenza A viruses contain a segmented, single-(-) stranded RNA genome encoding at least 10 different proteins and are highly diverse due to hypermutation and reassortment. In previous work, 56 viral genes from six different influenza A virus isolates had been cloned and genome-wide screened for virus-host protein interactions using yeast-two hybrid technology and several human and chicken cDNA libraries, leading to the identification of 127 high-confidence cellular interactors of which 40 have also been identified by RNA interference in other studies. In this thesis, two of the cellular interactors identified which both bound to the viral multifunctional protein NS1, TRBP and PACT, were further investigated with regard to their role in virus life cycle. These two proteins are known to be involved in miRNA silencing and PKR regulation.

Both interactions between NS1 and TRBP and NS1 and PACT were confirmed by co-immunoprecipitation, and both TRBP and PACT co-localized with NS1 in a cytosolic compartment. NS1 was also found to be present in the RISC complex in pull-down assays with the RISC core component Ago2. In functional assays, NS1 dose-dependently inhibited RNA silencing. Although no differences in TRBP-binding between NS1 proteins of various different influenza strains could be detected in direct mating Y2H assays, they varied with regard to their inhibitory activity on RNA silencing. TRBP and PACT alone were unable to restore NS1-induced inhibition of RNA silencing activity, however both together restored RNA silencing. Moreover, the siRNA knockdown of PACT abolished the association of NS1 with Ago2, and NS1 competitively inhibited the binding of TRBP and PACT to Ago2. The depletion of either TRBP or PACT led to an inhibition of influenza virus

replication. The depletion of TRBP also lifted cellular IFN β level without infection. However, the knockdown of TRBP but not PACT blocked IFN β production and increased cell viability post infection. These results indicate that NS1 inhibits the binding of PACT and TRBP to the RISC complex and thereby inhibits miRNA-induced gene silencing. The hypothesis that TRBP supports influenza replication potentially by regulating PKR regulation and IFN β induction requires further investigation. In conclusion, this study provides evidence for the complexity of virus-host interactions and the dual role of viral proteins in activating both positive and negative regulatory cellular mechanisms.

Lay Summary

Influenza A viruses are the cause of flu in human and is able to infect a number of different species including humans, birds and pigs. Seasonal flu and occasional pandemics threat public health and global economy. Like all the viruses, influenza A viruses rely on the host cells to support their life cycle. Biological processes within a cell are carried out by protein-protein interactions. Thus it is important to identify how the viral proteins interact with cellular proteins to understand the mechanisms of the virus life cycle. Protein-protein interaction screening by using yeast is one of the approaches to detect host-viral proteins interactions. In previous work, a large-scale screen was performed between viral proteins from six different influenza virus strains and host proteins libraries, a comprehensive set of virus-host interactions were obtained.

In the screen, influenza A virus protein Non-structural Protein 1 (NS1) is found to interact with cellular proteins TARBP2 (TRBP) and PACT (PRKRA). Both of TRBP and PACT are known regulators of cellular protein PKR, which is an important anti-viral protein. They are also involved in the host RNA interference, a process in which RNA molecules inhibit gene expression. The aim of the study was to elucidate the role of interaction of NS1 with TRBP and PACT in influenza virus replication. The interactions were validated by different methods. TRBP, PACT and NS1 were found to localise in the same area inside the cell. Investigation into role of NS1 in host RNA interference reveals that NS1 physically binds to multiprotein complex for host RNA interference and inhibits RNA interference by binding to TRBP and PACT. Role of TRBP and PACT in virus replication and host immune response was also investigated. Depletion of TRBP and PACT from the cells inhibits virus replication. Depletion of TRBP but not PACT from the cells limits production of antiviral protein interferon- β .

Overall, data presented in this work show the role of influenza A virus NS1 in host RNA interference, and the possible role of TRBP in supporting influenza virus replication by regulating PKR and interferon- β induction. The interactions of NS1 with TRBP and PACT are of great interest to improve our understanding of how influenza A virus interacts with the host, mediates potential cross talking between distinct cellular functions, which could be applied for novel therapeutic development and improved strategies of flu treatment.

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I didn't expect that I would have sit in A&E Royal Infirmary next to the lab and awaited treatment for my bad allergy back in September. It was a very difficult time for me. But when I was sick, the supports that I received from Jürgen, Sam, members of the lab, and Huizhong were tremendous. I also realise more and more these firm supports have been exactly the same and consistent all the way through my PhD journey, I am indeed blessed to be surrounded by these inspiring people. Thus I would like to take this opportunity to express my thanks as follows.

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Dedicated to my parents

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Abbreviations

% (v/v)	percentage concentration (volume per volume).
% (w/v)	percentage concentration (weight per volume).
°C	degrees Celsius (temperature).
2'5'-OAS	2'5'-oligoadenylate synthetase.
3'UTR	5' untranslated region.
5'UTR	5' untranslated region.
aa	amino acid.
Ab	antibody.
AD	GAL4 DNA activation domain.
att site	recombination attachment site.
bp	base-pair.
BSA	bovine serum albumin.
CARD	caspase recruitment domain.
Co-IP	co-immunoprecipitation
CPE	cytopathic effect.
CPSF30	30 kDa subunit of the cleavage and polyadenylation specificity factor.
cRNA	anti-genomic RNA.
DAPI	4', 6-diamidino-2-phenylindole.
DBD	DNA-binding domain.
DCs	dendritic cells.
DMEM	Dulbecco's modified Eagle's medium.
DMSO	dimethyl sulphoxide.
dNTP	deoxyribonucleotide triphosphate.
DNA	2' de-oxyribonucleic acid.
dsRBM	double-stranded RNA-binding motif.
dsRNA	double-stranded RNA.

ECL	enhanced chemiluminescence.
EDTA	ethylene diamine tetra-acetic acid.
eGFP	enhanced green fluorescent protein.
EGTA	ethylene glycol tetra-acetic acid.
eIF2 α	eukaryotic translation initiation factor 2 α .
ER	endoplasmic reticulum.
FBS	foetal bovine serum (FCS).
g	gram.
GFP	green fluorescent protein.
HA	influenza A virus haemagglutinin.
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid.
hpi	hours post infection.
HRP	horse-radish peroxidase.
IFN	interferon.
IgG	immunoglobulin G.
IL	interleukin.
IPTG	isopropyl- β -D-thiogalactopyranoside.
IRF3	interferon regulatory factor 3.
kDa	kilodalton.
LUMIER	luminiscence based mammalian interactome assay.
M	molar concentration (moles per litre).
mAb	monoclonal antibody.
MCS	multiple cloning site.
MDCK	Madin-Darby canine kidney.
mg	milli-gram.
MGC	mammalian gene collection.
MHC	major histocompatibility complex.
mins	minutes.

ml	milli-litre.
mM	milli-molar.
MOI	multiplicity of infection.
mRNA	messenger RNA.
miRNA	microRNA
MW	molecular weight.
NA	influenza A virus neuraminidase.
NEP	nuclear export protein.
NLS	nuclear localisation signal.
ng	nano-gram.
nm	nanometre.
NoLS	nucleolar localisation signal.
NP	influenza A virus nucleocapsid protein.
NS1	influenza A virus non-structural protein.
OD	optical density.
ORF	open reading frame.
PAB	poly(A) binding protein (I or II).
pAb	polyclonal antibody.
PACT	protein activator of the interferon-induced protein kinase.
PAMP	pathogen-associated molecular pattern.
PBS	phosphate-buffered saline.
PCR	polymerase chain reaction.
PFU	plaque forming units.
pH	$\log_{10}[\text{H}^+]$.
pi	post-infection.
PI3K	phosphoinositide 3-kinase.
PKR	double-stranded RNA activated protein kinase.
poly I:C	polyribinosinic-polyribocytidylic acid.

PR8	influenza A virus strain A/Puerto Rico/8/34.
RHGP	Random homozygous gene perturbation.
RIG-I	retinoic acid-inducible gene product I.
RISC	RNA-induced silencing complex.
RLU	relative light units.
RNA	ribonucleic acid.
RNAi	RNA interference.
RNP	ribonucleoprotein complex.
RPM	revolutions per minute.
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis.
siRNA	short-interfering RNA.
STAT1	signal transducer and activator of transcription 1.
TLR	Toll-like receptor.
TNF α	tumour necrosis factor α .
TPCK	L-1-tosylamido-2-phenylethylchloromethylketone.
TRBP	TAR (HIV-1) RNA binding protein.
Tris	tris-hydroxymethyl-aminomethane.
UAS	upstream activation sequence.
Ud	influenza A virus strain A/Udorn/72.
UV	ultra-violet.
V	volts.
vRNA	genomic viral RNA.
WSN	influenza A virus strain A/WSN/33.
WT	wild-type.
Y2H	Yeast two-hybrid.

Chapter 1: Introduction

1.1 *Influenza A viruses*

1.1.1 “Flu” and influenza A viruses

Influenza viruses cause annual epidemics and occasional pandemics with serious threats to public health and the global economy. Annual epidemics usually occur during winter and affect 5 – 15% of the human population resulting in 3 – 5 million cases of severe illness (Krammer and Palese, 2015, Knipe and Howley, 2013). Most people recover within 1-2 weeks without medical treatment but infections in the young, elderly, or chronically ill can sometimes result in serious consequences such as pneumonia, hospitalisation, or even death. An estimated 250,000 – 500,000 deaths per year worldwide are attributable to influenza (Krammer and Palese, 2015, Knipe and Howley, 2013).

Occasionally influenza strains cause pandemic outbreaks that can affect 20-40% of the global population in a single year. Four pandemic influenza outbreaks happened in 20th century: “Spanish Flu” in 1918, “Asian Flu” in 1957, “Hong Kong Flu” in 1968, and “Russian Flu” in 1977 (Kawaoka, 2006). The most recent pandemic was caused by “Swine Flu” in 2009 (see Figure 1.1) and resulted in approximately 60.8 million cases of infection worldwide (Webster and Govorkova, 2014). The 1918 “Spanish Flu” pandemic was the most severe in history. It is estimated that one-third of the world’s population was clinically affected, and that more than 40 million people were killed (Kawaoka, 2006).

In humans, influenza viruses infect the epithelial cells of the upper respiratory tract and cause acute and highly contagious respiratory disease. Typical symptoms include sudden onset of fever, headache, malaise, nasal congestion, sore throat and body aches. In addition, the highly pathogenic avian influenza A viruses can cross

the host species barriers and infect the lower respiratory tract, causing viral pneumonia and acute respiratory distress syndrome. A proinflammatory cytokine storm may result in multiple organ failure (Knipe and Howley, 2013, Kawaoka, 2006). In 2013, a novel avian influenza A H7N9 strain was identified in China which caused progressive pneumonia, respiratory failure, and acute respiratory distress syndrome, and had a fatality rate of 30% (Li *et al.*, 2014, Gao *et al.*, 2013).

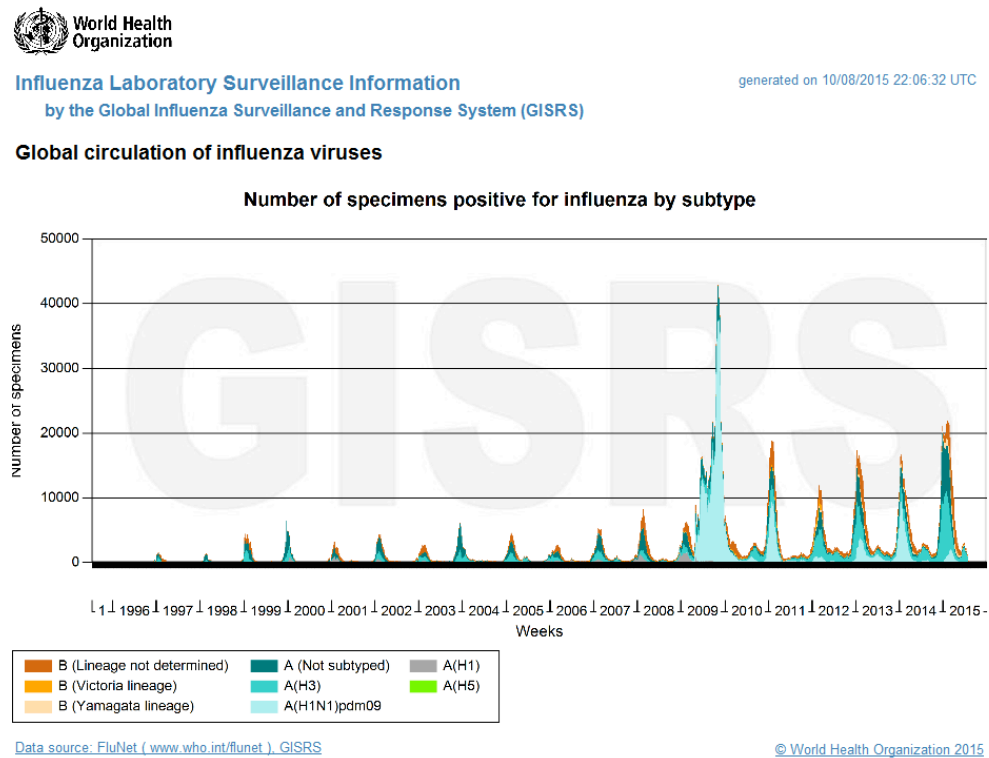


Figure 1.1 Number of specimens tested positive for influenza viruses by subtypes from 1995-2015. WHO GISRS laboratories tested specimens for influenza during the time from 1995 to 2005. Notably, the 2009 pandemic saw a significant increase in infection cases by “Swine Flu”. Figure was generated by WHO GISRS.

The actual agent that causes influenza is the influenza A virus and was first isolated from humans in 1933 by Wilson Smith, Sir Christopher Andrewes, and Sir Patrick Laidlaw at the National Institute for Medical Research, London, UK (Knipe and Howley, 2013). The nomenclature for individual influenza A virus strains usually includes reference to the host of origin except in humans, the geographic location of

first isolation, the strain number, and the year of isolation. In addition, subtype with antigenic description of the two viral surface glycoproteins hemagglutinin (HA) and neuraminidase (NA) is given as well (Kawaoka, 2006). For example, A/Chicken/Beijing/1/94 (H9N2) is a type A influenza virus strain first isolated from a chicken in Beijing in 1992. Of the 16 antigenically distinct HA subtypes and 9 antigenically distinct NA subtypes, this strain has H9 and N2. Similarly, A/Shanghai/1/2013 (H7N9) which lacks description of host of origin indicates that this H7N9 strain was isolated from a human in Shanghai in 2013.

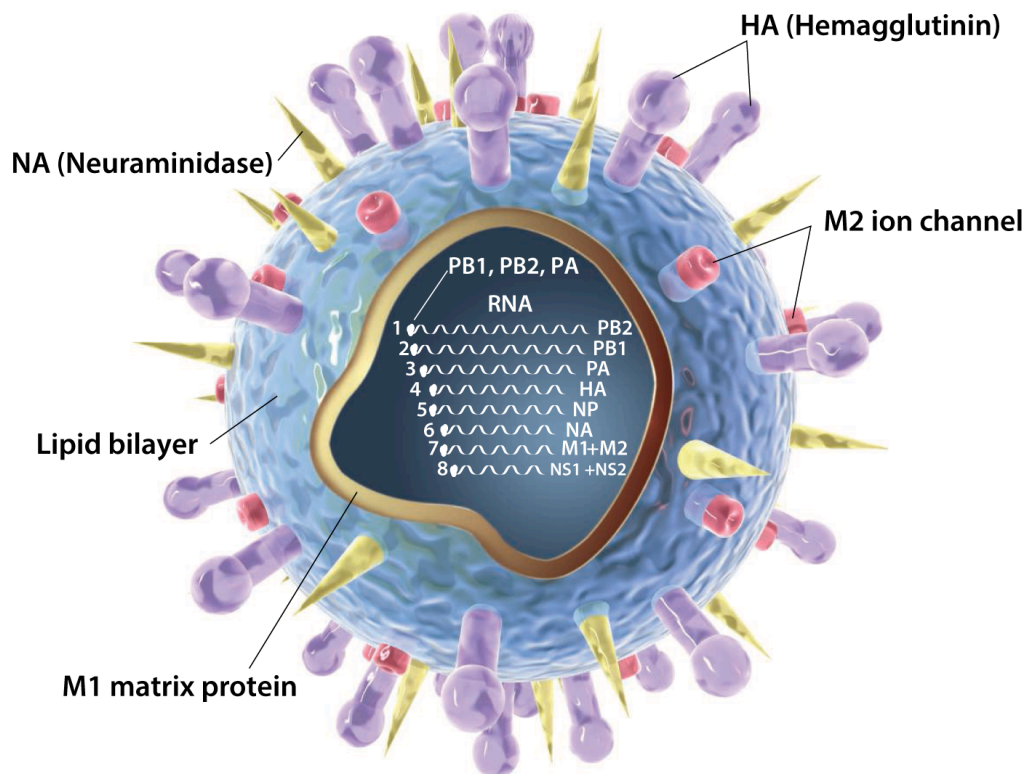


Figure 1.2 A schematic representation of the influenza A virus particle. The virions are enveloped by a lipid membrane. HA, NA, and the M2 ion channel are embedded in the envelope. A layer of M1 matrix protein is located beneath the envelope. The viral genome, in the form of vRNP, has eight segments of vRNA, coated with NP. The viral polymerase complex including PB1, PB2, and PA, is associated with each RNP. Small amounts of NEP encoded by segment 8, are found in purified virions. Figure is adapted from review by Kaiser (Kaiser, 2006).

1.1.2 Virion structure

Influenza A viruses belong to the *Orthomyxoviridae* family, which also includes the influenza B virus, influenza C virus, and thogotovirus genera. The *Orthomyxoviridae* are enveloped viruses containing a segmented, single-stranded, negative-sense RNA genome. Influenza A viruses can be distinguished from influenza B and C viruses by antigenic differences, morphological features, protein-encoding mechanisms, number of RNA segments, and sequence variability in their surface glycoproteins. In addition, influenza A viruses naturally infect a variety of animal species, including birds, humans, pigs, horses, seals, cats, and dogs, whilst influenza B and C viruses predominantly infect humans (Knipe and Howley, 2013).

Influenza A virus particles (virions) have a typical diameter of 80-160 nm, and are pleomorphic in shape; a characteristic dependent upon the viral strain, as well as the cell-type used for propagation (Knipe and Howley, 2013). Virions consist of a lipid envelope (derived from the host plasma membrane) out of which protrude two surface glycoproteins: haemagglutinin (HA) and neuraminidase (NA). Embedded within the lipid envelope bi-layer is the viral integral membrane protein (M2) whilst the viral matrix protein (M1) lies underneath the envelope and structurally supports the particle. Contained within this matrix shell is the segmented viral RNA genome which, for influenza A virus, consists of eight strands. Each strand is wrapped-up in multiple copies of the nucleocapsid protein (NP), and is associated with three proteins responsible for transcription and replication: PB1, PB2, and PA (the viral complex). Each NP-encapsidated RNA segment, together with its heterotrimeric polymerase, constitutes a viral ribonucleoprotein complex (RNP). A schematic representation of the influenza A virus particle, together with its RNA segments and encoded proteins, is shown in Figure 1.2.

Although the influenza A virus genome consists of only 8 single-stranded, negative-sense RNA segments, at least 11 viral proteins are produced during infection:

segment 1, PB2; segment 2, PB1 and PB1-F2; segment 3, PA; segment 4, HA; segment 5, NP; segment 6, NA; segment 7, M1 and M2; segment 8, NS1 and NEP (Knipe and Howley, 2013, Shi *et al.*, 2012). Some other accessory proteins are also produced. For example, segment 3 contains a second open reading frame ('X-ORF'). Ribosomal frameshift allows an alternative product PA-X (Jagger *et al.*, 2012, Shi *et al.*, 2012). The varied roles of these proteins are briefly outlined below in the context of the virus replication cycle.

1.2 Replication cycle

1.2.1 Virus entry

Virus particles bind to the apical surfaces of polarised epithelial cells via an interaction between the viral HA spike glycoprotein and sialic acid residues linked to the cell membrane (Kawaoka, 2006). Receptor-mediated endocytosis causes internalisation of the whole virus particle into intracellular, pre-lysosomal, low pH vesicles. Such internalisation at the cell periphery requires host GTPase dynamin, and involves actin rearrangements (Lakadamyali *et al.*, 2003, Roy *et al.*, 2000). The virus particle is then rapidly trafficked through both early and late endosomes in a dynein-dependent manner to the perinuclear region. A number of cellular kinases have also been implicated in this process, including protein kinase C, and phosphoinositide 3-kinase (Ehrhardt *et al.*, 2006, Sieczkarski *et al.*, 2003).

In order to release the viral genomic segments into the host-cell cytoplasm, the viral membrane and that of the late-endosome must first fuse. Fusion is mediated by a low pH induced conformational change in the viral HA protein, which usually requires an optimum pH of ~5.0-5.5, but can be temperature dependent (Knipe and Howley, 2013, Kawaoka, 2006). Mature endosomal compartments have an acidic pH of between 4.5 and 5.5, therefore localisation of influenza A virus particles into these specific vesicles is the physiological trigger for membrane fusion, and acts to direct

virion release to the perinuclear region. This sorting event alone is insufficient to deliver the individual viral genomic segments into the cytoplasm. However, the low pH activated ion channel activity of the viral M2 protein permits the flow of protons from the lumen of the endosomal compartment into the virion, acidifying the interior of the virion and consequently disrupting protein-protein interactions between both viral RNPs and M1, as well as HA and M1 (Kawaoka, 2006, Pinto *et al.*, 1992, Knipe and Howley, 2013). Dissociation of these interactions allows full egress of viral RNPs from the disassembled virion into the cytoplasm subsequent to membrane fusion. Regulation of virus uncoating/RNP release by ensuring prior translocation to low-pH endosomes near the perinuclear region efficiently targets RNPs to the site of transcription and replication. This mechanism also potentially minimises the contact time of “naked” RNPs with the cytoplasm, which may be beneficial to the virus for evading some host-cell viral sensors.

1.2.2 RNP nuclear import

Unlike most RNA viruses, transcription and replication of the influenza A virus genome occurs in the nucleus of the infected cell. This could be due to the requirements for viral transcription. For example initiation of viral mRNA synthesis requires the capped 5' ends of host mRNAs to act as primers, and two viral mRNAs require splicing. Thus, it has been suggested that localising within the nucleus may allow close contact between viral replication centres and high concentrations of essential host factors, such as cellular mRNAs whose export is blocked by the viral NS1 protein, and the cellular splicing machinery (Satterly *et al.*, 2007). Negative-sense, RNA viruses in the *Bunyaviridae* and *Arenaviridae* families also require host-derived 5' cap structures to initiate mRNA transcription, but these viruses replicate in the cytoplasm. Since mRNA splicing is a common protein-encoding mechanism among all members of the *Orthomyxoviridae* family, nuclear localisation of their replication could be due to the requirement for splicing (Knipe and Howley, 2013, Kawaoka, 2006). After uncoating, all viral RNPs are actively transported into the

nucleus. NP and the three polymerase subunits all contain nuclear localisation signals (NLSs). NP has multiple NLSs, and is both necessary and sufficient for mediating the nuclear import of each viral RNP segment, probably via its interaction with the cellular importin family. Within the nucleus, RNPs strongly associate with insoluble “nuclear matrix” or chromatin components (Hutchinson and Fodor, 2012, Hutchinson and Fodor, 2013).

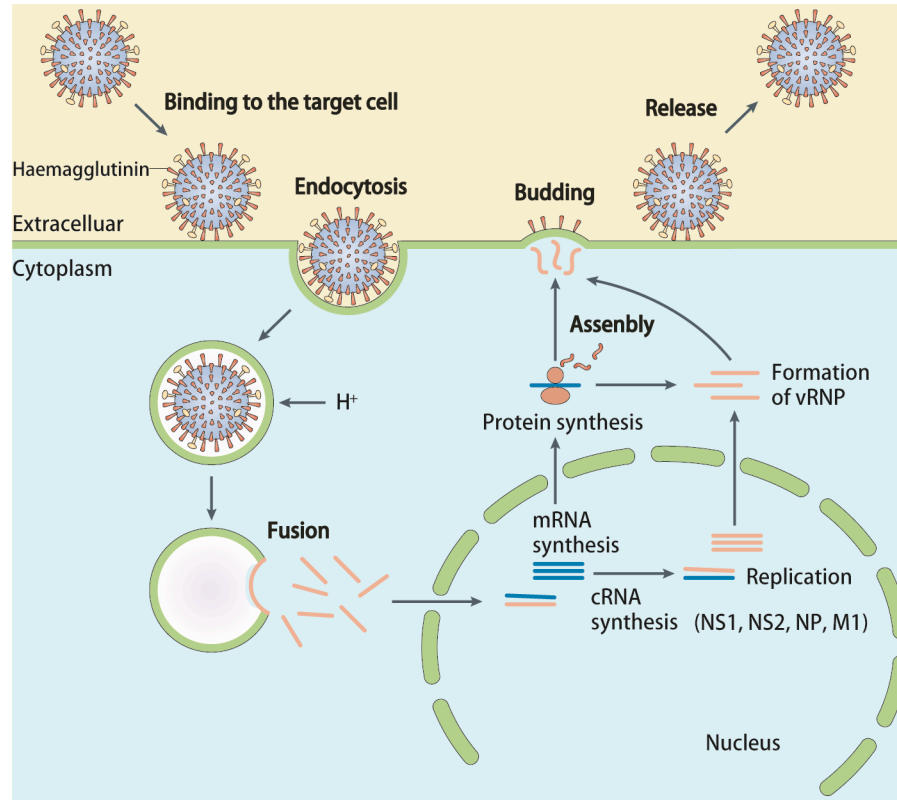


Figure 1.3 Life cycle of the influenza virus. The life cycle of Influenza virus can be divided into 5 steps: 1) entry into the host cell; 2) vRNPs entry into the nucleus; 3) viral genome transcription and replication; 4) vRNPs export from the nucleus; 5) virion assembly and budding. The diagram is adapted from review by von Itzstein. (von Itzstein, 2007).

1.2.3 Viral mRNA synthesis

Influenza A virus RNA synthesis requires NP, PA, PB1, and PB2, which make up the functional RNP together with viral RNA (Portela and Digard, 2002). Genomic viral RNA (vRNA) has conserved sequences at the 5' and 3' termini that share some sequence complementarity, and thus cause base-pairing leading to the proposed

formation of RNA structures. Each vRNA is encapsidated by NP, and is associated with a single heterotrimeric polymerase complex. The incoming RNP is sufficient to transcribe mRNA, as long as an appropriate 5' host-derived capped RNA is available as a primer (Kawaoka, 2006, Fodor, 2013, Neumann *et al.*, 2004).

During mRNA synthesis, the polymerase binds to the 5' end of the vRNA segment and thereby stimulates the cap-binding activity of PB2 (Cianci *et al.*, 1995, Hagen *et al.*, 1994, Li *et al.*, 1998a). Interaction of the 3' end of the vRNA template with the viral polymerase triggers cleavage of the cellular mRNA by PB2, 5'-methylated host cap structure together with 10-15 cellular mRNA nucleotides are cleaved. Interestingly, viral capped mRNAs are protected from cleavage when they interact with the viral polymerase (Shih and Krug, 1996). The cleaved capped RNA or dinucleotides for example ApG are employed by the virus as primers for transcription initiation by PB1, which is enhanced by the physical interaction of PB2 with the cap structure (Hagen *et al.*, 1995). After elongation according to the template vRNA, PB2 dissociates from the cap. Synthesis of viral mRNA terminates at 5-7 uridine residues about 17 nucleotides away from the 5' end of the vRNA, a poly(A) tail is then added to the viral mRNA (Robertson *et al.*, 1981, Poon *et al.*, 1998). This polyadenylation could be caused by viral RNA polymerase reiteratively coping the poly(U) at the 5' end of vRNA (Poon *et al.*, 1999).

1.2.4 Genome replication and viral protein expression

The replication of genomic vRNA can be divided into two stages: the first stage of initial synthesis of "template" RNA that is complementary to full-length vRNA and termed as cRNA, and the second stage of copying of cRNA into new vRNAs (Hay *et al.*, 1982, Kawaoka, 2006). Although viral mRNAs and cRNAs are both positive-sense, mRNA cannot act as a replicative intermediate in the formation of new vRNAs due to its 5' host-derived cap. Thus, the viral polymerase initiates cRNA synthesis in a cap-independent manner, which requires polyadenylation signal at the 5' end of vRNA (Resa-Infante *et al.*, 2010). NP encapsidates viral genome as an

RNP particle for the purposes of transcription and packaging. Different from mRNA transcription, the incoming viral RNP alone is insufficient to stimulate cRNA synthesis, and it has been shown that an initial round of mRNA and protein synthesis is required prior to initiation of cRNA synthesis (Beaton and Krug, 1984, del Rio *et al.*, 1985, Nagata *et al.*, 1989, Kawaoka, 2006). It is not clear what viral components constitute the replication polymerase complex. It has been shown that three polymerase subunits and NP are required for production of progeny RNPs, although without NP short recombinant RNA templates can be replicated *in vitro*, presence of NP improves the efficiency of replication and is vital for elongation of long templates. Roles of NP include regulation of transcription template, or direct regulation of interactions between polymerase subunits to favour a replication polymerase or a transcription polymerase (Turrell *et al.*, 2013). Cellular factors are also thought to play crucial roles in the regulation of transcription replication, which include hCLE, HSP90 and UAP56 (Shimizu *et al.*, 1994, Kawaoka, 2006, Nagata *et al.*, 2008). NS1 also directly interacts with the viral replication polymerase complex and regulates accumulation of vRNA and translation, which is discussed in 1.4.4.

Synthesis of viral proteins generally depends on the amount of viral mRNA transcribed in the infected cells (Shapiro *et al.*, 1987, Knipe and Howley, 2013). Immediate upon infection, all eight viral mRNAs are synthesized simultaneously in equal amounts. However, in subsequent transcription phase, NS1 and NP mRNA are preferentially synthesized, which results in predominant accumulations of NS1 and NP proteins (Hay *et al.*, 1977, Smith and Hay, 1982). The reason could be due to the essential role of NP in replication and transcription of vRNA and the role of NS1 in evasion of host defense and regulation of viral and cellular gene expression. At the late stage, production of NS1 reduces and again vRNAs are synthesized in equal amounts for progeny viral genome. In addition, structural proteins like HA, NA, and M1 are preferentially produced (Hay *et al.*, 1977, Mikulasova *et al.*, 2000, Shapiro *et al.*, 1987). Protein synthesis occurs in cytoplasm which requires transportation of capped and polyadenylated viral mRNAs from nucleus to cytoplasm. Upon

synthesis, membrane proteins HA, NA are transported into secretory pathway for protein post-translational modification and further to the cell surface for membrane integration.

1.2.5 Virion assembly and budding

Influenza NEP (nuclear export protein) is a 14 kDa protein synthesised by segment 8 and found in both virions and virus-infected cells, which plays an essential role in exporting newly synthesised vRNP from nucleus to cytoplasm (Kawaoka, 2006, Knipe and Howley, 2013, O'Neill *et al.*, 1998). NEP binds directly to the viral M1 protein, which is also able to form complexes with cellular proteins like nucleoporins, hCRM1 and Ran-GTP. At the late stage of virus life cycle, by binding to vRNPs, nuclear fraction of M1 is able to inhibit viral transcription. The interaction between M1 and NEP mediates vRNP nuclear export by recruiting cellular nucleocytoplasmic transport machinery (Neumann *et al.*, 2000, Boulo *et al.*, 2007, Akarsu *et al.*, 2003). NP also plays a role in regulating vRNP nuclear export by interacting with hCRM1 (Akarsu *et al.*, 2003, Elton *et al.*, 2001). Other cellular factors involved in vRNP nuclear export include mitogen-activated protein kinase and caspase cascades (Wurzer *et al.*, 2003, Pleschka *et al.*, 2001). Once exported to cytoplasm, by interacting with membrane-associated M1 or by cytoskeletal networks, newly synthesised RNPs are transported to cell surface (Digard *et al.*, 1999, Kawaoka, 2006).

Since assembly and budding of influenza virus occur at cellular plasma membrane, virions do not exist inside infected cells. All viral components are required to be transported to specific site of plasma membrane in polarized epithelial cells, which is the apical domain of the plasma membrane. Such polarity is due to preferential accumulation of HA, NA, and M2 at the virus assembly site (Hughey *et al.*, 1992, Kundu *et al.*, 1996, Kawaoka, 2006). It has been shown that HA and NA are specifically sorted to lipid raft domains at apical plasma membrane enriched with sphingolipid and cholesterol (Nayak and Barman, 2002). These lipid raft domains

provide space for initiation of signal transductions required for assembly and budding. Furthermore, accumulation of HA and NA in these spaces result in high concentrations of glycoproteins for newly budding viruses, which increase the infectivity of virus progeny (Takeda *et al.*, 2003, Scheiffele *et al.*, 1999). Once HA and NA are clustered in lipid raft domains, M1 binds to the cytoplasmic tails of HA and NA and serves as a docking site for the vRNPs. Polymerization of the M1 subsequently leads to elongation of the budding virion, which results in a polarized localization of the vRNPs within the virion. By interacting with M1, M2 is then recruited to the budding sites and stabilizes budding sites prior to finalization of assembly (Nayak *et al.*, 2004, Rossman and Lamb, 2011). Notably, incorporation of all 8 vRNPs during assembly is required for formation of infectious virus particles. It has been reported vRNP segments are selectively incorporated into each budding virion, since vRNAs are equimolar within viral particles even though their concentrations in infected cells may differ (Smith and Hay, 1982). The mechanism of vRNP selection is not well understood, random packaging has also been suggested (Bancroft and Parslow, 2002). After M2 is localized to the neck of the budding virion, insertion of the M2 amphipathic helix at the lipid phase boundary alters membrane curvature at the neck of the budding virion and leads to virus release (Nayak *et al.*, 2004, Rossman and Lamb, 2011). During budding, catalysis activity of NA removes sialic acid from the surface of infected cells and also from HA and NA molecules, which allows efficient virus egress, and prevents self-aggregation of virus progeny (Palese *et al.*, 1974, Knipe and Howley, 2013).

Among influenza viral proteins, NS1 and PB1-F2 are expressed but not packaged into virion progeny. NS1 is discussed in section 1.4. PB1-F2 is 87 aa long and encoded by an alternate reading frame within segment 2. Expressed at peak 5 hpi, PB1-F2 localizes to mitochondrial membranes (Chen *et al.*, 2001, Yamada *et al.*, 2004, Gibbs *et al.*, 2003). As PB1-F2 interacts with the mitochondrial apoptotic factors ANT3 and VDAC1, it induces cell death by apoptosis. Besides apoptosis, PB1-F2 also induce formation of non-specific pores within membranes. However, the PB1-

F2 ORF is not conserved in all influenza A virus genomes, for example, 2009 pandemic strain does not possess PB1-F2. Knockout of PB1-F2 does not significantly attenuate virus, but considering its pro-apoptosis role, PB1-F2 could contribute to viral pathogenesis by limiting virus clearance mediated immune Cells (Zamarin *et al.*, 2005, Zamarin *et al.*, 2006, Schnitzler and Schnitzler, 2009).

1.3 The yeast two-hybrid system for systematic analysis of protein-protein interactions between influenza virus and host cellular proteins

The yeast two-hybrid (Y2H) system is a well-established technique for the detection of protein-protein interactions. Since it was first described using the GAL4 transcription factor for yeast *S. cerevisiae*, it has proved to be effective in identifying many interactions in biological systems (Fields and Song, 1989). The system relies on two domains of transcription factor GAL4; a DNA-binding domain (DBD) and an activation domain (AD). In order to identify interactions between two proteins using the Y2H system, two hybrid proteins are generated. The DBD of GAL4 is fused to one protein of interest, known as the bait, and the AD is fused to the other protein of interest, known as the prey. The bait and prey are co-expressed in the yeast and if the two proteins of interest interact then the DBD and AD are brought together by the interaction and are able to initiate the transcription of reporter genes under the regulation of an up-stream activation sequence (Fields and Song, 1989). In this system, the reporter gene is activated by the interaction to produce histidine which allows the yeast to grow in medium lacking histidine. Another reporter gene, MEL1, encodes α -galactosidase, which causes the breakdown of X- α -gal and can be used for semi-quantitative analysis of interactions.

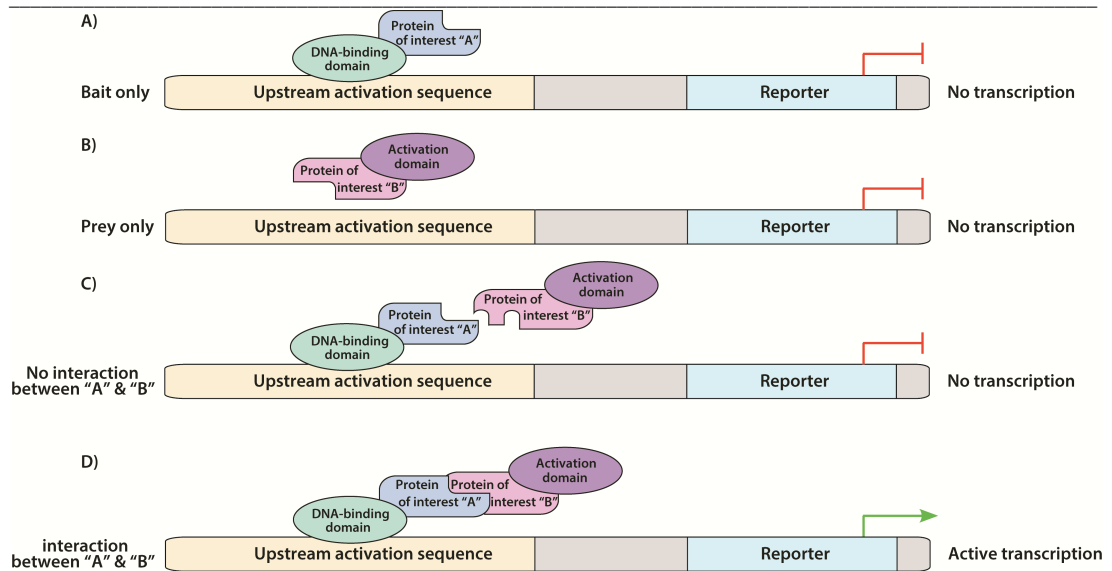


Figure 1.4 Schematic representation of Y2H system. The figure illustrates the mechanism of Y2H system. To detect an interaction between proteins of interest “A” and “B”, “A” and “B” are either fused with DNA-binding domain (DBD) of GAL4 (bait) or with activation domain (AD) of GAL4 (prey). The DBD binds to upstream activation sequence (UAS) of GAL4, while AD activates transcription of GAL4. When fusion proteins are expressed in the yeast, transcription of the reporter gene depends on the two domains being in close proximity. Bait or prey alone or no interaction between the two proteins of interest as shown in A, B and C does not activate reporter. The DBD and AD are brought together only when “A” interacts with “B”, allowing initiation of reporter transcription. Figure is adapted and redrawn from Fields *et al* (Fields and Song, 1989).

Y2H can be used either to detect direct interactions between two proteins of interest or to screen a bait protein with a library of prey proteins. Direct interactions provide a quick and easy method to detect and verify interactions between two proteins. One of the applications of direct interactions is the ability to map the interaction binding sites of the proteins using deletion mutants and domains of proteins. Also of importance is that semi-quantitative measurements can be made to calculate binding affinities of interactions (Bruckner *et al.*, 2009). Library screens are able to identify new interaction partners of a protein in a large-scale. They can be performed in a high-throughput manner and produce enormous interactome data. The prey library can consist of full-length cDNAs or domains of proteins. It has been reported that individual domains yield better results in Y2H screen which could be

due to protein domains being able to fold independently of each other (Van Crielinge and Beyaert, 1999).

Given that viruses rely on cellular machinery to replicate, a genome-scale yeast two-hybrid protein interaction screen was performed using 6 different influenza A virus strains to gain insight into the complex interplay between cellular proteins and influenza A viral proteins. The details of the screen and the analysis are presented in Chapter 3. A further investigation into two interactions of interest is discussed in Chapters 4-6.

1.4 Influenza non-structural protein 1 (NS1)

The genome-scale yeast two-hybrid protein interaction screen using 6 different influenza A virus strains identified interactions between NS1, TRBP and PACT. NS1 protein is widely regarded as a multifunctional virulence factor, and since TRBP and PACT are heavily involved in distinctive host cellular functions, it is of great interest to investigate the role of these two interactions. TRBP and PACT are introduced in detail in section 1.5. NS1 is expressed at high levels in virus-infected cells, but is not a structural component of the virion. During infection, NS1 performs a range of activities that each contribute to efficient virus replication, including temporal regulation of viral RNA synthesis, enhancement of viral mRNA translation, regulation of virus particle morphogenesis, and suppression of the host immune and apoptotic responses (Hale *et al.*, 2008, Knipe and Howley, 2013, Ayllon and Garcia-Sastre, 2015).

1.4.1 Synthesis of the NS1 protein

Transcription of segment 8 of influenza A virus, the smallest genomic segment, produces two separate mRNAs, which encode two viral proteins NS1 and NEP. While NS1 is translated from the colinear transcript, spliced mRNA encodes NEP

(Lamb and Choppin, 1979, Knipe and Howley, 2013, Lamb and Lai, 1980). Both transcripts share a 56nt leader sequence, which contains the AUG initiation codon and encodes 9 N-terminal amino acid residues in common. NEP mRNA contains a intron of 473nt which is removed before translation of NEP mRNA continues in the +1 ORF after the splicing site. The ORFs of NS1 and NEP overlap by the equivalent of 70 residues. When infection is established in a steady state, amount of spliced NEP mRNA is approximately 10% that of colinear NS1 mRNA (Lamb and Lai, 1980). In addition, splicing of NEP mRNA is regulated by NS1 itself (Garaigorta and Ortin, 2007).

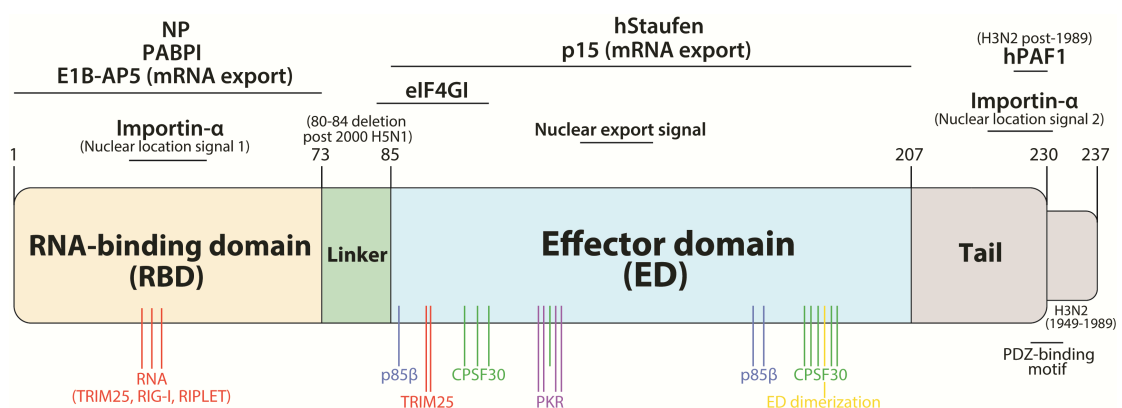


Figure 1.5 Domains of the NS1 protein. A Schematic representation of the primary structure of an NS1 monomer highlights its most representative features and the regions known to be required for its multiple interactions. The figure is modified from review by Ayllon and García-Sastre (Ayllon and Garcia-Sastre, 2015).

1.4.2 Biochemistry of NS1 protein

Molecular weight of NS1 protein is approximately 26 kDa, and the length ranges from 230aa to 237aa (Knipe and Howley, 2013). In infected cells, some NS1 proteins are phosphorylated. Considerable differences in charge and phosphorylation of NS1 between strains can be observed. While NS1 proteins from avian strains are predominantly acidic, NS1 proteins derived from human generally appear basic. Differences in sequence mainly contribute to this, however notably, homogeneity in charge among other viral proteins has been reported. Phosphorylation of NS1 also

highly depends on the strain, for example in tissue culture studies, phosphorylation was not observed among some NS1 proteins from avian strains (Petri and Dimmock, 1981, Petri *et al.*, 1982). Phosphorylation of NS1 occurs in the nucleus rapidly after translation. This is possibly due to NS1 can be phosphorylated by a kinase activity associated with purified vRNPs in vitro, and vRNP is usually located in the nucleus (Skorko *et al.*, 1991, Privalsky and Penhoet, 1981). Those NS1 proteins that can be phosphorylated could contain different distinct sites of modification, for example, serine-42, 48 and threonine-215. Phosphorylation of NS1 has been linked to RNA binding activity of NS1, maintaining conformational structure and efficient virus replication (Hale *et al.*, 2009, Hsiang *et al.*, 2012).

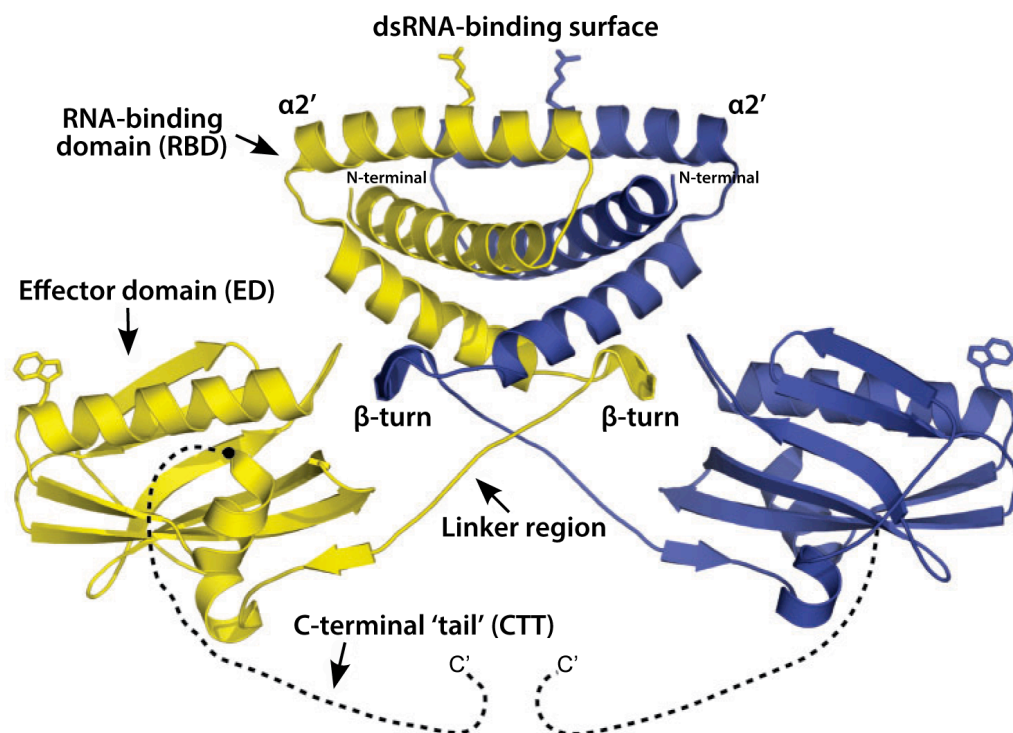


Figure 1.6 Crystal structure of a full-length dimeric NS1 protein. The two monomers of NS1 in yellow and blue respectively form a dimer. The N and C termini of each monomer and the four regions are labelled. As the C-terminal tails have not been observed in crystal structures, they are represented with dash lines. The figure is adapted from review by Hale (Hale, 2014), which was generated from ID 4OPH of Protein Data Bank (Carrillo *et al.*, 2014).

The NS1 protein comprises two functional domains, an N-terminal RNA-binding domain (residues 1-73) which binds to dsRNA in a sequence-independent manner, and a C-terminal effector domain (residues 74-230/237) which binds to a number of

cellular proteins as shown in figure 1.5 (Ayllon and Garcia-Sastre, 2015). In between RBD and ED, there is a linker, as well as a C-terminal tail after ED. NS1 exists as a homodimer, and both RBD and ED contribute to functional multimerisation. As shown in figure 1.6, the RBDs can form symmetrical homodimer, and each monomer consists of three α -helices. Dimerisation is essential for binding dsRNA, and the relative quantity of RBD dimer and dsRNA is 1:1. The C-terminal effector domain of NS1 is also a homodimer, and each monomer consists of seven β -strands and three α -helices (Carrillo *et al.*, 2014, Hale, 2014, Chien *et al.*, 2004, Bornholdt and Prasad, 2006, Lohrmann *et al.*, 2013). Interactions of this domain with cellular proteins are further discussed below.

1.4.3 Intracellular localization of NS1

Depending on several factors, which include virus strain, expression level of NS1, cell-type used for infection, cell polarity, time post-infection, as well as experimental procedure used for detection, nevertheless, intracellular localization of NS1 proteins in infected tissue-culture cells are predominantly in the nucleus, especially in the early time post infection. However, a significant proportion can also be found in the cytoplasm (Newby *et al.*, 2007, Li *et al.*, 1998a, Ayllon and Garcia-Sastre, 2015). NS1 proteins contain one or two nuclear localisation sequences (NLS) which mediate their active nuclear import by binding to the cellular protein karyopherin importin α (Greenspan *et al.*, 1988, Melen *et al.*, 2007). A monopartite NLS signal (NLS1) locates in N-terminus amino acid residues 35–41 of NS1, which overlaps with critical residues for dsRNA binding activity such as arginine-38 and lysine-41. NLS1 is well-conserved among NS1 proteins from all known influenza A virus (Melen *et al.*, 2007). In addition, a second bipartite NLS (NLS2) locates between residues 219–232 in C-terminal tail of NS1 proteins from H3N2 strain, which gained an elongated tail between 1950 and 1989 (Ayllon and Garcia-Sastre, 2015). NLS2 also encompasses a functional nucleolar location signal (NoLS), which requires residues 224 and 229, although NLS2 and NoLS are absent from the majority of influenza A

virus strains, it has been suggested that they play a role in the pathogenesis, and their function remain to be determined (Melen *et al.*, 2012).

NS1 also contains a latent nuclear export signal (NES) within residues 138-147 in NS1 which relocates NS1 from nuclear to cytoplasm (Li *et al.*, 1998b). The NES in the effector domain requires leucines at positions 144 and 146, however it is functionally masked by spatially adjacent residues 148-161. Relocation of NS1 back into the cytoplasm requires an unmasking process, possibly by interaction of residues 148-161 in NS1 with another viral protein. Thus potentially this could be regulated temporally by the relative expression levels of other viral proteins. On the other hand, interactions between newly synthesised NS1 and cellular or viral protein may mask the NLS and retain NS1 in the cytoplasm (Li *et al.*, 1998b). As some function of NS1 is required in the nucleus, for example, CPSF30 binding, while others should take place in the cytoplasm, for example, RIG-I signalling and PKR inhibition and PI3K activation, host factors clearly play an important role in determining the sub-cellular localisation of NS1 (Li *et al.*, 1998b, Ayllon and Garcia-Sastre, 2015).

1.4.4 NS1 in vRNA synthesis and viral mRNA translation

Influenza A virus gene expression can be divided into early and late stages. Immediately after nuclear import, all the viral segments appear to be equally transcribed into mRNA (Shapiro *et al.*, 1987, Knipe and Howley, 2013). However, segments 5 and 8 become preferentially synthesised, which leads to an increase in transcription of NS1 and NP mRNAs and high expression levels of NS1 and NP proteins. In contrast, at late stage of infection all the eight genomic segments show efficient replication and a high level of transcription and translation (Shapiro *et al.*, 1987, Hay *et al.*, 1977, Skehel, 1973).

NS1 has been reported to regulate temporal synthesis of both viral mRNAs and vRNAs. Alanine substitutions of highly conserved NS1 isoleucine-123 and

methionine-124 residues of the Ud strain result in a temporal deregulation of vRNA synthesis, with late genes being synthesised earlier and in greater amount, particularly both HA and M1 (Min *et al.*, 2007). Although residues 123/124 are required for binding and inhibition of PKR, this was unrelated to any activation of PKR, a cellular protein bound by NS1 that can regulate translation (further discussed in Section 1.4.5.2). The mechanism by which NS1 regulates viral vRNA, mRNA, and protein synthesis is not clear, however it has been linked to interaction of NS1 with the vRNP by direct binding to NP (Robb *et al.*, 2011). It has also been reported that mRNA levels of HA is impaired in PR8 virus that lacks NS1 ED domain, suggesting role of NS1 in regulating viral RNA synthesis (Maamary *et al.*, 2012). Further evidence suggests that NS1 inhibits cellular helicase DDX21, which interacts with PB1 and NS1/CPSF30, to block formation of viral polymerase complex (Chen *et al.*, 2014).

NS1 is an RNA-binding protein that is known to bind to various RNA species, including dsRNA, vRNA, viral mRNA, mRNA containing Poly(A), and snRNA (Qiu *et al.*, 1995, Qiu and Krug, 1994, Wang *et al.*, 1999, Hatada *et al.*, 1997, Marion *et al.*, 1997b). It has been reported that NS1 is able to antagonise host antiviral response by interacting with various RNA species (Kochs *et al.*, 2007b, Min *et al.*, 2007, Nemeroff *et al.*, 1998, Pichlmair *et al.*, 2006). Furthermore, the interaction between NS1 and the 5'UTR of viral mRNAs has been linked to role of NS1 in enhancing the translation initiation of viral mRNAs (Garfinkel and Katze, 1993, Park and Katze, 1995, de la Luna *et al.*, 1995). This also may involve formation of NS1 with RNA-protein complex which contains eukaryotic translation initiation factor eIF4GI, poly(A)-binding protein I (PABI), and viral mRNAs. Studies have shown that interactions of NS1 with PABI and eIF4GI require the N-terminal 81 and 113 residues of NS1 respectively, those mutant NS1 proteins unable to bind eIF4GI are incapable of enhancing viral mRNA translation (Aragon *et al.*, 2000, Burgui *et al.*, 2007, Marion *et al.*, 1997a). In addition, NS1 also interacts with hStaufen, a PKR related protein that binds to dsRNA and tubulin (Wickham *et al.*, 1999, Falcon *et al.*,

1999). This interaction is not mediated by RNA, but involves ED domain of NS1 and the dsRNA binding domains of hStaufen. As a cytoplasmic protein, hStaufen can be relocalised to the nucleus by binding with NS1. It has been suggested that the interaction between NS1 and hStaufen enhances viral protein synthesis from since hStaufen plays a role in microtubular transport of cellular mRNAs to cytoplasmic polysomes, where same mRNA is translated by multiple ribosomes (Wickham *et al.*, 1999, Falcon *et al.*, 1999, Marion *et al.*, 1999).

1.4.5 NS1 in host immune response

Viruses face great challenges from innate immunity immediately after infecting a host. Sophisticated antiviral mechanisms allow host cells to sense foreign pathogen products, and kick off signalling pathways that leads to production of cytokines including type I (IFN α and IFN β) and type III (IFN λ) interferons (IFNs) (Garcia-Sastre, 2011, McNab *et al.*, 2015). Secreted IFNs act in both an autocrine and paracrine fashion to establish antiviral state by upregulating over 300 interferon-stimulated genes (ISG) (Schneider *et al.*, 2014). Moreover, besides role in innate immunity, IFN system is also associated with adaptive immune response (Iwasaki and Medzhitov, 2010, Schmolke and Garcia-Sastre, 2010). Thus, influenza A virus has evolved various tactics to antagonize IFN system for a successful infection (Tripathi *et al.*, 2015).

It been revealed that the NS1 protein is a major contributor to antagonise innate immune response by repressing type I/III interferon system during infection (Krug, 2015). Studies have shown that influenza A virus lacking of NS1 replicates to much lower titres than wild type both in vitro and in vivo, and causes high transactivation of ISGs (Garcia-Sastre *et al.*, 1998). Although influenza A virus encodes several other factors such as PB1-F2 to antagonise innate immune response, PB1-F2 is not as effective as NS1, which is due to a much more important impact by NS1 on IFN pathway at multiple levels (Vasin *et al.*, 2014). However, more evidences have

demonstrated that the mechanisms by which NS1 achieves anti-IFN or other functions are not well-conserved across different strains. Details that how NS1 limits both the production and downstream effects of IFN are discussed below.

1.4.5.1 Limitation of interferon production by NS1

Recognition of molecules termed pathogen-associated molecular patterns (PAMPs) triggers interferon signalling cascade. PAMPs are characterized by being conserved among entire classes of pathogens, essential for the survival of the pathogen, and distinguishable from “self”. Presence of PAMPs alerts cell of an invading “non-self” agent (Schmidt *et al.*, 2012). PAMPs are recognized by pattern recognition receptors (PRRs). These PRRs can be classified into different families according to their subcellular location, the PAMPs they recognize and the origin of those PAMPs (Takeuchi and Akira, 2009). The PRR responsible for detecting influenza A virus is the cytosolic RNA helicase RIG-I (retinoic acid-inducible gene I), which can sensor viral RNA upon the infection that is not found in cytoplasmic cellular RNAs (Yoneyama *et al.*, 2004, Pichlmair *et al.*, 2006). Binding of RIG-I to viral dsRNA leads to an ATP-dependent conformational change on RIG-I. As a result of conformational change, two N-terminal caspase recruiting domains (CARD) of RIG-I are exposed, and different residues of RIG-I are ubiquitinated by the E3 ligases TRIM25 and RIPLET (Myong *et al.*, 2009, Gack *et al.*, 2007, Oshiumi *et al.*, 2009, Zeng *et al.*, 2010). Ubiquitinated RIG-Is are further oligomerized. Modified CARDS of RIG-I can then bind to the CARDS of the mitochondrial adaptor MAVS (mitochondrial antiviral signalling), which leads to MAVS oligomerization to stimulate the phosphorylation of transcription factors IRF3, IRF7, c-Jun/ATF-2 and NFkB (Wu and Hur, 2015). Once translocated into nucleus, these activated transcription factors can turn on expression of the type I IFNs (Kell and Gale, 2015, McWhirter *et al.*, 2005).

Studies have shown that NS1 protein can block activation of IRF-3, NFkB, and c-Jun/ATF-2 mediated by dsRNA and virus, thus limiting expression of IFN at pre-transcription level (Ludwig *et al.*, 2002, Talon *et al.*, 2000, Wang *et al.*, 2000). This inhibition occurs at very upper step of the RIG-I signalling pathway. Co-immunoprecipitation shows that NS1 forms a complex with RIG-I, however it is unclear whether any other player is involved in this complex (Pichlmair *et al.*, 2006, Guo *et al.*, 2007, Opitz *et al.*, 2007, Mibayashi *et al.*, 2007). It has been reported that the ubiquitin ligases TRIM25 and RIPLET which are activators of RIG-I have been associated with NS1 (Gack *et al.*, 2009, Rajsbaum *et al.*, 2012). NS1 can inhibit oligomerization of TRIM25 by binding to its coiled coil domain and thus inhibit E3 ligase activity on the CARD domains of RIG-I. Substitution of E96/E97 residues in ED of NS1 that result in failure binding between NS1 and TRIM25 can cause viral attenuation and higher IFN induction (Gack *et al.*, 2009). Interaction between NS1 and RIPLET also blocks RIPLET from activating RIG-I, although E96/E97 residues here in this interaction do not play a role. It is unclear whether the same residues in the C-terminal CARD domains of RIG-I can be ubiquitinated by both RIPLET and TRIM25. It has been suggested that RIPLET first mediates a conformational change of RIG-I by modifying its N-terminal, which allows RIG-I subsequently activated by TRIM25 (Rajsbaum *et al.*, 2012). Thus, NS1 can inhibit activation of RIG-I in two independent steps. This multiple-level inhibition could be due to viral strain or host species specific for example, human NS1, which can bind both human RIPLET and TRIM25 but are unable to bind murine TRIM25, relies on its interaction with RIPLET to achieve inhibition of RIG-I in mice. Furthermore, different NS1 could also interact with RIG-I pathway components depending on the host species, which allows virus to overcome host species barrier. In addition to E96/E97 residues in ED of NS1, R38/K41 in RBD also play a role in pre-transcriptional inhibition of the interferon production activated by PAMPs through RIG-I pathway. R38/K41 are known essential residues for RNA binding activity, substitution of R38/K41 abolishes ability of NS1 in binding RNA but as well as its interaction with RIG-I, TRIM25 and RIPLET (Wang *et al.*, 1999, Gack *et al.*, 2009, Rajsbaum *et al.*, 2012).

Furthermore, introduction of R38/K41 mutation in NS1 significantly impairs its inhibition of interferon production (Pichlmair *et al.*, 2006). However, to date, it is not fully understood how RNA-binding activity of NS1 contributes to limit interferon production through RIG-I.

Several strains circulating in human have been reported unable to block RIG-I pathway efficiently at the pre-transcriptional level (Hayman *et al.*, 2006, Kochs *et al.*, 2007a, Kuo *et al.*, 2010). Thus different strategy is also employed by NS1 to shut off IFN and IFN-stimulated gene expression at a post-transcriptional level. Primary transcripts require post-transcriptional processing to mature in eukaryotic cells. The 3' ends of primary transcripts are cleaved and polyadenylated into mature mRNA by Cleavage and Polyadenylation Specificity Factor (CPSF), which is a polyprotein complex formed by four subunits (Wahle and Keller, 1996, Colgan and Manley, 1997). The smallest subunit of CPSF is a 30 kDa protein (CPSF30) which contains five C3H zinc fingers (Barabino *et al.*, 1997). The C-terminal effector domain of NS1 binds directly to two of the CPSF30 zinc fingers, F2 and F3 region. The interaction then prevents pre-mRNAs binding to CPSF complex and inhibits correct cleavage and polyadenylation of host mRNA (Nemeroff *et al.*, 1998, Twu *et al.*, 2006, Das *et al.*, 2008). As NS1 also binds poly(A)-binding protein II (PABII), it blocks nuclear export of those mRNAs which escape from the inhibition of 3' end cleavage and polyadenylation (Chen *et al.*, 1999). Cellular pre-mRNAs accumulated in the nucleus of infected cells are later on degraded, it has also been suggested these pre-mRNA provides materials for cap snatching by the viral polymerase (Knipe and Howley, 2013). However notably, viral transcripts are not affected by the inhibition of CPSF activity since they are polyadenylated by the viral polymerase (Knipe and Howley, 2013). Mutagenesis and X-ray crystal structure studies revealed highly conserved hydrophobic 184-188 residues of Ud/NS1 mediates its interaction with CPSF30, whereas the PABII interaction site requires residues 223-237 (Das *et al.*, 2008, Noah *et al.*, 2003). A mutation at G184 position abolishes Ud/NS1 binding to CPSF30 (Das *et al.*, 2008). Residue W187 which is required for dimerization of ED is also important

(Aramini *et al.*, 2011). Furthermore, this interaction is stabilised by residues F103 and M106, and vRNP subunits PB1, PB2, PA and NP are also reported to contribute to stabilize this interaction too (Twu *et al.*, 2007, Kochs *et al.*, 2007a). Thus by interacting with CPSF30 and PABII, NS1 blocks global cellular mRNA processing and expression, including those anti-viral IFN and ISG.

Depending on the strain, NS1 protein can limit the IFN production by both pre-transcriptional and post-transcriptional processes or by one of the processes. In other words, pre-transcriptional inhibition of the RIG-I signalling and the suppression of global gene expression are both variable features in a strain-specific manner. While NS1 from some of the human strains fail to block pre-transcriptional IFN signalling cascade but can bind strongly to CPSF30, NS1 from 2009 pandemic (pH1N1) influenza virus is defective in binding to CPSF30 (Hayman *et al.*, 2006, Kochs *et al.*, 2007a, Kuo *et al.*, 2010, Hale *et al.*, 2010b). The existence and evolution of two mechanisms may increase the capacity of influenza A viruses to adapt to new environments and hosts. It is also possible that different experiment approaches and laboratory adaptation of some strains result in viruses employing these mechanisms to inhibit IFN in distinctive ways.

1.4.5.2 Inhibition of cellular mRNA processing by NS1

As discussed above, NS1 proteins can retain host mRNAs but not viral mRNAs in the nucleus by interacting with CPSF30 and PABII. Besides these two cellular mRNA processing components, NS1 has been reported to form a complex with key components of the mRNA export machinery that interact with both mRNAs and nucleoporins to direct mRNAs through the nuclear pore complex. These components include NXF1/TAP, p15/NXT, Rae1/mrnp41, and E1B-AP5 (Satterly *et al.*, 2007). Moreover, Nup98 protein, which is a nucleoporin serving as a docking site for mRNA export factors, is down-regulated during infection (Satterly *et al.*, 2007). Both the RBD and ED of NS1 are required for complex formation with NXF1, Rae1,

and E1B-AP5, although treatment with RNase A only affects the pulldown of E1B-AP5 with NS1. On contrast, interaction between NS1 and p15 only involves C-terminal ED (Satterly *et al.*, 2007). NS1-mediated block on mRNA nucleocytoplasmic transport can be reversed by overexpressing mRNA export components, suggesting NS1 can act directly on nuclear mRNA export factors to inhibit host protein synthesis (Satterly *et al.*, 2007). Wolff *et al.* also identified a novel human 70-kDa protein that interacts with NS1, which was subsequently termed as NS1-BP. NS1-BP localises in the nuclear regions enriched with the spliceosome assembly factor SC35, suggesting that NS1-BP plays a role in the cellular mRNA splicing machinery. Upon infection, NS1-BP is dispersed from speckles, redistributed throughout the nucleus and co-localises with NS1 (Wolff *et al.*, 1998). As NS1 was shown to inhibit pre-mRNA splicing of a reporter gene *in vitro* and change nuclear localisation of some other cellular splicing factors, it is proposed that the interaction between NS1 and NS1-BP may contribute to the inhibition of host-cell mRNA processing (Fortes *et al.*, 1995, Wolff *et al.*, 1998). NS1-BP was also shown to interact with the heterogeneous nuclear ribonucleoprotein (hnRNP) K to promote splicing of M1 mRNA, which produces the viral M2 mRNA (Tsai *et al.*, 2013). Notably, in contrast to the inhibitory effect of NS1 on host cellular mRNAs, NS1 does not suppress the nuclear export of most viral mRNAs, the only exception is NS1 own mRNA (Garaigorta and Ortin, 2007, Satterly *et al.*, 2007).

1.4.5.3 Inhibition of IFN-stimulated genes by NS1

Besides antagonising interferon genes at pre- and post-transcriptional level, NS1 also directly inhibits antiviral proteins produced by interferon-stimulated gene (ISG), including dsRNA-dependent serine/threonine protein kinase R (PKR), and 2'-5'-oligoadenylate synthetase (2'-5'-OAS). PKR is constitutively expressed in an inactive conformation in mammalian cells as a sensor activated by dsRNA and other stimuli (Garcia *et al.*, 2006). Double-strand RNA binds to N-terminal dsRNA-binding domain of PKR, which results in dimerization-induced

autophosphorylation of PKR and thereby activation of its kinase activity (Garcia *et al.*, 2006). PKR can be also activated by IFN treatment or by cellular protein PACT. Activated PKR can then phosphorylate α -subunit of eIF2 (eukaryotic Translation Initiation Factor 2), a process which causes the inhibition of all protein synthesis in the cell (Garcia *et al.*, 2006). Apart from its role in host shut off, PKR was considered a major cytoplasmic PRR involved in the transcriptional activation of IFN before the discovery of the RIG-I, it activates several signalling pathways, including the transcription factor IRF3 and NFkB that is crucial to IFN production (Pichlmair and Reis e Sousa, 2007, Diebold, 2010, Williams, 2001, Munir and Berg, 2013).

Influenza A viruses have developed two direct mechanisms to antagonise the powerful antiviral activity of PKR, firstly, by regulating function of p58^{IPK}, a cellular factor that is able to physically interact with PKR and inhibit activation and activity of PKR (Melville *et al.*, 1997, Melville *et al.*, 1999, Goodman *et al.*, 2007). Influenza virus activates p58^{IPK} to block the activity of PKR to repress translation in the infected cells. In the uninfected cells, p58^{IPK} forms a complex with its own inhibitor, Hsp40, which keeps p58^{IPK} in an inactive state (Melville *et al.*, 1997). Upon infection, viral protein NP interacts with P58^{IPK} and dissociates P58^{IPK} from its complex with Hsp40 which subsequently decreases PKR phosphorylation (Sharma *et al.*, 2011). Second mechanism influenza virus employs to counteract PKR is by action of NS1. NS1 knockout recombinant PR8 viruses are able to replicate efficiently in mice that are lack of PKR (Bergmann *et al.*, 2000). Early studies suggested NS1 inhibits PKR by competitive binding to dsRNA (Hatada and Fukuda, 1992, Lu *et al.*, 1995). However, recent work revealed that as for RIG-I inhibition, the affinity of NS1 for dsRNA is much lower than the affinity of PKR for dsRNA, thus model of competitive binding to dsRNA appears unlikely (Li *et al.*, 2006a). Further evidences showed that PKR is effectively suppressed by a recombinant influenza virus containing RNA binding-defective R38A NS1. NS1 also blocks activation of PKR by PACT, a process which is independent of dsRNA binding (Li *et al.*, 2006a). Co-immunoprecipitation studies suggested amino acids 123–127 in the C-terminal effector domain of NS1 and the N-

terminal region of PKR are responsible for their interaction (Min *et al.*, 2007). Taking above findings together, it is proposed that NS1 blocks PKR binding to dsRNA by preventing activating conformational change in PKR (Li *et al.*, 2006a). Thus, inhibition of PKR by NS1 during infection allows efficient viral protein synthesis as well as antagonising of IFN induction and production IFN.

The 2'-5'-oligo(A) synthetase (2'-5'-OAS) is an enzyme produced upon IFN stimulation, which catalyses synthesis of 2'-5' oligoadenylate (2'-5' oligo(A)) chains in 2'-specific nucleotidyl transfer reactions from ATP (Sen, 2000). These 2'-5' oligo(A) molecules cause dimerisation and the activation of the latent the latent RNase L, which is a potent inhibitor of viral infection by degrading single-stranded RNA, such as mRNAs and ribosomal RNAs (Sen, 2000). Pattern recognition receptors like RIG-I also can detect by-products of this degradation by RNase L, which provides additional positive feedback to IFN response (Silverman, 2007). Using a rUd virus expressing NS1-R38A revealed that RNA-binding activity by NS1 plays a major role in circumventing the host IFN-induced antiviral state (Min and Krug, 2006). Compared to WT, A549 cells pre-treated with IFN β resulted in a dramatic decrease in the yield of rUd NS1-R38A virus. However, knockdown of RNase L by siRNA restores some degree of the yield of rUd NS1-R38A virus, suggesting this effect is mediated mostly by RNase L (Min and Krug, 2006). As no direct interaction has been found between NS1 and OAS and/or RNase L, given that RNase L is activated solely by 2'-5'-OAS, and NS1 likely has a higher binding affinity for dsRNA than 2'-5'-OAS, it has been postulated that the mechanism of this inhibition is by NS1 out-competing binding with dsRNA away from 2'-5'-OAS, thus blocking the activation of this antiviral enzyme (Min and Krug, 2006).

1.4.5.4 NS1 and adaptive immune response

Dendritic cells (DC) play an important role in both innate and adaptive immune responses against influenza virus (Summerfield and McCullough, 2009). Upon

infection, maturation of DCs results in upregulation of major histocompatibility complex (MHC) class II molecules and the production of pro-inflammatory cytokines and chemokines (Fernandez-Sesma *et al.*, 2006). Mature DCs migrate to the draining lymph nodes of the lung where they present peptide pathogen-specific to virus-specific naïve T cells. This leads to proliferation and differentiation of T cells into effector cells, thus stimulates initiation of specific adaptive immune responses to the virus (Summerfield and McCullough, 2009). Influenza viruses are able to interfere with the maturation of DCs, it has been shown that DCs infected with influenza virus display reduced upregulation of MHC class II and costimulatory receptors like CD80 and CD86, which are essential for host T cell activation (Fernandez-Sesma *et al.*, 2006). Expression of genes involved in DC maturation and migration induced by the virus is limited by NS1, these genes include MHC, IL-8, IL-12 p35, IL-23 p19, IRF-7, RANTES, TRIF, MIP-1, and CCR7. DCs infected with PR8 virus lacking NS1 can produce higher amounts of type I IFN, TNF α and IL-6. Thus, infected DCs are unable to mature, and thereby fail to stimulate the secretion of IFN from T cells. (Fernandez-Sesma *et al.*, 2006). Moreover, presentation of an endogenous viral antigen by mature DCs can also be interfered with by influenza virus (Young *et al.*, 2007). Taken together, as NS1 limits initial innate host response, it results in a profound inhibition of the priming and the quality of the adaptive response.

1.4.6 NS1 and other host responses and signalling pathways

1.4.6.1 NS1 and the host apoptotic response

Apoptosis is a tightly controlled process by which cells activate pathways leading to their self-destruction in response to a wide range of stimuli, including virus infection (Lowy, 2003, Iwai *et al.*, 2013). Influenza A viruses induce cell death by apoptosis during infection. Some studies showed that cellular pro-apoptotic factors including caspase-3, NFkB/FasL/TRAIL elevate efficient replication of influenza A

viruses (Wurzer *et al.*, 2003, Wurzer *et al.*, 2004). However induction of cell death is regulated into a temporal balance between pro- and anti-apoptotic viral proteins, as well as other factors that contribute to apoptosis, such as dsRNA (Morris *et al.*, 2005). Throughout each stage of infection, the kinetics of apoptosis induction appear to be important for virus replication. Several influenza virus proteins have been reported to directly regulate apoptosis. NA can activate transforming growth factor (TGF), which induces apoptosis in epithelial cell (Morris *et al.*, 1999, Schultz-Cherry and Hinshaw, 1996). M1 can interact with caspase-8 to promote apoptosis (Zhirnov *et al.*, 2002b). Also virulence factor PB1-F2 forms ion channels in the inner mitochondrial membrane either by itself or by interacting with ANT3 and VDAC to induce apoptosis (Chanturiya *et al.*, 2004, Zamarin *et al.*, 2005).

NS1 can have both pro- and anti- apoptotic functions, but the exact role of NS1 in virus-induced apoptosis/cell-death is not entirely clear (Schultz-Cherry *et al.*, 2001, Zhirnov *et al.*, 2002a). It has been suggested it could be in a specific host-cell factors or virus strain-dependent manner. Ud/NS1 but not PR8/NS1 was reported to inhibit the processing of cellular pre-mRNAs, which appears to be toxic to the cell. Ud/NS1 also shows toxicity to MDCK cells but not HeLa cells (Schultz-Cherry *et al.*, 2001). Transiently expressed Ud/NS1 in MDCK cells revealed RNA-binding domain of NS1 can amplify apoptosis induced by NS1, which is independent of other virus-induced factors. On the other hand, NS1 also shows anti-apoptotic functions, which could be due to its ability to antagonise IFN system. Another example is inhibition of active PKR by NS1 which phosphorylates α subunit of eIF2 and leads to host shut off. Direct binding between PKR and NS1 may contribute to suppression cell death (Li *et al.*, 2006a). This could also apply to pro-apoptotic functions of 2'-5' OAS/RNaseL which are inhibited by NS1. Overall, during infection NS1 may achieve a balance in favour of virus survival between both early active suppression of apoptosis and late toxic induction cell-death.

1.4.6.2 NS1 and phosphoinositide 3-kinase (PI3K) signalling

Phosphoinositide-3-kinase (PI3K) is a ubiquitously expressed heterodimeric enzyme, which catalyses the production of phosphatidylinositol-3,4,5-triphosphate (PIP3) by phosphorylating phosphatidylinositol (PI), phosphatidylinositol-4-phosphate (PIP), and phosphatidylinositol-4,5-bisphosphate (PIP2) ((Engelman *et al.*, 2006)). PI3K is composed of a catalytic subunit p110 and a regulatory subunit p85. The product PIP3 is a membrane-embedded lipid second messenger, which activates downstream signalling components, the best known is Ser/Thr kinase Akt. PI3K signalling is involved in cellular survival, proliferation, trafficking, and regulation of the immune function (Engelman *et al.*, 2006).

By characterizing phosphorylation of Akt, Influenza A virus was reported to activate PI3K signalling twice during infection (Ehrhardt and Ludwig, 2009, Ayllon *et al.*, 2012a). The first transient activation of PI3K happens during early stage of infection when the virus attaches to the cell surface and enters into the cell. It was suggested the early activation is involved in endocytosis (Ehrhardt *et al.*, 2006). The second sustained activation of PI3K happened when NS1 is expressed which binds to PI3K (Hale *et al.*, 2006, Shin *et al.*, 2007, Zhirnov and Klenk, 2007). When NS1 binds to p85 of PI3K, it relocates its N-terminal SH2 domain from its position on the heterodimer, then releases p110 subunit from inhibition. A highly conserved tyrosine in position 89 of NS1 effector domain is responsible for the interaction between NS1 and p85 interphase (Hale *et al.*, 2010a). A NS1 mutant with Y89F substitution failed to bind p85 and trigger PI3K/Akt activation (Hale *et al.*, 2010a).

PI3K activation has been shown to improve virus replication in vitro and virulence in vivo of PR8 strain, but not WSN, which indicates it works in a strain-dependent manner (Hrincius *et al.*, 2012, Ayllon *et al.*, 2012b). As PI3K signalling has anti-apoptotic function, by using viruses lacking NS1 protein it was first suggested virus fitness is benefited from better cell survival induced by NS1, however NS1 was also

reported to play a role in promoting apoptosis (Zhirnov and Klenk, 2007, Shin *et al.*, 2007, Schultz-Cherry *et al.*, 2001). Other function of PI3K activation by NS1 involves regulation of cationic currents in the airway epithelium, where influenza virus primality targets. As a result, it affects liquid accumulation and contributes to pathogenicity (Gallacher *et al.*, 2009). Interestingly, as p110 subunit of PI3K has three isotypes p110a, p110 β , and p110d, and p85 has two p85a and p85 β , the kinase can be comprised with different combination of isotypes (Engelman *et al.*, 2006). But NS1 only binds specifically to p85 β isotype (Hale *et al.*, 2006). Investigation of this specific targeting could be a key to our understanding of the biological relevance of PI3K activation by NS1.

1.5 The cellular proteins TRBP and PACT

1.5.1 Identification of TRBP and PACT

The cDNAs coding for TRBP1 and TRBP2 were cloned based on a protein-RNA binding screen using the highly structured human immunodeficiency virus type 1 (HIV-1) TAR RNA as a probe (Gatignol *et al.*, 1991). Due to the alternative translation of the first exon, TRBP2 has an extra 21 amino acids at its N-terminal in comparison to TRBP1. The rest of the two proteins are identical. The cDNA coding for the homolog of TRBP in mouse was cloned from mouse male germ cell cDNA expression libraries. The murine counterpart was identified as Prm-1 RNA binding protein (PRBP) which plays a crucial role in translational control during spermatogenesis (Lee *et al.*, 1996, Braun, 2000). TRBP2 and PRBP are highly homologous, with a homology of 94-96%. Homologs of TRBP are present in many other organisms including fish, reptiles, crustaceans, insects and worms. Functions of homologs of TRBP in *Drosophila* and *Caenorhabditis elegans* (RDE-4) have been well studied. A homolog of TRBP was also found in invertebrate tunicates, the closest relative of vertebrates (Daniels and Gatignol, 2012). TRBP is encoded by the *tarbp2* gene located on human chromosome 12 (12p12.1-q13.1). One pseudogene of TRBP is

also located on chromosome 8q22 in human genome. There are three alternatively spliced variants of human TRBP2 mRNA. Transcript variant 1 is full length and named isoform A. Isoform B is encoded by transcript variant 2, which, in comparison to transcript 1, lacks 253 aa in C-terminal due to the loss of 17 nt at the beginning of a coding exon. Transcript variant 3 encodes isoform C which lacks the first 21 aa as compared to isoform A (Kozak *et al.*, 1995, Duarte *et al.*, 2000).

PACT is a dsRNA binding protein that was cloned based on its interaction with PKR in a yeast two-hybrid screen of a human cDNA library using inactive PKR as bait (Patel and Sen, 1998). PACT gene is located at chromosome 2q31.3 (Rowe and Sen, 2001). In contrast to the inhibition of PKR by TRBP, PACT plays a role in enhancing phosphorylation of PKR and eIF2 α , which leads to translation inhibition and host shut off. PACT is also an activator of RIG-I, the interaction between PACT and RIG-I stimulates RIG-I-induced type I interferon production (Kok *et al.*, 2011). In addition, PACT-knockout mice have abnormal craniofacial phenotypes such as rounded rostrum, shorten nose, hypoplastic turbinates, and small outer ear, suggesting a role for PACT in ear development (Rowe *et al.*, 2006). PACT is expressed in all kinds of tissues independent of IFNs. Murine ortholog of PACT is called RAX which is encoded by *prkra* gene. PACT and RAX are almost identical that only 6 out of 313 residues are different with 4 similar substitutions (Ito *et al.*, 1999).

1.5.2 Structural domains of TRBP and PACT

Among the first proteins identified to have dsRBDs, TRBP contains two dsRBDs and a third C-terminal region. The C-terminal Medipal (Merlin-Dicer-PKR activator Liaison) region mediates protein-protein interactions (Daniels *et al.*, 2009, Laraki *et al.*, 2008). Different from isoforms A and C of TRBP, isoform B of TRBP has only the first dsRBD. The first dsRBD is located between aa 31 and 96 in TRBP2, and the second one is located between aa 160 and 226. The third domain is located between

aa 298 to 366. Similar to TRBP, PACT contains three structural domains, including two N-terminal dsRBDs, dsRBD1 and dsRBD2, and the third C-terminal domain called Domain 3. dsRBD1, 2 and 3 of TRBP and PACT share 56, 62 and 57% identical amino acid residues, respectively. dsRBD1 and 2 of PACT have been suggested to interact with each other. For TRBP-PACT, PACT-Dicer and TRBP-Dicer interactions, the C-terminal domain is sufficient for binding. In TRBP, dsRBD2 has a stronger dsRNA binding activity than dsRBD1 (Laraki *et al.*, 2008, Daniels *et al.*, 2009). This is due to dsRBD2 has a 15-aa KR-helix motif, which can bind dsRNA by itself. The entire protein, or the two dsRBDs expressed in tandem, have a much higher affinity for dsRNA than either dsRBD alone, suggesting that the two domains are both required for optimal dsRNA binding. Mutations of H37, K59, and K63 in dsRBD1 or H167, K189, and K193 in dsRBD2 of TRBP1 abolish its dsRNA binding ability. In TRBP2, the position numbers are increased by 21 aa (Daviet *et al.*, 2000).

1.5.3 The role of TRBP in HIV-1 biology

TRBP was originally identified as a transactivator of HIV-1 LTR by binding to TAR RNA in the 5' end of LTR (Gatignol *et al.*, 1991). In murine cells and human astrocytes, HIV-1 replicates at very low level, but overexpression of TRBP can enhance viral expression and thus virus replication (Ong *et al.*, 2005). Knockdown of TRBP by siRNA in HeLa cells decreases the level of HIV-1 production, whereas transduction of shRNA against TRBP in human SupT1 lymphocytic cells results in long-term inhibition of HIV-1 replication (Eekels *et al.*, 2011, Christensen *et al.*, 2007). The mechanisms by which TRBP contributes to viral expression and replication are ascribed to the inhibition of PKR activation by TRBP and to a direct activity of TRBP that relieves TAR RNA structure-mediated translational block. The binding of TRBP with TAR RNA has been extensively studied. A short lysine- and arginine-rich motif in TRBP was identified to be responsible for high-affinity binding with TAR as well as other GC-rich dsRNAs (Gatignol *et al.*, 1993, Erard *et al.*, 1998).

1.5.4 Role of TRBP and PACT in regulating PKR activity

As described in section 1.4.5.3, PKR plays a central role in the host innate immune response as an IFN-inducible dsRBP that has a serine/threonine kinase activity. The activation of PKR is highly regulated by viral and cellular proteins and RNAs (Li *et al.*, 2006b). PACT was identified as a cellular protein activator of PKR. PACT forms heterodimer with PKR and activates it in the absence of dsRNA *in vivo*. Overexpression of PACT also activates PKR in mammalian cells. Moreover, co-expression of PACT and PKR enhances the anti-growth effect of PKR in the yeast. The inhibition of PKR activation by TRBP was discovered as TRBP promotes growth of a mutant vaccinia virus which lacks E3L protein, an inhibitor of PKR (Park *et al.*, 1994). Further studies showed TRBP and PKR bind directly through their dsRBDs, which leads to inhibition of PKR activation (Benkirane *et al.*, 1997). Therefore, it was suggested that TRBP inhibits PKR activation by competitive binding to dsRNA as well as direct protein-protein interaction. Without any stimuli, binding between TRBP and PACT keeps PACT in an inactive state, thereby unable to activate PKR. It has been reported stress-induced phosphorylation of PACT at its serine 287 position significantly increases its ability to activate PKR, which is due to weaker interaction between TRBP and PACT (Singh *et al.*, 2011). Substitution by alanine in this position results in enhanced interaction of PACT with TRBP, which leads to inefficient PKR activation by PACT. Furthermore, overexpression of TRBP is unable to block apoptosis induced by a stably expressing PACT in the cell. Another study shows that interaction between PACT dimers can be enhanced by phosphorylation of serines at positions 246 and 287 of PACT, which is essential for efficient PKR activation. PACT mutants which are unable to form dimers are inefficient to activate PKR (Singh and Patel, 2012). In summary, TRBP and PACT are important regulators of PKR which could have profound implication during virus infection.

1.5.5 The role of TRBP and PACT in host RNA interference

RNA interference (RNAi) plays a crucial regulatory role in all eukaryotic cells, which have been implicated in various cellular function, proliferation, differentiation and host defense (Carthew and Sontheimer, 2009). Since RNAi was first described in *C. elegans*, Dicer, Argonaute (Ago), and a small dsRNA binding protein were identified as the key proteins of the RNA-induced silencing complex (RISC). These three proteins are highly conserved among eukaryotes and are required for minimal RISC-loading complex (RLC). The dsRBPs associated with Ago are different from one organism to another, in *C. elegans*, this dsRBP is RDE-4 while in *Drosophila*, it is either R2D2 or Loquacious (Loqs), and in humans, this dsRBP is identified as TRBP (Fire *et al.*, 1998, Liu *et al.*, 2003, Liu *et al.*, 2006, Tabara *et al.*, 2002, Haase *et al.*, 2005). Further studies report TRBP, Dicer, and Ago2 are able to spontaneously assemble, form the RLC, and execute RISC activity by processing precursor miRNA (pre-miRNA) and then cleaving target mRNAs (Lau *et al.*, 2009, MacRae *et al.*, 2008). Whereas some suggested that this complex remains intact with multiple rounds of cleavage, others indicated that Ago2 is associated more with mature miRNA and appears to dissociate after miRNA loading (Gu *et al.*, 2011, Maniataki and Mourelatos, 2005). This implies that TRBP and Dicer play an essential role in processing shRNAs/miRNAs but they do not contribute to target cleavage. In addition, the C4 domain of TRBP, which mediates interaction between TRBP and Dicer, is essential in efficient RNAi activity. Furthermore, TRBP and Dicer colocalise in the cytoplasm but not in processing bodies, suggesting they are more involved in RLC formation and miRNA processing rather than target mRNA cleavage mediated by Ago2 (Pare *et al.*, 2009, Daniels *et al.*, 2009). PACT, which is also found in Dicer-containing complexes later, contributes to the stabilization and activity of Dicer together with TRBP (Lee *et al.*, 2006). Although it remains unclear whether the recruitment of PACT to Dicer is mediated by TRBP or dsRNA, PACT promotes pre-miRNA processing activity and the efficiency of siRNA. While study by Lee *et al.* reported knockdown of TRBP exerts more influence on siRNA-initiated

gene silencing than knockdown of PACT, results by Kok *et al.* indicated siRNA-initiated gene silencing is affected similarly by knockdown of either TRBP or PACT (Lee *et al.*, 2013, Kok *et al.*, 2007). Moreover, it has been shown that mRNA targeted by miRNAs overall appears to be favoured for complexes containing PACT but not TRBP (Noland and Doudna, 2013). Nevertheless, PACT forms complex with TRBP, Dicer, and Ago2 and contributes to the processing by Dicer and the function of miRNAs and shRNAs (Figure 1.7). Although these proteins undoubtedly form a complex, it is yet to be determined whether TRBP and PACT directly bind Ago2 or whether Dicer serves as a bridge between them, or other proteins could also take part in the complex formation.

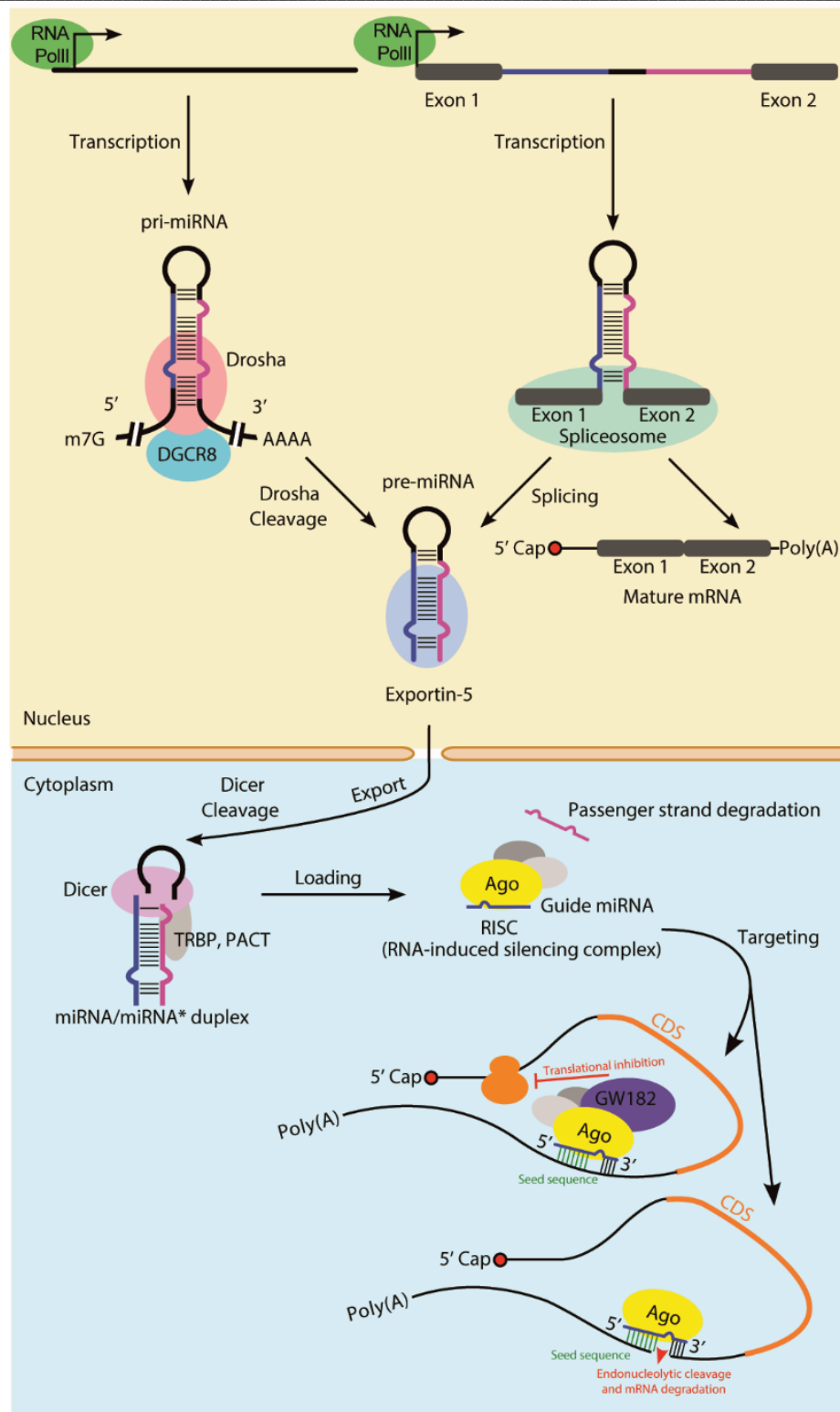


Figure 1.7 Dicer mediated RNA silencing pathway, participated by TRBP and PACT. Schematic representation showing how RNA is transcribed and processed into mature miRNA, which is loaded Ago2-contained RNA-induced silencing complex, thereafter results in RNA silencing activity.

1.6 Aim of the study

Like all viruses, influenza viruses rely on the host cellular machinery for their life cycle. Accordingly, it is of great interest and essential to identify host factors involved in virus replication to better understand the mechanisms of the virus life cycle and to search for potential targets for the development of novel diagnostics and antiviral therapeutics. One of the ways of achieving this is to investigate protein-protein interactions between host and virus. Thus the aim of this PhD project is to analyse the interactome generated by genome-wide approach Y2H screen and then to elucidate the roles of specific interactions identified in the screen between influenza virus NS1 protein and cellular proteins TRBP and PACT.

Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Plasmids

	Vector name	Feature	Source
1	pDONR207	Gateway Donor vector	Invitrogen
2	pDONR223	Gateway Donor vector	Invitrogen
3	pGADT7	Y2H prey	Clontech
4	pGBKT7	Y2H bait	Clontech
5	pCR3-dest	Mammalian expression vector	Invitrogen
6	pCR3-N-Myc-dest	N-terminal Myc tag	Invitrogen
7	pCR3-N-HA-dest	N-terminal HA tag	Invitrogen
8	pCDNA	Mammalian expression vector	Invitrogen
9	pCDNA-miRK10/12	pCDNA plasmid with 10 out of 12 KSHV encoded miRNAs	Dr Georg Malterer, Max von Pettenkofer Institut
10	psiCHECK-2-miRK4	psiCHECK-2 plasmid constructed with KSHV miRNA binding sequences	Dr Georg Malterer, Max von Pettenkofer Institut
11	pEGFP-C2-dest	N-terminal GFP tagged mammalian expression vector	Clontech
12	pEGFP-N1-PR8/NS1	C-terminal GFP tagged pEGFP-N1 plasmid constructed with PR8/NS1	Professor Paul Digard, University of Edinburgh

Table 2.1 List of plasmids used in this study.

2.1.2 Bacterial and yeast strains

	Strains	Source
1	Electrocompetent <i>Escherichia coli</i> DH10 β	Invitrogen
2	Chemically competent <i>Escherichia coli</i> DH10 β	Invitrogen

3	AH109 <i>Saccharomyces cerevisiae</i> mating type α	Clontech
4	Y187- <i>Saccharomyces cerevisiae</i> mating type a	Clontech

Table 2.2 List of bacterial and yeast strains used in this study.

2.1.3 Cell lines

	Cell lines	Media
1	HEK293	DMEM (Lonza)/ 10 % FCS (Gibco)/ 1 % Penicillin/streptomycin (Lonza)
2	HEK293T	DMEM (Lonza)/ 10 % FCS (Gibco)/ 1 % Penicillin/streptomycin (Lonza)
3	293T-Ago2-Myc	DMEM (Lonza)/ 10 % FCS (Gibco)/1 % Penicillin/streptomycin (Lonza)
4	A549	DMEM (Lonza)/ 10 % FCS (Gibco)/1 % Penicillin/streptomycin (Lonza)
5	MDCK	DMEM (Lonza)/ 10 % FCS (Gibco)/1 % Penicillin/streptomycin (Lonza)

Table 2.3 List of mammalian cell lines and media. The cell lines used in this study and their media and supplements are listed above.

2.1.4 Chemicals and reagents

2.1.4.1 General chemicals

	Chemicals	Source
1	Prolong Gold anti-fade reagent with DAPI	Invitrogen Cat #P-36931
2	Protein G Sepharose 4 Fast Flow	GE Healthcare Cat #17-0618-01
3	3-amino-1, 2, 4-triazole (3-AT)	Sigma Aldrich Cat #A8056
4	40 % acrylamide/bisacrylamide solution	Sigma Aldrich Cat #A7802
5	Paraformaldehyde	Thermo Scientific Cat #28906
6	4-methylumbelliferyl- β -xylobioside	Sigma Aldrich Cat #M6018
7	Ammonium persulfate	Sigma Aldrich Cat #A3678
8	Coelenterazine	PJK Cat #260350
9	Benzonase purity >90%	Novagen Cat #70746

10	Bicine	Sigma Aldrich Cat #163791
11	Sorbitol	Sigma Aldrich Cat #S1876
12	Polyethyleneglycol (PEG)	Sigma Aldrich Cat #88440
13	Goat serum	Sigma Aldrich Cat #G9023
14	Skim milk powder	Fluka Cat #70166

Table 2.4 List of chemicals used in the study.

2.1.4.2 Molecular weight markers

	Reagent	Source
1	GeneRuler™ DNA 1 kb ladder	MBI Fermentas Cat #SM0313
2	GeneRuler™ DNA 100 bp ladder	MBI Fermentas Cat #SM0241
3	PageRuler™ prestained protein Ladder	Thermo Scientific Cat #26616

Table 2.5 List of markers used in the study.

2.1.4.3 DNA dyes

	Reagent	Source
1	6X DNA loading dye	MBI Fermentas Cat #R0611
2	SYBR® Safe DNA gel stain	Invitrogen Cat #S33102

Table 2.6 List of DNA dyes used in the study.

2.1.4.4 Enzymes and PCR reagents

	Reagent	Source
1	Restriction enzyme BamHI	NEB Cat #R0136
2	Restriction enzyme BanII	NEB Cat #R0119
3	Restriction enzyme EcoRI	NEB Cat #0101
4	Restriction enzyme EcoRV	NEB Cat #R0195
5	Restriction enzyme XhoI	NEB Cat #R0146

6	Restriction enzyme XbaI	NEB Cat #R0145
7	Restriction enzyme NheI	NEB Cat #R0131
8	Restriction enzyme DpnI	NEB Cat #R0176
9	Gateway® LR clonase II enzyme mixture	Invitrogen, Cat #15224041
10	Gateway® BP clonase II enzyme mixture	Invitrogen, Cat #12536017
11	Exonuclease III	NEB Cat #M0206
12	Ribonuclease A	Sigma Aldrich Cat #R4875
13	Expand Long Template PCR System	Roche Diagnostics Cat #11681834001
14	KOD DNA polymerase	Novagen Cat #71086
15	BioMix™ red Taq polymerase	Bioline Cat #BIO-25006
16	Taq DNA polymerase	NEB Cat # M0273
17	Desoxyribonucleotides dATP, dCTP, dGTP, dTTP mix	Roche diagnostics Cat #03622614001
18	Oligonucleotides, HPLC-purified	Metabion, Germany
19	Universal probe library (UPL)	Roche Cat #04683641001

Table 2.7 List of Enzymes and PCR reagents used in the study.

2.1.4.5 Antibodies

	Reagent	Source
1	Anti-HA high affinity, rat monoclonal antibody, clone 3F10	Roche Diagnostics Cat #11867423001
2	Anti-c-Myc, mouse monoclonal antibody, clone 9E11	Santa Cruz Biotechnology Cat #SC-47694
3	Anti-NS1, mouse monoclonal antibody, clone NS1-23-1	Santa Cruz biotechnology Cat#SC-130568
4	Anti-β-actin, rabbit monoclonal antibody, clone 13E5	Cell Signalling Cat#5125
5	Goat anti-mouse peroxidase coupled	Jackson Cat #115035062
6	Goat anti-rat peroxidase coupled	Jackson Cat #112035003
7	Goat anti-rabbit peroxidase coupled	Cell signalling Cat#7074
8	Alexa fluor®488 goat anti-mouse IgG	Invitrogen Cat #A-11001
9	Alexa fluor®594 goat anti-rat IgG	Invitrogen Cat #A-11007

10	Rat IgG2 α isotype control	Invitrogen Cat #R2A00
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Table 2.8 List of antibodies used in the study.

2.1.4.6 Yeast reagents

	Reagent	Source
1	Yeast Extract, Bacto™	BD Cat #288260
2	Tryptone, Bacto™	BD Cat #211705
3	SD base	Clonetechn Cat #630411
4	Glucose	Sigma Aldrich Cat #G7528
5	Tryptophan	Sigma Aldrich Cat# T90204
6	Leucine	Sigma Aldrich Cat#L8000

Table 2.9 List of yeast reagents used in the study.

2.1.4.7 Antibiotics

	Reagent	Source
1	Ampicillin	Sigma-Aldrich Cat#A9393
2	Kanamycin	Sigma-Aldrich Cat# K1377
3	Gentamycin	Sigma-Aldrich Cat# G1397

Table 2.10 List of antibiotics used in the study.

2.1.4.8 Transfection reagents

	Reagent	Source
1	Lipofectamine 2000	Thermo Fisher Scientific Cat# 11668019
2	Lipofectamine LTX	Thermo Fisher Scientific Cat# 15338100
3	DharmaFECT 1	GE Cat# T-2001-03

Table 2.11 List of transfection reagents used in the study.

2.1.4.9 Culture media

	Reagent	Source
1	DMEM	Lonza Cat# BE12-604F/12
2	RPMI	Thermo Fisher Scientific Cat# 12633
3	Fetal calf serum	HyClone - Multiple catalog numbers used
4	Penicillin/Streptomycin	Thermo Fisher Scientific Cat# 15140
5	Hepes buffer	Sigma-Aldrich Cat# 83264
6	Glutamine	Lonza Cat# 17-605

Table 2.12 List of culture media used in the study.

2.1.5 Kits

	Kits	Source
1	Pierce™ BCA Protein Assay Kit	Thermo Fisher Scientific Cat# 23225
2	Brilliant II PCR kit	Agilent technologies Cat# 600804
3	Dual-Luciferase® Reporter Assay System	Promega, UK Cat# E1910
4	Pierce™ ECL Western Blotting Substrate	Thermo Fisher Scientific Cat# 32106
5	QIAprep Spin Miniprep Kit	Qiagen Cat #28704
6	PureYield™ Plasmid Midiprep System Kit	Promega Cat #A2492
7	Verso 1-step QRT-PCR Low ROX Mix	Thermo Scientific Cat #AB4102
8	Verso 1-Step QRT-PCR SYBR® Green Low ROX Kit	Thermo Scientific Cat #AB4106

Table 2.13 List of kits used in the study.

2.1.6 Plastic and other consumables

Reagent	Source
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1	Falcon® 8-well culture slide	Fisher Scientific Cat #08-774-26
2	Corning® 96 well plates, opaque bottom	Sigma-Aldrich Cat# CLS3917-100EA
3	Corning® Costar® 6/24/48/96 cell culture plates	Sigma-Aldrich Cat # CLS3516/ CLS3524/ CLS3548/ CLS3596
4	Corning® cell culture flask, 25cm ² , 75 cm ² , 175cm ² straight neck, vented cap	Sigma-Aldrich Cat # CLS3056/ CLS430641/ CLS431080
5	Eppendorf® 1.5ml Safe-Lock microcentrifuge tubes	Sigma-Aldrich Cat # T9661-1000EA
6	Electroporation cuvette 2 mm	Cell Projects Cat #EP-202*
7	Corning® 15ml/50ml PP Centrifuge Tubes	Corning® Cat #430052/ 430290
8	FUJIFILM Super RX X-Ray film	Laborversand Cat #RF12
9	Amersham Protran 0.45 NC nitrocellulose membranes	GE Cat #10600002

Table 2.14 List of consumables used in the study.

2.1.7 Buffers and solutions

	Buffer/Solution	Contents
1	1X TAE	0.04 M Tris–Acetate, 0.001 M EDTA, 5.7 % glacial acetic acid
2	2x HBS:	50 mM HEPES, 10 mM KCl, 12 mM dextrose, 280 mM NaCl, 1.5 mM Na ₂ PO ₄ , pH 7.05
3	alkaline lysis extraction solution I	50 mM glucose, 25 mM tris-Cl (pH 8.0) and 10 mM EDTA (pH 8.0), 100 µg/ml RNase just before use
4	alkaline lysis extraction solution II	0.2 N sodium hydroxide, 1 % SDS
5	alkaline lysis extraction solution III	3 M potassium acetate and 11.5 % glacial acetic acid
6	acrylamide/bisacrylamide solution	40% bought ready-to-use
7	3-Amino-1, 2, 4-triazole (3-AT)	1 M stock solution in H ₂ O
8	paraformaldehyde (PFA)	4% diluted in PBS, degassed
9	4-methylumbelliferyl-β-xylobioside	1000 X stock in dimethylformamide
10	APS	10% in H ₂ O
11	Coelenterazine	diluted 1 mg coelenterazine in 2.36 ml of deoxygenated methanol/ HCl (8.7 ml of 35% HCl in 1l methanol [deoxygenated by pumping nitrogen gas through it on ice for 30 mins])

12	HIRT buffer	10 mM EDTA, 0.6 % SDS
13	immunofluorescence antibody dilution buffer	1% BSA in PBS
14	immunofluorescence blocking buffer	5% normal goat serum (Sigma Aldrich®) in PBS
15	2x Laemmli running buffer:	4% SDS, 20% glycerol, 10% 2-mercaptoethanol, 0.004% bromphenol blue and 0.125 M Tris HCl, pH 6.8
16	1x LUMIER lysis buffer stock	22mM Tris pH 7.5, 275 mM NaCl, 1 % TritonX-100, 11 mM EDTA.
17	LUMIER lysis buffer per sample: NB Buffer: 0.15 M NaCl (Sigma Aldrich®), 10 mM Bicine (Sigma Aldrich®)	900 µl LUMIER lysis buffer stock, 10 mM DTT, with cOmplete inhibitor tablets and phosStop, 0.5 µl Benzonase (Novagen 70746, puritiy >90%)
18	NP-40 lysis buffer	20 mM tris (pH 7.5), 150 mM NaCl, 5 mM MgCl ₂ and 1 % NP40 (IGEPAL-CA, Sigma Aldrich®), one tablet of cOmplete inhibitor tablets (Roche, UK) and one tablet of phosStop (Roche, UK) were added to 50 ml of NP40 buffer before use
19	PEG/Bicine solution	40% Polyethylene glycol (PEG) 200 mM Bicine
20	SBEG	1 M Sorbitol (Sigma Aldrich®), 10 mM Bicine, pH 8.35 with NaOH, 3% PEG (Sigma Aldrich®)
21	1X TBST	10 mM Tris-Cl (pH 7.5), 100 mM NaCl, 0.2% Tween20
22	Western blot blocking buffer	5 % skimmed milk powder dissolved in TBST
23	1x Western blot transfer buffer	240 mM tris, 190 mM glycine, 1 mM SDS, 20 % methanol
24	Tryptophan	20 mg/l in H ₂ O
25	Leucine	100 mg/l in H ₂ O
26	2YT	1.6 % Bacto-tryptone, 1 % yeast extract, 0.5% sodium chloride (NaCl)
27	SD-LW medium	2.67% minimal SD base, 0.064% Leucine and Tryptophan drop-out supplement
28	SD-LWH medium	2.67 % minimal SD base, 0.064 % Leucine and Tryptophan and Histidine dropout supplement
29	YPD medium	2% (w/v) Bacto-peptone, 1% (w/v) yeast extract, 2% glucose
30	YPDA medium	YPD medium, 0.002% (w/v) adenine hemisulphate
31	YPD agar plates	YPD medium, 1.5% (w/v) agar
32	LB broth	1 % Bacto-tryptone, 0.5% yeast extract, 1% sodium chloride (NaCl)
33	LB-agar plates	LB broth, 1.5% agar

Table 2.15 List of buffers and solutions used in the study.

2.1.8 Equipment

	Equipment	Source
1	RC5B superspeed centrifuge	Sorvall
2	Thermocycler	Techne TC512
3	Nanodrop ND1000	Labtech
4	GenePulser	Biorad
5	MX3000-P™ real-time PCR cycler	Stratagene
6	X-Ray Film Processor	Optimax
7	Polarstar Optima plate reader	BMG Biotech
8	Shaking incubator	Infors
9	Tabletop centrifuge 5415D	Eppendorf
10	Twin vertical electrophoresis system Galileo	Bioscience
11	Gel Doc 1000, UV transilluminator	Biorad

Table 2.16 List of equipment used in the study.

2.2 Methods

2.2.1 DNA techniques

2.2.1.1 Gateway® recombinatorial cloning

Gateway® cloning system (Invitrogen®) was used to clone all genes of interest (GOI) into vectors for use in assays. Genes of interest were amplified by polymerase chain reaction and purified before cloning into the Gateway® donor vector pDONR207. The reaction mixture consisted of 300 ng of purified PCR product, 300 ng of pDONR207, 1 µl of water and 1 µl of BP clonase® II enzyme. The reaction mixture was incubated overnight at 25°C before 1 µl of the reaction mixture was transformed into electrochemically competent *E.coli* DH10β (see section 2.2.1.6.7). Colonies were screened by DNA isolation using alkaline lysis method and verified

by restriction digestion and sequencing (see section 2.2.1.6). Human cellular genes were obtained from the MGC library in pDONR223 plasmid.

2.2.1.2 Sequencing

All pDONR207 and pDONR223 entry clones were verified by DNA sequencing, by GATC biotech, Konstanz, Germany. The sequencing primers are listed below in Table. Sequencing results obtained were aligned with published sequences with the respective accession numbers using EBI Pairwise align software. Clones with silent mutations or missense mutations were considered positive.

Primer	Sequence
pDONR207 Fwd	TAACGCTAGCATGGATCTC
pDONR207 Rev	GCAATGTAAACATCAGAGAT
pDONR223 Fwd	TCGCGTTAACGCTAGCATG
pDONR223 Rev	TAATACGACTCACTATAGGG

Table 2.17 Sequencing primers. The forward (Fwd) and reverse (Rev) primers used for sequencing reactions of pDONR207 and pDONR223 plasmids are listed.

2.2.1.3 Agarose gel electrophoresis

Agarose gel electrophoresis was used to separate DNA fragments based on size. A 1% agarose gel was made by adding 1 g agarose per 100 ml 1x TAE buffer and heating in a microwave oven until fully dissolved. After cooling the gel, the DNA stain SYBR® safe was added (2 µl per 100ml gel) and the gel poured into a casting chamber of appropriate size with the appropriate well-forming comb. The DNA sample was mixed with 6x loading dye and loaded on the gel. Molecular weight markers were added along with each set of samples and electrophoresis was performed horizontally at 80-150 V for 30-60 minutes following which the gels were illuminated by a short-wave UV transilluminator for visualisation.

2.2.1.4 Determination of DNA concentration

The concentration and purity of DNA was determined by measuring the UV absorbance at 260 and 280 nm using a Nanodrop. The DNA concentration was calculated with the OD at 260 nm (1 OD₂₆₀ = 50 µg/ml dsDNA). The purity was estimated by the OD₂₆₀/OD₂₈₀ ratio, with a ratio of approximately 1.8, indicating sufficiently pure DNA.

2.2.1.5 Restriction digest

Restriction digestion was performed according to the manufacturer's recommendations. In general, 1 µg of DNA was digested for 2 hours with 10-20 U of restriction enzyme at 37°C.

2.2.1.6 Plasmid DNA extraction

2.2.1.6.1 Alkaline lysis small scale plasmid DNA extraction

Following transformation, individual bacterial colonies were picked from LB agar plates and grown overnight in 1.2 ml LB broth with appropriate antibiotic in 96-deep-well block, shaking at 250 rpm at 37°C. The block was centrifuged at 4000 rpm for 10 mins and the bacterial cell pellet was resuspended in 300 µl of solution I using a multi-channel pipette, lysed with 300 µl of solution II and neutralised by adding 300 µl of solution III and incubating on ice for 5 min. The blocks were centrifuged for 30 min in a pre-cooled centrifuge (4°C) at 4000 rpm. Supernatant was transferred to a clean 96 deep-well block and 580 µl of isopropanol was added before incubating at -20 °C for at least 1 hour. DNA was pelleted by centrifuging for 45 min at 4000 rpm in a pre-cooled centrifuge (4°C), and the pellet was washed with

500 µl of 70% ethanol. The block was allowed to dry for about 30 min and the DNA pellet was re-suspended in 50 µl of water.

2.2.1.6.2 Affinity column small-scale plasmid DNA extraction

DNA was isolated from overnight cultures of individual bacterial colonies grown in 5 ml LB broth with appropriate antibiotic done using a QIAprep Spin Miniprep Kit, according to manufacturer's guidelines.

2.2.1.6.3 Affinity column large-scale plasmid DNA extraction

Larger quantities of DNA were prepared from 100-250 ml bacterial cultures using the PureYield™ Plasmid Midiprep System Kit according to manufacturer's guidelines.

2.2.1.6.4 Preparation of chemically competent *E.coli* DH10β

DH10β were streaked on an antibiotic-free LB agar plate overnight, and a 5 ml pre-culture grown from a single colony overnight at 37°C in a shaking incubator at 200 rpm. This pre-culture was used to inoculate 1 litre LB broth. This was incubated at 37°C shaking at 250 rpm until an OD600 (measuring density of bacteria) of 0.5 when bacteria are on exponential growth phase was reached. Cell growth was arrested by swirling vigorously on ice for 20 minutes after which cells were pelleted in a pre-cooled centrifuge at 4000 g for 10 minutes. Cell pellets were washed twice with 100 mM CaCl₂ before resuspending in 3-6 ml of 100 mM CaCl₂ with 15% glycerol. Cells were aliquoted in 50 µl volumes and snap-frozen in liquid nitrogen before storing at -80°C. Transformation efficiency was determined by transforming 1 ng of undigested plasmid and calculating the number of colonies per µg of DNA.

2.2.1.6.5 Preparation of electrocompetent *E.coli* DH10 β

A litre of 2YT media was inoculated with 10 ml overnight culture of a single colony from a freshly streaked plate and incubated at 37°C shaking at 250 rpm until an OD₆₀₀ of 0.5 was reached. After cell growth was arrested by swirling vigorously on ice for 20 minutes, cells were pelleted in a pre-cooled centrifuge at 4000 g for 10 minutes. Cell pellets were washed once with water and once with 10% glycerol. The cell pellet was then resuspended in 3-6 ml of 10% glycerol, aliquoted in 50 μ l volumes and immediately frozen in liquid nitrogen before storing at -80°C. Transformation efficiency was measured by transforming 1 ng of undigested plasmid and calculating the number of colonies per μ g of DNA.

2.2.1.6.6 Transformation of chemically competent *E.coli*

1-2 μ l of reaction mixture or DNA was mixed with 50 μ l thawed competent DH10 β cells and incubated on ice for 30 minutes. A heat shock of 42°C was given in a water bath for 45 sec followed by incubation on ice for 2 minutes. Cells were recovered by adding 500 μ l LB media and incubating for one hour shaking at 1200 rpm in a Thermomixer. 250 μ l of the culture was plated on LB-agar plates with the appropriate antibiotic. The plates were incubated overnight in a dry incubator at 37°C. Colonies were picked in appropriate volume of LB media with antibiotics for plasmid DNA extraction.

2.2.1.6.7 Transformation of electrocompetent *E.coli*

1-2 μ l of reaction mixture or DNA was mixed with 50 μ l thawed electrocompetent DH10 β . Cells were then transferred to a long electrode electroporation cuvette with 2 mM path length. An electrical pulse of 2.5kV was provided to the cells followed by recovery of cells by adding 500 μ l LB media. Cells were incubated for one hour shaking at 1200 rpm and 37°C before 50 μ l of the culture was plated on LB-agar

plates with the appropriate antibiotic. The plates were incubated overnight in a dry incubator at 37°C before colonies picked and DNA extracted.

2.2.2 RNA techniques

2.2.2.1 RNA isolation and purification

Bench and pipettes were cleaned by RNaseZap before extraction. Mammalian cells for RNA extraction were lysed using appropriate volume of TRIZOL® depending on the well type (100 µl for 96-well plate to 500 µl for 6-well plate). 1/5 total sample volume of chloroform was added and the sample was centrifuged for 15 mins at 12 g at 4°C. The top aqueous phase was transferred to a new tube and 2.5 V of aqueous phase of 75 % ethanol, 0.1 V of 3 M sodium acetate (pH 5.2) and 20 µg glycogen were added to the aqueous solution and incubated at -80°C for at least two hours before centrifuging for 30 min at 12 g at 4°C. RNA pellets were washed twice with 75% ethanol and dried at room temperature before resuspending in 30 µl of RNase-free water by heating at 55°C for 10 minutes.

2.2.2.2 Quantitative real-time polymerase chain reaction (qRT-PCR)

Quantitative real time PCR (qRT-PCR) was performed using Verso 1-Step Taqman QRT-PCR Low ROX Kit. Primers and probe used for qRT-PCR for NP, IFNB and HRPT2 are listed below in Table 2.18.

2.2.3 Protein techniques

2.2.3.1 Preparation of protein extracts for Western blot analysis

Cells were washed once with PBS before being lysed in NP40 lysis buffer on ice for 30 minutes. After incubation on ice, cell lysates were centrifuged for 10 minutes at

1000 rpm and 4 °C to remove unsolubilised material. The supernatant was resuspended in 2x SDS protein sample buffer and boiled for 10 minutes at 95 °C and analysed by SDS-PAGE.

Primer/ Probe	Sequence
NP Fwd primer	TTCAAACCCAGCCAAATAA
NP Rev primer	TGGCTCAGTTTCTTCACCTG
IFN β Fwd primer	CGACACTGTTCGTGTTGTCA
IFN β Rev primer	GAAGCACAACAGGAGAGCAA
IFN β probe	UPL mouse #16
NP probe	UPL #78
HRPT2 Fwd primer	TGACCTTGATTTATTTTGCATACC
HRPT2 Rev primer	CGAGCAAGACGTTTCAGTCCT
HRPT2 probe	UPL #73

Table 2.18 Sequences of primers and probes used for QPCR analysis.

The sequences of DNA primers for qPCR analysis of SPZ1 and HRPT2 and the UPL probe numbers used are listed.

PCR components	Reaction conditions:
0.1 μ l Verso Enzyme Mix	95 °C for 10 mins
1 μ M Forward Primer	95 °C for 30 sec
1 μ M Reverse Primer	60 °C for 1 min
0.5 μ l Enhancer	} 40 cycles
20 ng Template RNA	
5 μ l 1-Step QPCR low ROX Mix (2x)	
Up to 10 μ l with water	

Table 2.19 QPCR conditions. The PCR reaction components and conditions for the QPCR amplification of RNA are shown.

2.2.3.2 Quantification of protein

Protein was quantified using BCA assay kit according to manufacturer's guidelines. Absorbance was measured and protein quantified using a polar star plate reader (Polar Star Optima, BMG Biotech).

2.2.2.3 Co-immunoprecipitation

Protein interactions in mammalian cells were determined by expressing the two proteins of interest in HEK293 cells. The appropriate quantity of protein was removed for each sample and lysates were pre-cleared with 50 μ l of pre-equilibrated Protein G Sepharose beads by incubating in an over-head rotator for 1 hour at 4°C followed by centrifugation at 2000g. The supernatant was divided into two equal parts and proteins precipitated with 1 μ g of each antibody (anti-c-Myc Ab for bait protein and anti-HA for prey protein with 50 μ l protein G-Sepharose beads. Beads were washed three times with ice-cold NP-40 buffer and were resuspended in 2X Laemmli sample buffer. Samples were boiled for 10 minutes at 95°C and directly analysed by SDS-PAGE.

2.2.2.4 SDS-PAGE

Gel electrophoresis was performed using a twin vertical electrophoresis system with 10-15% resolving gel, depending on molecular weight of the proteins of interest, and 6% stacking gel. The resolving gels were prepared with acrylamide mix to the desired concentration, 375 mM Tris (pH 8.8) and 0.1% SDS. Prior to pouring the gel, 0.1% ammonium persulfate (APS) and 0.01% TEMED were added, mixed and, after pouring, the gel was overlaid with isopropanol to level gel surface. After polymerisation, the isopropanol was removed and the stacking gels were prepared with 6% acrylamide mix, 125 mM Tris (pH 6.8) and 0.01 % SDS. Prior to pouring the

gel 0.1 % ammonium persulfate and 0.01% TEMED were added. The stacking gel solution was poured on top of the resolving gel and a comb with the required number of wells was inserted. After polymerisation, the glass plates containing the gel were assembled in the gel electrophoresis apparatus. The central chamber and base of electrophoresis apparatus was filled with 1x running buffer, and samples were loaded on the gel together with a molecular weight marker ladder. Separation was performed at 75V constant current through stacking gel for 1h and increased voltage to 125V for resolving gel separation for another hour.

2.2.2.5 Western blot

Proteins were transferred to nitrocellulose membranes using the Trans-Blot Semi-dry Transfer Cell (Bio-Rad, UK). A sandwich was formed on the platinum anode as described, with all layers pre-soaked in transfer buffer with 20% methanol (v/v): two layers of filter paper (Whatman, UK), a layer of nitrocellulose membrane, acrylamide gel and two layers of filter paper. Air bubbles were removed by rolling a pipette over the stack and subsequently the cathode was placed followed by the safety cover. Electrophoresis was performed at 15 V for 1 hr. The membrane was incubated in blocking buffer (5% skimmed milk in TBST) for 1 hr at room temperature or overnight at 4°C. Membranes were then incubated for 1 hour on an orbital shaker with primary antibody diluted (usually 1/1000) in blocking buffer. Primary antibody was washed off by incubating three times for 10 minutes in TBST buffer before incubating for one hour with the appropriate secondary antibody diluted (usually 1/3000) in blocking buffer. The membrane was washed a further three times for ten minutes in TBST before proteins were detected using the ECL Western blotting detection reagent according to the manufacturer's instructions. The membranes were exposed to X-ray film for different exposure times and films were developed using X-Ray Film Processor.

2.2.2.6 Co immunoprecipitation

1×10^6 HEK293T cells were seeded in 10 cm dishes a day prior to transfection. Myc and HA amino-terminal fusion proteins (bait and prey plasmids, respectively) were used for co-immunoprecipitation. Since the bait and prey plasmids have a T7 bacteriophage promoter, cells were first infected with recombinant vaccinia virus (NIH AIDS repository) expressing T7 polymerase at an MOI of 10 in DMEM with 1% FCS and no penicillin/streptomycin. Virus was removed after one hour and replaced with complete media. 10 μ g each of Bait and Prey plasmid, of the required interaction partners, were transfected by calcium phosphate transfection. After 48 hours, cells were rinsed in PBS and lysed with 2 ml NP-40 lysis buffer per 10 cm dish on ice for 30 mins. Lysates were centrifuged for 10 minutes at 1000 g and 4°C to remove cell debris and supernatant was used for immunoprecipitation.

2.2.2.7 Immunofluorescence

1×10^5 A549 cells were grown in 8-well chamber slide and transfected with the desired plasmid. 48 hours post transfection, cells were washed three times with PBS and fixed with 4% paraformaldehyde for 15 minutes at room temperature. Cells were washed a further three times with PBS and incubated with 50 mM NH_4Cl for 10 min to quench background fluorescence signals. After quenching, cells were washed twice with PBS and permeabilised with 50 μ l of 0.5% Triton X-100 for 3 minutes. After two washes with PBS, cells were blocked by immunofluorescence blocking buffer for 1 hour at room temperature, then stained overnight with primary antibody diluted 1/100 in antibody dilution buffer. Cells were washed three times with PBS and then incubated for 1 hour with fluorescent secondary antibody diluted 1/500 in antibody dilution buffer at room temperature in the dark. Cells were washed three times with and mounted with Prolong Gold Anti-fade reagent with DAPI and examined using Zeiss LSM 510 META confocal microscope.

2.2.3 Yeast-two-hybrid assay

2.2.3.1 Preparation of competent yeast cells

Two strains of compatible *Saccharomyces cerevisia* yeast strains, AH109 (mating type α) and Y187 (mating type α) were streaked on YPDA plates and grown for 2-3 days before single colonies used to inoculate 50 ml liquid YPDA broth. This pre-culture was grown overnight in a shaking incubator at 30°C and 200 rpm, before 250 ml of fresh YPDA was inoculated with a volume of overnight culture to produce an OD600 value of 0.15. Cultures were incubated at 30°C shaking at 250 rpm until an OD600 of 0.6 was reached. Cell growth was stopped by shaking cells on ice for??, and yeast were pelleted by centrifuging at 2000g for 5 minutes at room temperature, washed once in SBEG and resuspended in 5 ml SBEG, aliquoted in 100 μ l volumes and stored at -80°C until use.

2.2.3.2 Transformation of competent yeast cells

1 μ g plasmid DNA was added to 100 μ l of the appropriate competent yeast strain (bait plasmids were usually transformed into AH109 cells, whilst prey were usually transformed into Y187 cells) and 750 μ l of PEG/Bicine solution was added. Yeast were incubated for one hour at 30°C shaking at 900 rpm before heat-shocking at 45°C for 5 min. Cells were pelleted by centrifuging for 2 min at 3000 rpm and the pellet was washed in 1 ml NB buffer by centrifuging for 5 min at 3000 rpm. Cells were resuspended in 200 μ l NB buffer and plated on SD-agar plates lacking the appropriate selective amino acid (minus leucine for prey; minus tryptophan for bait). Plates were incubated at 30°C for 3 days until colonies appear.

2.2.3.3 Protein interaction assay

Direct yeast two-hybrid screen was done to test for interactions between two proteins. Yeast colonies transformed with the appropriate bait and prey plasmids were picked and grown overnight at 30°C in single selective SD media [minus tryptophan (-W) for Bait, and minus Leucine (-L) for prey]. Bait and prey strains were mated in double selection media (SD-LW) supplemented with 5% YPDA broth and incubated overnight at 30°C. The following day, diploid yeast were selected by transferring a small volume of mated yeast into diploid selection media (SD-LW). After a further 2-day incubation at 30°C, yeast containing interacting proteins were selected by transferring into triple selective SD-LWH media and incubated at 30°C for 6 days for selection of interactions. An inhibitor of interaction 3-Amino-1, 2, 4-triazole (3-AT) was used in increasing concentrations (0 3-AT, 1 mM 3-AT, 2.5 mM 3-AT, 5 mM 3-AT, and 10 mM 3-AT) to competitively inhibit the interaction. Auto-activation was tested for every bait and prey by testing against empty prey or bait. Interaction was detected by growth on agar plate or fluorescence measurement in liquid culture. A substrate 4-methylumbelliferyl- β -xylobioside (4-MuX) was added to SD-LWH broth to measure fluorescence readout (excitation at 365nm; emission at 448nm).

2.2.4 Mammalian cell culture, infection and cellular reporter assays

2.2.4.1 *Maintenance of cell lines*

All cell lines were maintained in humidified incubators at 37°C and 5% CO₂. Cultures were passaged when they reached 90-95% confluency by washing with PBS and treating with trypsin/EDTA solution at 37°C until all cells were detached from the flask. Cells were recovered by adding complete growth media and centrifuged at 1000 rpm for 10 minutes in a benchtop centrifuge. The cell pellets were resuspended in growth medium and counted by use of a haemocytometer and passaged into new flasks in the ratio of 1:3 – 1:5 or seeded as required for experiments.

2.2.4.2 Freezing of cell lines

Cells were trypsinised and counted as described above. Cells were frozen at a density of 1×10^7 cells/ ml in pre-cooled freezing media (70% growth media, 20% FCS and 10% DMSO) in sterile cryovials (Nunc, UK). Cells were transferred to a cryopreservation box (Mr Frosty, Nalgene) and frozen overnight at -80°C , at a rate of 1 degree per minute, before transferring into liquid nitrogen tank for long-term storage.

2.2.4.3 Thawing of frozen cell lines

Cells were thawed rapidly by warming in a 37°C water bath until only just thawed. Cells were transferred into 15 ml centrifuge tubes and recovered by adding growth media dropwise before centrifuging at 1000 rpm for 10 minutes. The cell pellet was then resuspended in complete growth media and transferred to a culture flask.

2.2.4.4 Determination of the cell number

Cells were trypsinised and concentrated in 10 ml appropriate media and 10 μl of cells were added into a slide chamber (HYCOR: KOVA, cat. no. 87144) and the number of cells in 3 squares was counted under a microscope at 10x magnification. The number of cells per ml was calculated as follows.

$\text{Cells/ml} = \text{average number of cells per square} \times 1000$

2.2.4.5 Transfection of plasmids into cells

2.2.4.5.1 Calcium phosphate transfection

Calcium phosphate transfection into 10 cm dishes was done as follows. 6×10^5 to 2×10^6 HEK293 cells were seeded one day prior to transfection and incubated at 37°C . A total of 10 μg DNA to be transfected was mixed with 50 μl 2.5M CaCl_2 and made up

to a volume of 500 µl with 0.1x TE. The DNA-CaCl₂ mixture was added dropwise on a vortex into a bijoux containing 500 µl of 2x HBS and incubated at room temperature for 20 minutes. The transfection mix was added to the cells and incubated at 37°C for 24 hours. Transfection into different sizes of tissue culture dishes were done by scaling down the reaction volumes. 2.2.4.5.2 Lipid transfection

For each cell line, optimal lipofection reagent and transfection conditions were first determined. A range of different lipofection reagents were used according to manufacturer's instructions.

2.2.4.6 Preparation of virus stocks

2.2.4.6.1 Preparation of Influenza A virus

To grow influenza A viruses, 90% confluent MDCK cells in T75 were washed twice in PBS to remove serum, and then infected in serum-free DMEM at an MOI of 0.001 PFU/cell. After 2 h rocking at 37°C, the inoculum was removed and monolayer were washed with 5 ml serum-free DMEM. 12 ml of serum-free DMEM supplemented with 2 µg/ml TPCK-treated trypsin was added to the cells. Cells were incubated at 37°C with 5% CO₂ for 36 h to 48 h, and supernatants were harvested when 50-70% cytopathic effect was observed. Debris was removed by centrifugation at 3000g for 10 mins, and aliquots were frozen at -80°C. Titres were determined by plaque assay.

2.2.4.6.2 Preparation of modified Vaccinia Ankara (MVA)

To grow modified Vaccinia virus Ankara (MVA), 46 T175 flasks of BHK-21 cells were infected with a crude stock of MVA at an MOI of 0.5 for 2 hours in pen-strep free DMEM containing 5% FCS. Fresh complete medium containing 10 % FCS and 1% pen-strep was added to the flasks and incubated for 48 h to 72 h at 37°C until a cytopathic effect of 70%-80% was observed. The flasks were frozen at -80 °C

overnight before thawing and shaking forcefully when the ice layer loosened. The contents of the flasks were pooled into sterile 250 ml centrifuge bottles and centrifuged at 16000 g for 90 min. The pellets were resuspended in total of 50 ml 10 mM tris resulting in a crude stock of virus. The crude stock was freeze-thawed three times followed by sonication for 4 x 15 sec. 15 ml of 36% sucrose was added into six 35 ml centrifuge tubes and 15 ml of virus suspension was slowly pipetted on top. These were centrifuged for 90 min at 70000 g and each pellet was resuspended in 0.5 ml 1 mM tris, aliquoted in 100 µl volumes and stored at -80°C.

2.2.4.7 Titration of Influenza viruses by plaque assay

MDCK cells were grown in 6-well plates until 80-90% confluent, and washed twice in serum-free DMEM to remove serum. Virus preparations were serially diluted 10-fold in serum-free DMEM, and cells were inoculated with 1 ml of each virus dilution per well. After 2h on a rocking platform at 37°C, the virus inoculum was removed and cells overlaid with 6 ml of DMEM containing 0.2% BSA, 2µg/ml TPCK-trypsin mixed with an equal volume of 1% agarose dissolved in water. After the agarose overlay had set, monolayers were incubated at 37°C with 5% CO₂ for 3-4 days without agitation until distinct plaques had formed. Cells were fixed by adding 5% formaldehyde in PBS on top of the agarose plugs, and the monolayers were left room temperature to fix overnight. Plaques were stained with 0.1% Toluidine Blue O in water and titres calculated as plaque-forming units per ml (PFU/ml).

2.2.4.8 Infection assays

The influenza A virus strain used in this study is mainly recombinant wild-type influenza A virus (A/Udorn/72). For the experiments that determination of infectious virus was not required, no trypsin was added to the medium. Cells were infected with virus diluted in serum-free DMEM at required multiplicity of infection

(MOI). Monolayers were washed prior to infection in PBS to remove all traces of serum. After an adsorption period of 1h on a rocking platform at 37°C, the virus inoculum was removed and replaced with serum-free DMEM. Cells were incubated at 37°C with 5% CO₂ until harvested. To determine infectious titres over multiple replication cycles, 90% confluent cells were washed twice in PBS to remove serum and infected at required MOI in serum-free DMEM for 1h on a rocking platform at 37°C. After the adsorption period, the virus inoculum was removed by washing twice in PBS, and the cultures were subsequently incubated in serum-free DMEM containing 0.2% BSA and 2µg/ml TPCK-treated trypsin. Samples of the culture supernatants were removed at different time points post infection, and virus titres were determined by plaque assay as described below.

2.2.2.9 Dual luciferase reporter assay

HEK293T cells were plated on 24-well plate 24 hours before transfection. Transfection reactions were prepared using the desired volumes of DNA for each experiment. pRL-TK expressing Renilla luciferase was included if required. Cells were washed and lysed 48 hours post transfection with 1X Passive Lysis Buffer for 15 mins at room temperature. 20 µl of cell lysate were transferred into 96-well black plate with inner white wells. 30 µl of LARII reagent from Promega Dual luciferase assay kit was added to each well and relative luciferase units measured on a plate reader on the luminescence setting. After the plate was read, 30 µl of Stop and Glo reagent was added to each well to quench the firefly luciferase activity and read again on luminescence settings to measure Renilla luciferase activity. Relative luciferase activity was calculated by normalising the firefly luciferase value to that of the Renilla luciferase per sample.

Chapter 3: Virus-host protein interaction analysis between Influenza and cellular proteins

3.1 Introduction

Viruses employ numerous host cellular machineries to replicate despite their simple structure. In every step of their life cycle, including attachment to host cell surface receptors and entry into the cells, uncoating of the viral nucleic acid and genome replication, synthesis of new copies of viral proteins, assembly of progeny virions and finally cellular egress, host cellular functions are involved (Watanabe *et al.*, 2010). Interactions between viruses and the host cell machinery are therefore vital for their survival and replication. The availability of genome-wide approaches has enabled studies to systematically identify host factors involved in virus life cycle. Among these genome-wide approaches, high-throughput assays such as Y2H, proteomics, luminescence based mammalian interactome assay (LUMIER) provide a valuable tool to look at virus-host protein interactions. In the last ten years, Y2H has proved to be one of the most powerful tools to understand the interplay between viruses and their host, some of which are HSV, KSHV, HIV, vaccinia virus (Griffiths *et al.*, 2013, Uetz *et al.*, 2006, König *et al.*, 2008, McCraith *et al.*, 2000).

In influenza viruses, loss-of-function RNAi-based studies by Brass *et al.*, Hao *et al.*, Karlas *et al.*, König *et al.* and Shapira *et al.* provided insight into host factors affecting viral replication, whilst the genome-wide yeast-two-hybrid screen by Shapira *et al.* provided information about physical interaction partners of host-influenza A proteins. A study by Watanabe *et al.*, generated influenza virus-host interactome by combining liquid chromatography–mass spectrometry (Table 3.1) (Brass *et al.*, 2009, Hao *et al.*, 2008, Karlas *et al.*, 2010, König *et al.*, 2010, Shapira *et al.*, 2009, Watanabe *et al.*, 2014, Watanabe *et al.*, 2010). However, only a few influenza A virus strains like PR8 H1N1, WSN H1N1 or Ud H3N2 were employed in these studies, and the

overlap between the proteins identified in these studies is generally quite small. Moreover, due to the diversity of influenza A viruses there is a huge gap in our understanding of key cellular factors involved in life cycle of distinct strains. The group of thesis supervisor thus performed a genome-scale yeast two-hybrid protein interaction screen using 6 different influenza A virus strains to gain insight into common and distinct host interaction partners. The aim of the work presented in this Chapter was to analyse Y2H screen. In this Chapter the results of the Y2H screen which was done at the Preclinical Target Development and Genomics and Proteomics Core Facility of German Cancer Research Center in the laboratory of Dr Manfred Koegl are analysed and interpreted to select host interactors for further follow-up studies.

3.2 Results

3.2.1 Y2H to identify cellular binding partners of influenza proteins

In order to identify interactions between cellular proteins and of influenza viral proteins, 53 ORFs from 6 different influenza A virus strains were cloned into bait vector by Dr Suzanne Esper (Figure 3.1). An automated genome-scale yeast two-hybrid screen was subsequently performed at the Preclinical Target Development and Genomics and Proteomics Core Facility of German Cancer Research Center in the laboratory of Dr Manfred Koegl. The influenza bait proteins were screened against several different cDNA prey libraries from human brain, testis and HeLa epithelial cell line (commercial libraries purchased from Clontech Inc.), as well as a normalised hORFeome v3.1 library generated by pooling 12,381 clones derived from the Mammalian Genome Collection (MGC), and a chicken library. Since the Y2H system generally suffers from a high rate of false negatives, orthologous viral proteins from at least two different strains were screened against several different human and chicken libraries. For NS1, NP and PB1-F2, proteins from strains of

avian and swine origins, as well as high and low pathogenicity variants were also included. The avian influenza virus proteins were additionally screened against a chicken prey library.

Reference	Method	Cells used for RNAi Screen	Virus Strain	Detection Methods	Steps in the Viral Life Cycle Covered by the Screen	No. of Hits/ Genes Tested
Brass <i>et al.</i> (2009)	RNAi screen	Osteosarcoma cells (U2OS)	PR8; H1N1	HA expression on cell surface	entry, uncoating, nuclear import, transcription, translation, HA trafficking to cell surface	133/17,877
Hao <i>et al.</i> (2008)	RNAi screen	Drosophila cells	WSN; H1N1 virus possessing VSV glycoprotein G and NA-Luc genes	luciferase activity	uncoating, nuclear import, transcription, translation	110/13,071
König <i>et al.</i> (2010)	RNAi screen	human lung cells (A549)	recombinant WSN possessing HA-Luc reporter gene	luciferase activity	entry, uncoating, nuclear import, transcription, translation	295/19,628
Karlas <i>et al.</i> (2010)	RNAi screen	human lung cells (A549)	WSN (H1N1); H1N1 2009	virus yield determined by NP staining and luciferase activity	entry, uncoating, nuclear import, transcription, translation, nuclear export, protein transportation/processing, assembly, budding	287/22,843
Shapira <i>et al.</i> (2009)	Y2H; microarray; RNAi screen	HBEC	PR8 (H1N1); Udorn (H3N2)	virus yield determined by luciferase activity	entry, uncoating, nuclear import, transcription, translation, nuclear export, protein transportation/processing assembly, budding	616/1,745 (RNAi screen)
Sui <i>et al.</i> (2009)	RHGP	MDCK cells	Udorn (H3N2)	cloning of cells resistant to influenza virus infection	entry, uncoating, nuclear import, transcription, translation, nuclear export, protein transportation/processing assembly, budding	110 clones, sequenced
Watanabe <i>et al.</i> (2014)	Co-IP + LC/MS; RNAi screen	HEK293 cells	WSN (H1N1)	MS analysis, plaque assay, RT-qPCR	Early steps of viral life cycle, viral genome replication/transcription, virus-like particle (VLP) formation, vRNP incorporation into virions	323/1,292

Table 3.1 Summary of genome-wide screens for host factors involved in influenza virus life cycle. The table is adapted from review by Watanabe (Watanabe *et al.*, 2010, Watanabe *et al.*, 2014, Brass *et al.*, 2009, Hao *et al.*, 2008, König *et al.*, 2010, Karlas *et al.*, 2010, Sui *et al.*, 2009).

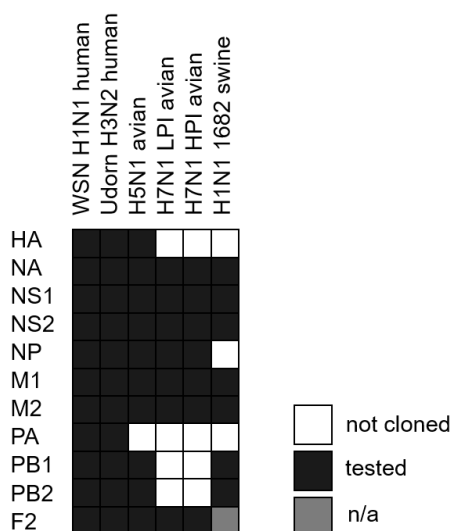


Figure 3.1 Influenza viral ORFs cloned and tested by Y2H interaction screen. In total 53 ORFs from 6 different influenza A virus strains were cloned and tested by Y2H analysis. Some viral ORFs could not be cloned. The 2009 pandemic H1N1 strain 1682 lacks PB1-F2. All the clones were generated by Dr Suzanne Esper.

All bait plasmids were transformed into the yeast strain AH109 (α mating type) and the prey libraries into the yeast strain Y187 (β mating type). Each bait was titrated against different concentrations (0-10mM) of the competitive inhibitor 3-aminotriazole (3-AT) to identify the optimal concentration for the subsequent Y2H screen to decrease the number of false positives and get a high confidence interaction dataset. The screen was performed using the pre-determined 3-AT concentration by plating mated yeast in ten 96-well dishes per bait. Individual colonies were picked and grown on SD-LWH agar plates. The interactor was identified by amplifying the prey gene by PCR, sequencing and NCBI BLAST screen. In total 2741 interaction events and 920 distinct interactions were observed. By filtering for specificity and reproducibility, the number was reduced to 127 high-confidence interactions between viral orthologues and cellular proteins (Table 3.2).

gene id	Prey gene symbol	M2	UDORN WSN	M1	UDORN WSN	NA	UDORN WSN	H5N1	H7N1-HP	H7N1-LP	UDORN	WSN	H7N1-HP	SWINE	H7N1-LP	UDORN	WSN	UDORN	WSN	UDORN
3198	HOXA1										67	149								
10606	PAICS							34	16		46	38								
6895	TARBP2												24	28	73	59	53			
219402	MTIF3	1	87										43	12	26	13	33			
8575	PRKRA												8	2	40					
1995	ELAVL3									58										
1398	CRK																			
7157	TP53							3	4		11	11								
64393	ZMAT3															7	16			
5721	PSME2								3		4	15								
9097	USP14												6	7	7					
64979	MRPL36							9			3	6								
79698	ZMAT4												44	23	51	1	17			
5702	PSMC3																			
6472	SHMT2		15																	
91283	C9orf30							3	2		3	5								
2275	FHL3																			
51144	HSD17B12															7	6			
8503	PIK3R3															12	1			
8106	PABPN1							4	3		1	4								
129831	RBM45							4	8											
3839	KPNA3							1	8			1								
93426	SYCE1															10				
90843	TCEAL8																			
55856	ACOT13					1		4	5		1	4		9						
23633	Kpna6										6	1								
28958	CCDC56								2									8		
8557	TCAP															8				
1635	DCTD							5	2											
6780	STAU1												1	2	3					
23567	ZNF346												4		2					
51727	CMPK1			2	4															
2495	FTH1							1			4	1								
60491	NIF3L1							1	1		1	3								
6428	SFRS3											2						4		
79622	SNRNP25										6									
6651	SON												1	2	2	3				
10040	TOM1L1			6																
284338	PRR19												1	2	2					
816	CAMK2B							3	2											
54810	GIPC2										3	2								
3615	IMPDH2							2	1		1	1								
84236	RHBDD1										3	2								
6241	RRM2	2	3																	
7126	TNFAIP1											5								
372	ARCN1			2							1								1	
9793	CKAP5					2					2									
1739	DLG1														4					
23421	ITGB3BP		4																	
3836	KPNA1							1			3									
23639	LRRC6															2			2	
4101	MAGEA2																		4	
266740	MAGEA2B																		4	
5074	PAWR								3			1								
29990	PILRB																		4	
10616	RBCK1										4									
23029	RBM34							1							3					
10138	YAF2											4								

Table 3.2 List of the 50 most frequently isolated interactions observed in the Y2H interaction screen. The numbers indicate the number of times an interaction was detected. Viral proteins for which no interactions were observed are not listed. Promiscuous proteins which are frequently isolated from the respective libraries and are likely to represent false positives are not displayed.

The Y2H screen was done in German Cancer Research Center in the laboratory of Dr Manfred Koegl.

To validate our results and receive an independent estimate for the reliability of the dataset, LUMIER pull-down assays were performed for 47 selected interactions. ORF clones for 47 cellular interactors (in the pDONR223 entry vector) could be obtained, and were subsequently subcloned into the LUMIER vectors, pRenilla and pTREX. HEK293 cells were transfected with pairs of pRenilla and pTREX vectors expressing the viral and cellular proteins that were found to interact in the Y2H system. Cells were lysed 40 hours post transfection in lysis buffer containing sheep anti-rabbit IgG-coated magnetic beads. Lysates were incubated on ice for 15 minutes. Luciferase activity was measured in the lysate as well as in washed beads. Cells transfected with the plasmid expressing the luciferase fusion protein and a vector expressing a dimer of protein A were used as negative control. Normalised interaction signals were z-transformed by subtracting the population mean and dividing through the standard deviation. The mean and standard deviation were calculated from large datasets of protein pairs which were not expected to interact, i.e. from negative reference sets. Interactions were considered positive if the z-score was >1 in at least one of the tested species. Overall, 38% (18/47) of the tested interactions could be validated in these assays with at least one influenza strain. The LUMIER assay results were largely similar between proteins of different influenza strains as shown in Figure 3.2.

3.2.2 Analysis between the Y2H screen and other genome-wide screens

Similar to our data, Shapira *et al.* reported a large number of interactions between influenza virus proteins and host cellular proteins based on a genome-scale Y2H screen (Shapira *et al.*, 2009). A comparative analysis of the two datasets identified 13 common virus-host interactions, in particular with NS1 and NP, and thus revealed a highly significant ($p < 10^{-9}$) overlap. However, while Shapira *et al.* report several

additional viral proteins binding to these host proteins in most cases, we only observed single viral proteins interacting with the host cell protein. Two interactions that were also identified by Shapira *et al.* were excluded from our further analysis since they included cellular proteins known to be highly promiscuous binders. In summary, our Y2H screen yielded 114 novel and 13 previously reported interactions.

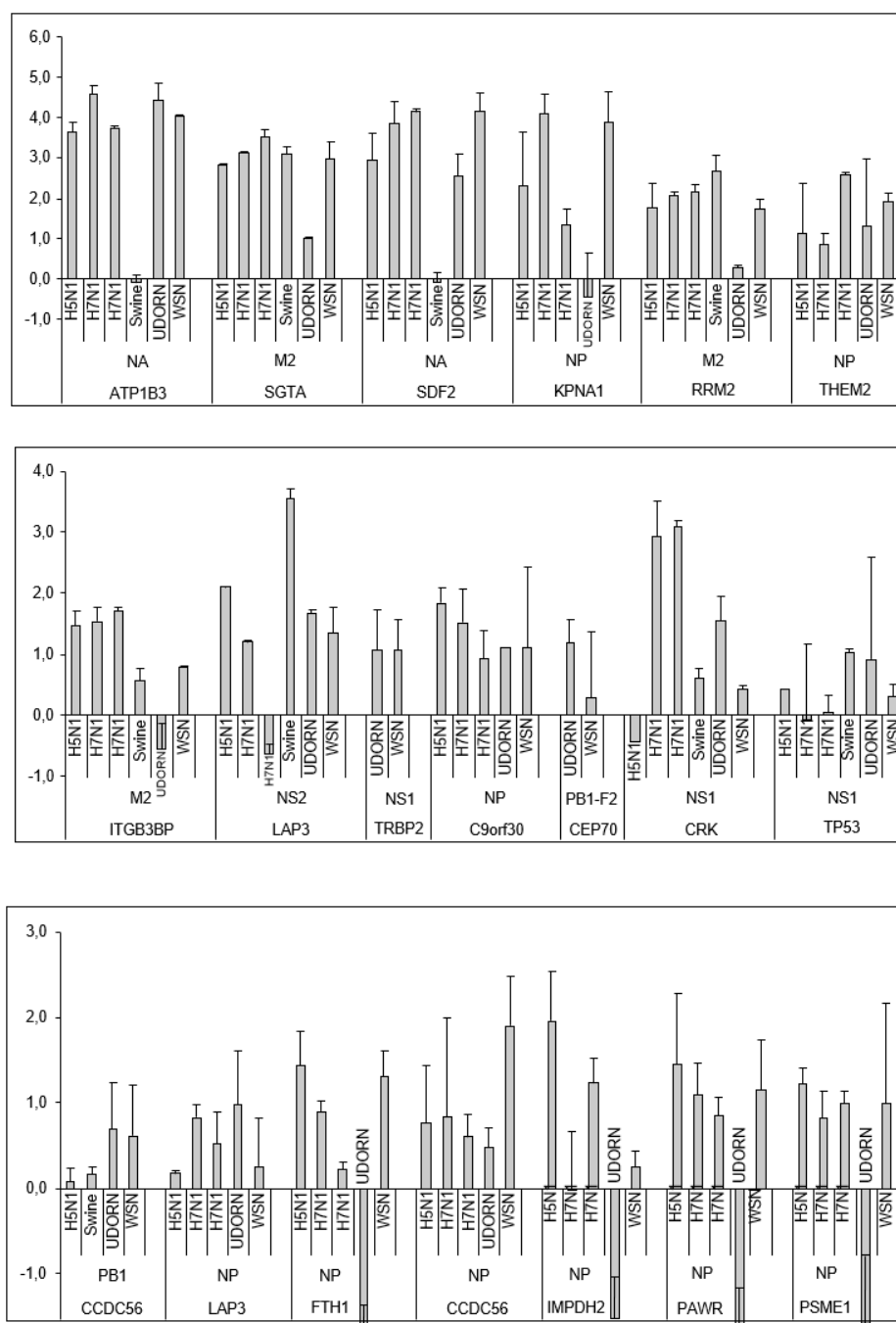


Figure 3.2 Validation of protein interactions found in Y2H Screens by LUMIER assays. Validation by LUMIER assays was done in German Cancer

Research Center in the laboratory of Dr Manfred Koegl. 20 interactions are shown, including all positives, and two orthologous groups that scored negative (TP53-NS1 and CCDC56-PB1). Y Axis represents z-scores. Results from triplicates are shown +/- standard deviations. Lack of interactions in some cases may be due to technical reasons, e.g. the propensity of Ud/NP to yield strong signals with the negative control resulted in negative values in several tests.

A: Overlap of datasets

		1557	51	127	97	251	86	287	295	526
		all	data-bases	Koegl	Shapira Y2H	Brass	Hao	Karlas	König	Shapira siRNA
1557	all									
51	databases	14								
127	Koegl	30	6							
97	Shapira Y2H	34		13						
251	Brass	42	1	3	0					
86	Hao	30	2	2	0	13				
287	Karlas	65	3	3	0	15	15			
295	König	58	3	4	1	10	7	34		
526	Shapira siRNA	73	4	10	25	16	5	15	14	

B: Enrichment of overlapping genes (fold over the number of genes expected by chance)

		all	data-bases	Koegl	Shapira Y2H	Brass	Hao	Karlas	König	Shapira siRNA
	all									
	databases	4								
	Koegl	3	20							
	Shapira Y2H	5		23						
	Brass	2	2	2	0					
	Hao	5	10	4	0	13				
	Karlas	3	5	2	0	5	13			
	König	3	4	2	1	3	6	9		
	Shapira siRNA	2	3	3	11	3	2	2	2	

Table 3.3 Overlap of human host factors found to affect influenza replication or interact with influenza proteins in large-scale genomic studies.

Analysis was done in German Cancer Research Center in the laboratory of Dr Manfred Koegl. Numbers in the top row and left column indicate total number of proteins identified. Y2H screen in this study was highlighted in red. Table A shows numbers of genes that overlap in the two respective datasets. Table B shows the enrichment of genes over the number of genes expected to overlap by chance. P-values were calculated by Wilcoxon signed rank test with continuity correction. All enrichments were highly significant ($p < 10^{-9}$), except for the overlap of the Shapira Y2H dataset with those of the siRNA screens other than the tests done in the same lab.

A combined dataset of interactions between influenza virus and human proteins was assembled from two public databases (VirusMint and VirusHosNet), including

the Y2H dataset reported by Shapira *et al.* and the Y2H results presented in this work (Shapira *et al.*, 2009). A functional GO-term analysis of the combined set of 255 host proteins interacting with influenza virus proteins showed several enriched categories in Figure 3.3. While most of the pathways were already significantly enriched in one of the two large Y2H-based datasets, the statistical significance became greatly enhanced in the combined dataset. For example the category “cell cycle” was not significantly enriched in either of the two single datasets, but highly significant enriched in the combined dataset. Shown in Figure 3.3, enriched categories fell into the areas of intracellular transport, RNA-processing, protein folding, cell cycle and signal transduction.

	GO-Term	all interactions	Koegl	Shapira	all siRNA hits	overlap all
intracellular transport	intracellular protein transport					
	NLS-bearing substrate import into nucleus					
	nuclear lumen					
	nuclear transport					
	nuclear pore					
	nucleolus					
RNA processing	double-stranded RNA binding					
	RNA processing					
protein folding	response to unfolded protein					
	protein complex biogenesis					
	identical protein binding					
cell cycle	cell cycle					
	DNA replication initiation					
signal transduction	phosphoinositide 3-kinase regulator activity					

Figure 3.3 Functional annotation of influenza virus host factors by the DAVID algorithm. Analysis was done in German Cancer Research Center in the laboratory of Dr Manfred Koegl. Enrichment analysis represents host proteins identified in this Y2H study, Y2H study by Shapira *et al.*, siRNA screens studies and two datasets VirusMint and VirusHostNet (all interactions), as well as a list of proteins which were

observed in at least two independent studies (overlap all). Significance is color-coded as follows: $p < 0.05$, light grey, $p < 0.01$, dark grey, $p < 0.001$, black.

Five published studies reported genome-scale siRNA knock-down screens for host factors affecting the influenza virus life cycle in human and fly cells (Brass *et al.*, 2009, Karlas *et al.*, 2010, König *et al.*, 2010, Shapira *et al.*, 2009, Hao *et al.*, 2008). A comparison of the results of these screens shows a low but significant overlap of the identified genes. A similarly low overlap was also observed in three siRNA screens for host factors that play a role in HIV life cycle (Zhou *et al.*, 2008, König *et al.*, 2008, Brass *et al.*, 2008). Sixteen of the 114 high-confidence host factors identified in our Y2H screen, have also been identified in at least one siRNA study screen, which is a significantly higher number than expected by chance ($p < 10^{-9}$). Among these 16 proteins, 11 were also identified by Shapira *et al.* and 5 in one or more of the four RNAi studies. Within the 97 host proteins isolated by Y2H screen by Shapira *et al.*, 25 were overlapped with the genes identified in the siRNA screen in the same study, but only one of them was positive in any of the other four siRNA studies. This could be due to methodological differences in the siRNA screens. Interestingly, the extent of overlap of host cell proteins is similar when comparing Y2H studies to siRNA screens, or siRNA screens amongst each other. The set of 1,345 genes implicated to have a role in influenza virus life cycle by siRNA screens shares 46 genes (APP, ARCN1, ATP6V0B, C10orf57, CALM2, CAMK2B, COL1A1, COPB1, COPS6, CRK, CUL1, DAAM1, DSP, EEF1A1, EIF4A3, EPRS, FBXL5, FHOD1, GPR146, HECTD1, IFI16, IL1RAP, ISL1, KPNA5, MT-ATP8, PAICS, PDGFRA, PIK3R3, PRKRA, PTS, RBCK1, RBM34, SFRS5, SFRS7, SNW1, SON, STAU1, TARBP2, TCAP, TCF20, TP53, UBE2A, UBR4, VEGFB, ZMAT3, ZMAT4) with the set of 255 influenza virus-host interactions, which is highly significant compared to 16 genes expected by chance ($p < 10^{-9}$). The interaction of NS1 with TARBP2 (TRBP) and with PRKRA (PACT) were among the 6 most frequently identified interactions and were found independent of the influenza virus strain (Table 3.2). A GO-term analysis of the combined set of siRNA hits from the five studies highlights 12 classes of enriched terms. Four of these (intracellular transport, RNA processing, protein folding, cell

cycle and signal transduction) overlap with those enriched within the set of host cell interactors (Figure 3.3). Interestingly, for a consensus list of 165 genes that were reported in at least two independent siRNA or protein interaction screens, the enrichment in most of the above categories is also significant. However, several classes of GO categories including “protein phosphorylation”, “regulation of cell death”, “ribosomal translation”, “vesicular trafficking”, “proton transport”, “transcription” and “ubiquitination” were only highly enriched in the siRNA screens but not in the protein-interaction datasets.

3.3 Discussion

Systematic, genome-wide screens are powerful tools for identifying host factors involved in the viral life cycle. The Y2H screen presented here identified 127 interactions between influenza viruses and host cellular proteins. The observed interactions are in many cases highly reproducible between viral orthologues, i.e. when an interaction was observed with a bait from one viral strain, it was also observed with other orthologous proteins from the other strains. The interactions isolated from the different libraries did not overlap in most cases, with the exception of PAICS binding to NP, as well as RBCK1, TARBP2 and SON binding to NS1. The binding of SON to NS1 was observed in screens of both human and chicken libraries. Notably, all of these 4 host proteins were also reported as relevant factors in recent genome-wide siRNA screens (Karlás *et al.*, 2010, König *et al.*, 2010, Shapira *et al.*, 2009). However, genome-wide approaches have limitations. The Y2H approach employed here potentially has a fairly high rate of false negatives due to several reasons, for example it has difficulty in identifying interactions of membrane proteins. In terms of the published studies based on RNAi screen, siRNA library does not cover entire human genome, the knockdown efficiency of target gene expression by siRNA could differ, and knockdown of certain genes may lead to cytotoxicity effects that these genes are eliminated from identification even if they

may be involved in virus life cycle (Watanabe *et al.*, 2010). Therefore, the identification of host gene candidates involved in virus replication by any primary screen really represents a start, more detailed functional studies on host factors identified in genome-wide screens are essential.

One of the top hits from our Y2H screen was between the influenza virus NP protein and PAICS (Table 3.2). All the NPs interact with PAICS except the NP from the H7N1 LP strain. NP/H7N1LP also fails to interact with a number of other cellular proteins including TP53, C9orf30, PABBP1, COT13, etc., which interacted with the NP from the other strains tested. Interestingly, amino acid sequence alignment of all six NP proteins (Figure 3.4) shows that NPs from both HP and LP H7N1 strains are shorter in the C-terminal than others. However, the only difference between HP and LP NPs is at residue 350. This residue in NP of H7N1 HP strain is replaced by an alanine that could be responsible for the interactions between NPs and a number of cellular proteins in N7N1 avian strains. That these interactions were not observed in NP of H7N1 LP strain in Y2H screen might be due to the mutation of this residue.

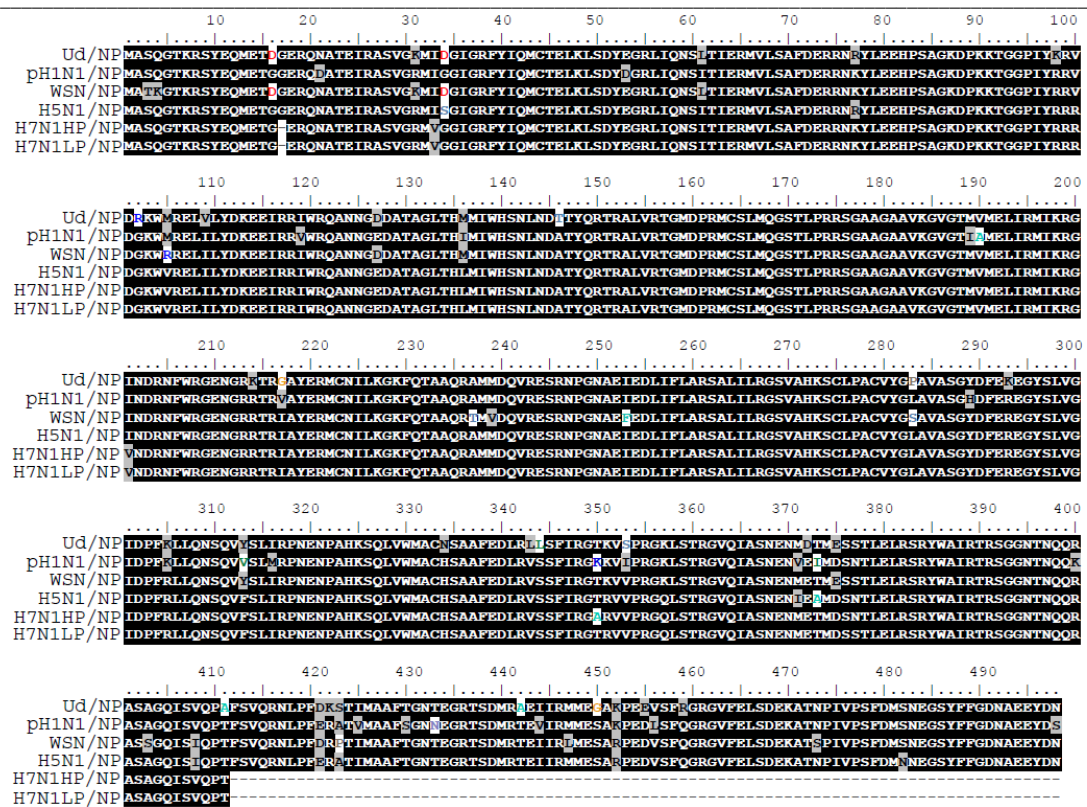


Figure 3.4 Amino acid sequence alignment of NP proteins used in Y2H screen. The accession number of NP proteins are CY009639, GQ332644, CY034135, CY021520, GU052933 and GU052941. Alignment was performed by Clustal Omega program (<http://www.ebi.ac.uk/>), figure was generated by using BioEdit.

PAICS is a bifunctional enzyme which is involved in purine biosynthesis that contains phosphoribosylaminoimidazole carboxylase activity in its N-terminal part and phosphoribosylaminoimidazole succinocarboxamide synthetase in its C-terminal region (Li *et al.*, 2007, Taschner *et al.*, 2013). It catalyses steps 6 and 7 of purine biosynthesis. Whilst rapidly dividing cancer cells rely heavily on the purine de novo pathway for synthesis of adenine and guanine, normal cells favour the salvage pathway. By using SILAC, Kroeker, *et al.* reported that groups of proteins consistently up-regulated by PR8 virus infection are associated with protein metabolism and modification, purine biosynthesis, cytoskeletal proteins and carbohydrate metabolism, proteins involved in purine biosynthesis including PAICS are upregulated (Kroeker *et al.*, 2013). In A549 cells, transient overexpression of PAICS increases replication of Ud virus as shown by an increased expression of

NP (Figure 3.5). It is not known yet whether PAICS possesses other functions other than its role in purine biosynthesis or NP is involved in host metabolic pathways. Thus interaction between PAICS and NP may shed light on novel function of PAICS. It would also be interesting to confirm whether PAICS has an impact on viral polymerase activity. In future investigation, a minigenome reporter can be used to check viral polymerase activity. This system contains the firefly luciferase gene flanked by the non-coding regions of the influenza NS gene segment, transcribed from a species-specific polI plasmid with a mouse terminator sequence (Pleschka *et al.*, 1996) and makes it possible to assess viral polymerase activity *in vitro*.

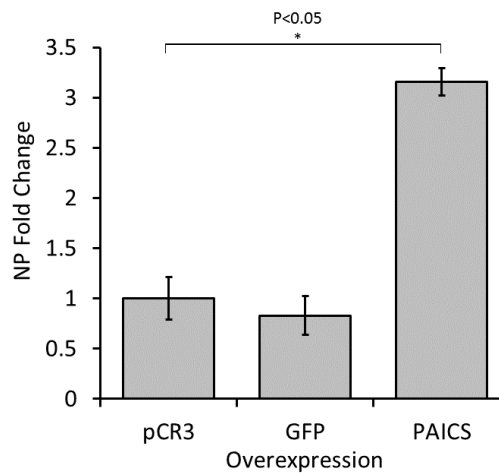


Figure 3.5 Overexpression of PAICS enhances influenza replication as measured by NP expression. A549 cells in 96-well plate were transfected with 100ng empty pCR3 or pCR3 plasmid expressing GFP or PAICS. Cells were infected with Ud virus at MOI=1 24h post transfection. Cells were fixed 9 hours post infection and stained with NP primary antibody and fluorescence secondary antibody. Fluorescence intensity was analysed by LICOR system. Error bars represents standard deviations from triplicate. Statistically significant are indicated with p-value calculated against pCR3 control.

Some of interactions identified in our Y2H screen may provide mechanistic insight into host factors identified that have been published in siRNA screens. König *et al.* discuss the role of ARCN1, subunit δ of COPI complex in influenza virus infection (König *et al.*, 2010). König *et al.* suggested knockdown of ARCN1 and the other subunit COPG by siRNA blocked virus entry (König *et al.*, 2010). Sun *et al.* suggested

that long-term siRNA COPI depletion in cells has an indirect effects on viral entry through defective cargo uptake and vesicular trafficking to late endosomes, while COPI complex inhibitors impair viral membrane protein expression and assembly (Sun *et al.*, 2013). M1 can bind to lipid, it lays underneath the viral membrane and forms the critical bridge between the viral envelope and vRNP. M1 interacts with the cytoplasmic tails of HA and NA. M1 also interacts with M2, vRNA and NP. M1 has a critical role in many steps of the virus replication which includes regulation of uncoating and disassembly, nuclear import and export of vRNPs, transcription and replication of vRNA, transport and assembly of vRNPs and virus budding and morphogenesis (Nayak *et al.*, 2013). Particularly after fusion M1 coimmunoprecipitates with HDAC6, a component of the aggresome pathway that interacts with the motor protein dynein and microtubules. Aggresome formation and disassembly machinery promote viron uncoating (Banerjee *et al.*, 2014, Rajsbaum and Garcia-Sastre, 2014). As interaction between M1 and ARCN1 was identified in our screen, although ARCN1 could be involved in viral entry, considering other overlapping roles of M1 and ARCN1, this interaction may provide additional mechanistic insight into steps of the virus replication which involve M1. CAMK2B and KPNB1 are discussed by König *et al.* for a role in viral RNA transcription and nuclear accumulation of NP, respectively (König *et al.*, 2010). We show that KPNB1 and other karyopherins as well as CAMK2B directly bind to NP, which may provide the molecular link for this observation. Karlas *et al.* suggest SON plays a role in trafficking of virions to late endosomes early in infection (Karlas *et al.*, 2010). However, we observed binding of SON1 to NS1 in screens with human and chicken NS1. NS1 is a non-structural viral protein, in other words, during trafficking of virions by SON cannot involve NS1. Our data suggest that SON could be involved in other stage of virus replication. SON is an RNA-binding protein which acts as a mRNA splicing cofactor by promoting efficient splicing of transcripts that possess weak splice sites. It specifically promotes splicing of many cell-cycle and DNA-repair transcripts that possess weak splice sites. SON can also bind to a specific DNA sequence upstream of the upstream regulatory sequence of

the core promoter and second enhancer of HBV. This binding represses HBV core promoter activity, thus transcription of HBV genes, and production of HBV virions. SON shows sequence similarities with other DNA-binding structural proteins such as gallin, oncoproteins of the MYC family, and the oncoprotein MOS, which implicated to be involved in protecting cells from apoptosis and in pre-mRNA splicing. Thus the interaction between NS1 and SON could contribute to apoptosis and pre-mRNA splicing regulated by NS1.

In summary, the Y2H screen performed by us provided a large number of interesting new interactions between Influenza virus and cellular host proteins. In pull-down validation experiment, about 40% of the interactions identified could be confirmed for a subset of interactions. NS1 from all six influenza virus strains including A/swine/New York/1682-WC_RG1/2009 (H1N1), A/chicken/Ivory coast/1787-35/2006 (H5N1), A/turkey/Italy/4580/1999 (H7N1 highly pathogenic strain), A/chicken/Italy/1067/1999 (H7N1 low pathogenic strain), A/Udorn/1972 (H3N2) and A/WSN/1933 (H1N1) was found to interact with two cellular proteins, TRBP and PACT. These two interactions were further investigated functionally in next three chapters.

Chapter 4: Influenza NS1 interacts with TRBP and PACT

4.1 Introduction

The yeast two-hybrid (Y2H) system is a very powerful tool for determining binary interactions between proteins on a genomic scale. However, it can produce a significant number of false positive and false negative results. False positive interactions do not occur physiologically and are usually not reproducible in an independent system (Van Crielinge and Beyaert, 1999). The majority of false positives are caused by auto-activating baits. In the absence of any interacting partners, these proteins fused with a DNA binding domain can activate transcription of reporter. Other important factors that contribute to false positives can be “sticky” proteins leading to non-specific binding (Van Crielinge and Beyaert, 1999).

In previous studies, NS1 of influenza A/Udorn/72 was found to interact with domain 1 of PACT (Li *et al.*, 2006a). Quantitative mass spectrometry analysis of proteins interacting with NS1 by Tawaratsumida *et al* also identified an interaction between Influenza NS1 (strain A/PR8/34) and PACT (Tawaratsumida *et al.*, 2014). To date, there is little published evidence of the interaction of NS1 with TRBP. Although an Y2H screen of Influenza strain PR8 proteins against the human host library identified interactions between both NS1 and PACT as well as NS1 and TRBP, it has not been validated and no further work has been carried out based on the screen (Shapira *et al.*, 2009). Whilst our study found NS1 from all six influenza strains including A/swine/New York/1682-WC_RG1/2009 (H1N1), A/chicken/Ivory coast/1787-35/2006 (H5N1), A/turkey/Italy/4580/1999 (H7N1 highly pathogenic strain), A/chicken/Italy/1067/1999 (H7N1 low pathogenic strain), A/Udorn/1972 (H3N2) and A/WSN/1933 (H1N1) to interact with both TRBP and PACT with high confidence - NS1:TRBP and NS1:PACT interactions occurred 237 and 127 times, respectively - it is crucial to validate interactions identified by a large-scale Y2H

library screen. To avoid the possible occurrence of false positive interactions in the Y2H library screen, and to confirm the validity of interactions, all positive Y2H interactions were tested independently by direct Y2H methods, and confirmed with two influenza strains in mammalian cells by co-immunoprecipitation.

4.2 Results

4.2.1 Validation of interactions of TRBP and PACT with NS1 by yeast two-hybrid

In order to validate the interactions of NS1 from 6 influenza A virus strains with PACT and TRBP, a liquid-based direct mating Y2H assay was performed. All the NS1 genes were subcloned from pDONR207 into the bait vector, and TRBP and PACT were subcloned from pDONR207 into the prey vector. Haploid yeast strains AH109 (mating type α) and Y187 (mating type α) were transformed with bait and prey plasmids respectively. For each pairwise interaction, the haploid yeast were mated and diploids were selected. The diploids were then transferred to interaction detection media lacking the amino acid histidine, one of the reporters of Y2H system, and supplemented with the histidine biosynthesis inhibitor 3-AT and the substrate for fluorescence detection, 4-Mux. A pre-determined high concentration of 10 mM of 3-AT was used for the specificity of interactions. If an interaction occurs, histidine biosynthesis allows the yeast to grow in media that lacks histidine. Interaction also allows the production of α -galactosidase. The activity of α -galactosidase was measured by the fluorescence generated by degradation of the substrate 4-Mux.

To check for auto-activation of the GAL4 promoter in the Y2H system, all NS1 proteins expressed as baits were tested against the empty prey plasmid that expresses only the GAL4 activation domain. Fluorescence levels were comparable to those observed with the negative control (empty bait plasmid tested against empty prey plasmid), indicating the NS1 proteins do not induce autoactivation of GAL4

transcription (Fig. 4.1). In comparison, fluorescence was significantly higher when NS1 prey proteins from 6 strains of influenza were tested against both TRBP and PACT. Although no variation in fluorescence intensity between NS1s from all 6 strains against TRBP and PACT was observed, due to the limitation by detection methods, it cannot be concluded that there is difference in binding affinity between strains.

4.2.2 Co-immunoprecipitation of TRBP and PACT with NS1 from mammalian cells

Whilst protein interactions observed in yeast cells provide good evidence for a genuine interaction, it is important to confirm them in mammalian cells. Co-immunoprecipitation assays were performed to confirm the observed interactions between NS1 and TRBP, and NS1 and PACT. NS1 proteins from two strains of influenza, A/Udorn/72 and A/WSN/1933, were selected here for the binding assay. Ud/NS1 and WSN/NS1 were subcloned from pDONR207 into N-terminal GFP-tagged plasmid pEGFP-C2. TRBP and PACT were subcloned from pDONR207 into N-terminal HA epitope-tagged plasmid pCR3-N-HA.

Plasmids expressing NS1s, TRBP and PACT were co-transfected into HEK293T cells, and proteins were co-immunoprecipitated with anti-HA Ab (Fig. 4.2). Expression of input protein was confirmed by blotting with anti-HA (TRBP; PACT) or GFP (NS1) antibodies. In order to compare expression levels, all samples were stained for β -actin, and RNase A was used to treat cell lysate at room temperature for 1 hour to exclude potential interaction mediated by RNA bridge.

Precipitation with anti-HA antibodies found a low level non-specific interaction between NS1 and pCR3-N-HA (Fig. 4.2). However, the increased intensity of both Ud/NS1 and WSN/NS1 bands precipitated with both TRBP-HA and PACT-HA confirm these specific and significant interactions.

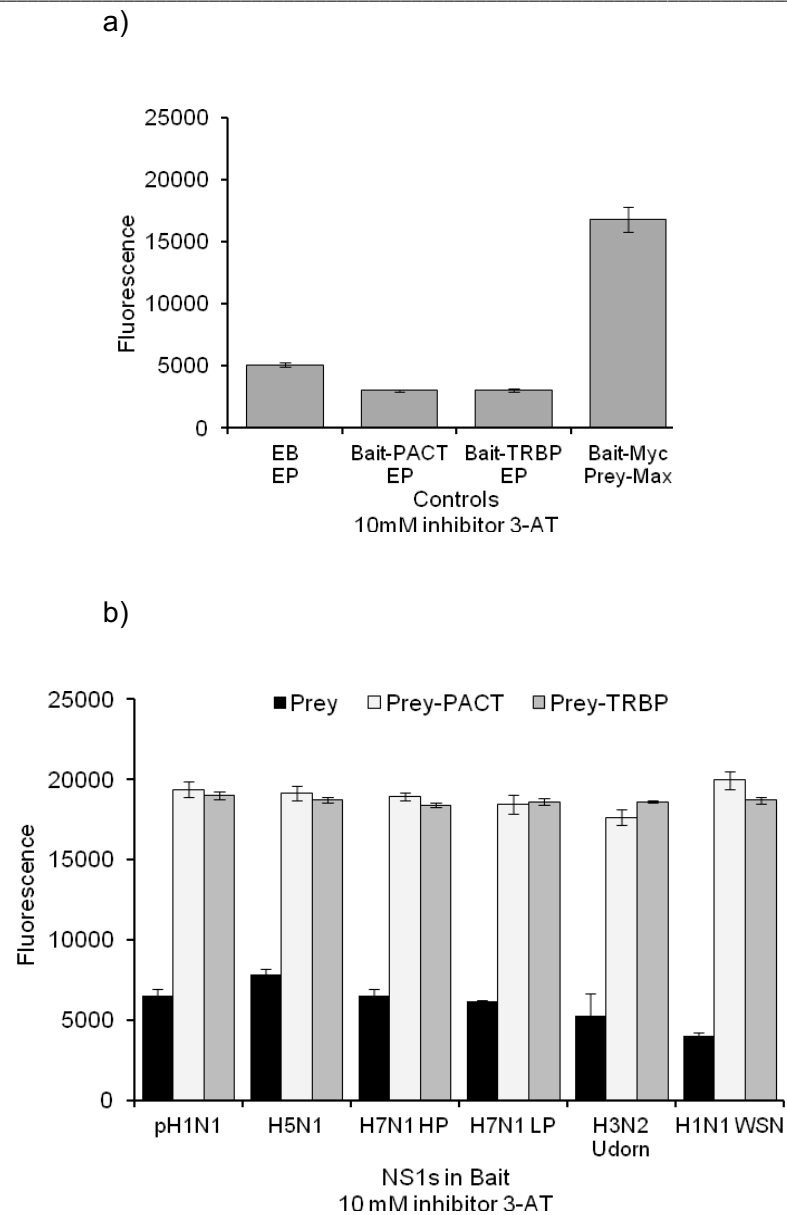


Figure 4.1 NS1 proteins of various different Influenza strains interact with TRBP in a direct mating Y2H assay. (a) Empty bait (EB) and Empty prey (EP) were used as negative controls. As Myc is known to interact with Max, bait-Myc and prey-Max were used as positive controls. (b) NS1s in bait were tested against TRBP and PACT in prey. Yeast cells of strain AH109 and Y187 were transformed with Bait and Prey and grown in SD-Trp and SD-Leu media. After 24h growth, two strains of yeast were mated and diploid cells were selected by SD-LW media. Interactions were detected by adding 4-MuX and 3-AT after 7-day culture in SD-LWH media. Error bars represent standard deviation of triplicates in one representative experiment.

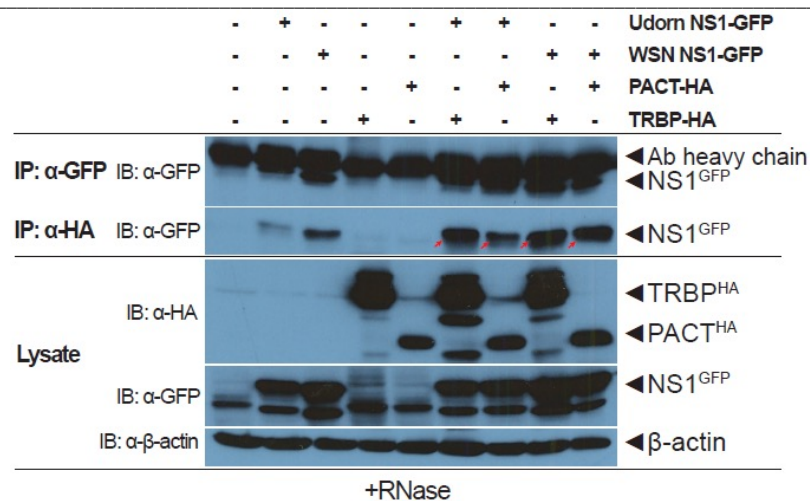


Figure 4.2 Validation of NS1:TRBP and NS1:PACT interactions by co-immunoprecipitation. Cell lysates treated with RNase and immunoprecipitated with anti-HA or anti-GFP antibody were boiled with 4X Laemmli buffer, separated on 12% polyacrylamide gels and immunoblotted with either anti-HA or anti-GFP antibody. Empty pCR3-N-HA and pEGFP-C2 were used as negative controls. Non-specific bindings were ruled out by co-transfection of NS1s with empty pCR3-N-HA and TRBP, PACT with empty pEGFP-C2. Specific bands corresponding to NS1s are indicated with red arrows.

4.2.3 TRBP, PACT and NS1 co-localise in A549 cells

A number of studies have reported various patterns of intracellular localization of NS1, TRBP and PACT. Predominantly NS1 localizes to nucleus, however a significant proportion of NS1 also appears in the cytoplasm. Virus strain, cell type, and time post-infection have all been suggested to contribute to the cellular distribution of NS1 (Hale *et al.*, 2008). TRBP and PACT are involved in RNAi pathways and PKR regulation and are mainly localised in the cytoplasm (Kok *et al.*, 2007).

To determine whether NS1, TRBP and PACT co-localise, proteins were co-expressed and confocal microscopy was performed. GFP-PR8/NS1 was co-transfected with HA-TRBP and Myc-PACT into A549 cells. PR8/NS1 was found to localise in both nucleus and cytoplasm, whereas TRBP and PACT were restricted to the cytoplasm (Fig. 4.3). Moreover, TRBP and PACT were found to co-localize, confirming the

interaction between TRBP and PACT reported by Kok *et al* (Kok *et al.*, 2007). As anticipated, the cytoplasmically located NS1 also co-localized with TRBP and PACT. Taken together, these data suggest NS1 interacts with both TRBP and PACT, that this localization is independent of RNA bridges, and that these three proteins co-localize in cytoplasm.

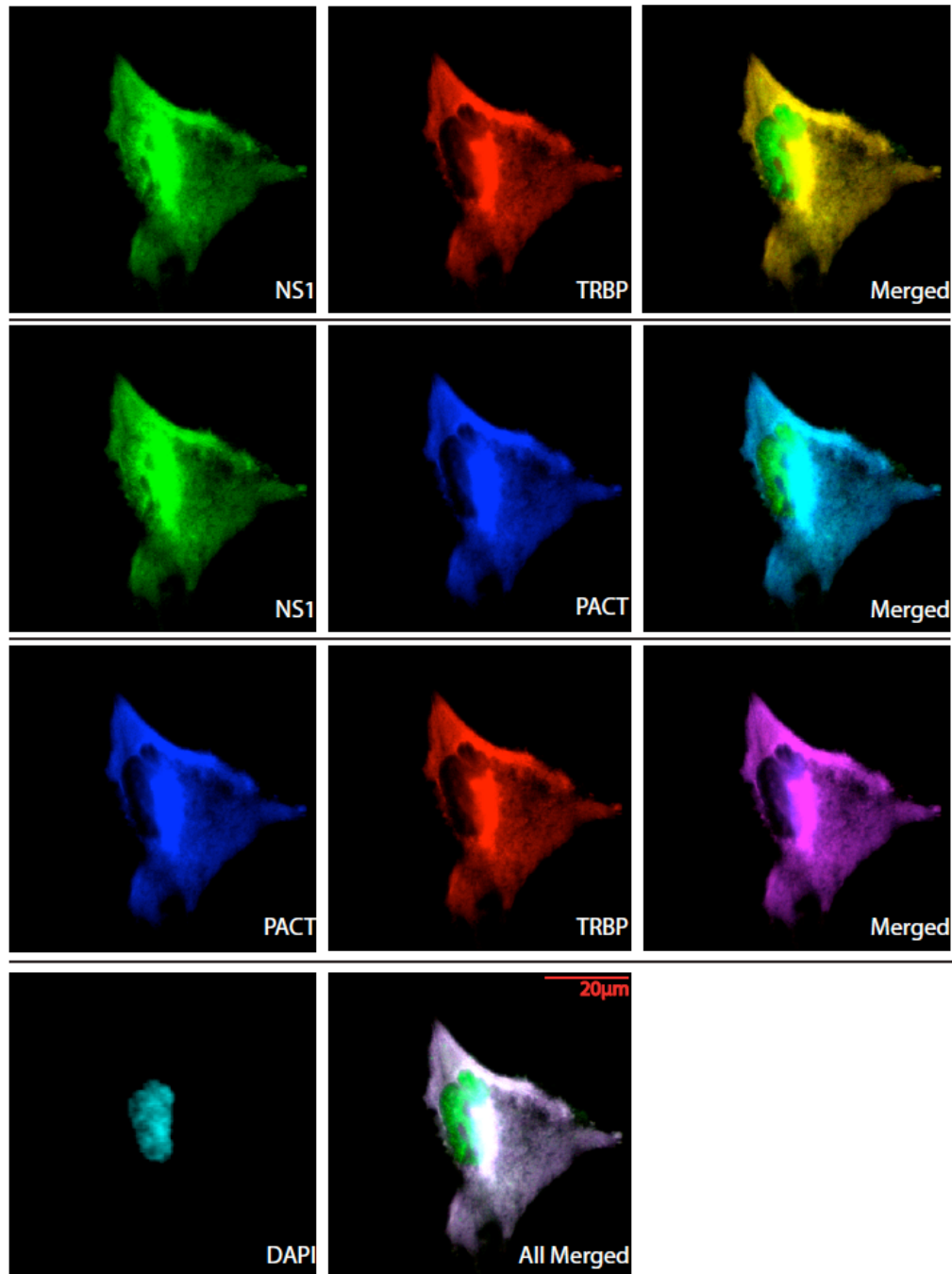


Figure 4.3 Co-localization of NS1, TRBP and PACT. A549 cells transfected with pEGFP-N1-PR8/NS1, pCR3-N-HA-TRBP and pCR3-N-Myc-PACT were

fixed 48 hours post transfection. Cells were stained with anti-HA primary antibody with Alexa fluor[®]594 goat anti-rat IgG as secondary antibody (Displayed as red by Zeiss system), and anti-Myc primary antibody with MycAlexa fluor[®]488 goat anti-mouse IgG as secondary antibody (Displayed as blue by Zeiss system). DAPI staining was performed as indication of nucleus. All the images were taken by Zeiss LSM 510 META system.

4.3 Discussion

Genome-wide Y2H analysis of host and virus interactions for several different viruses including HSV-1, KSHV, influenza A/PR8/34 has contributed to a comprehensive understanding of their life cycle (Griffiths *et al.*, 2013, Uetz *et al.*, 2006, Shapira *et al.*, 2009). A similar approach was employed in our study to identify interactions between influenza A virus proteins from different strains and the host proteome. In our study, NS1 from all six influenza virus strains tested, including A/swine/New York/1682-WC_RG1/2009 (H1N1), A/chicken/Ivory coast/1787-35/2006 (H5N1), A/turkey/Italy/4580/1999 (H7N1 highly pathogenic strain), A/chicken/Italy/1067/1999 (H7N1 low pathogenic strain), A/Udorn/1972 (H3N2) and A/WSN/1933 (H1N1), were found to interact with TRBP and PACT. These two interactions appeared most frequently, suggesting they could be involved in influenza virus life cycle.

Interactions of NS1 with TRBP and PACT were first confirmed in an Y2H direct mating assay. Interestingly, direct mating Y2H data indicates that the difference in affinity of interaction of NS1 with TRBP and PACT was not observed in the presence high concentration of 3-AT. However, in published studies, NS1 displays different binding affinity for its ligands. In addition, the binding affinity between NS1 from different influenza strains and the same ligand differ (Carrillo *et al.*, 2014). In a study by Thomas *et al.*, the PDZ-binding motif at the C-terminus of human and avian NS1 proteins show different PDZ-binding activity (Thomas *et al.*, 2011, Lee and Zheng, 2010). Carrillo reports that the binding affinity between NS1 and ligands such as RNA or CPSF30 could be modulated in a strain-dependent manner (Carrillo

et al., 2014). This modulation could be due to a preferred state of conformation and in this state the structural plasticity for each strain (Carrillo *et al.*, 2014). It could also be due to subtle sequence changes in the ligand binding interfaces. Further experiments to confirm the affinities between NS1s, TRBP and PACT might reveal differences between the human and avian strains. It would also be interesting to clarify whether the difference in binding affinity lead to differences in high- and low-pathogenicity of the virus.

NS1, TRBP and PACT are all known to be double-stranded RNA-binding proteins. The pattern recognition receptor RIG-I forms a complex with NS1 through N-terminal RNA-binding domain of NS1, although it is unclear if other proteins are involved (Ayllon and Garcia-Sastre, 2015). Furthermore, NS1 is reported to associate with the ubiquitin ligases TRIM25 and RIPLET via its RNA-binding domain, both of which act as positive regulators of RIG-I (Ayllon and Garcia-Sastre, 2015). Both TRBP and PACT consist of three copies of dsRBMs. Whilst the two N-terminal dsRBMs are capable of binding dsRNA, the C-terminal dsRBM mediates protein-protein interactions with proteins including dicer and merlin. Therefore, in the co-immunoprecipitation assay to validate interactions of Ud/NS1 and WSN/NS1 with TRBP and PACT, RNase A was used to exclude the potential mediation of interactions via an RNA bridge. Without binding to RNA, both Ud/NS1 and WSN/NS1 interact with TRBP and PACT. The lysate used in co-immunoprecipitation was obtained from HEK293T cells transfected with plasmids to overexpress proteins. It is possible that other cellular proteins such as Dicer and Ago2 join the observed complex with NS1, TRBP and PACT, and that RNA is protected from RNase within this complex. Weak nonspecific interactions were found in GFP-tagged NS1 precipitated with the empty pCR3-N-HA control plasmid. This suggests NS1 non-specifically binds to the anti-HA antibody coated Protein G Sepharose beads. However, specific bands of both Ud/NS1 and WSN/NS1 were significantly stronger, confirming the NS1-TRBP and NS1-PACT interactions as genuine, and able to occur in mammalian cells. In the assay, 150mM NaCl was used

in washing buffer after precipitation step, and although washing with a higher stringency buffer (increased NaCl concentration) was undertaken, the non-specific binding of NS1 to Protein G Sepharose beads was still observed. Alternatively, utilising other types of beads could eliminate non-specific binding.

As discussed in the Introduction, depended on virus strain, expression level, cell types used for infection, time post infection and some other factors, patterns of intracellular localization for NS1 can differ (Hale *et al.*, 2008). However NS1, which at least contains one nuclear localization sequence, distributes predominantly in the nucleus, but distribution of NS1 in cytoplasm can also be found (Hale *et al.*, 2008). Intracellular localization of TRBP and PACT, two components of RISC, was reported to be primarily in the cytoplasm, as along with their interactor Dicer (Lee *et al.*, 2006, Kok *et al.*, 2007). According to a study by Daniels *et al.*, TRBP colocalises with Dicer in the perinuclear space (Daniels *et al.*, 2009). PKR, as a translation regulator, is known to interact with TRBP and PACT, and whilst PKR predominantly localizes in the cytoplasm due to its association with ribosomes, it is also detectable in the nucleus (Garcia *et al.*, 2006). Taken together, as NS1 is validated to interact with TRBP and PACT, it is not surprising to observe here NS1, TRBP and PACT colocalisation in the cytoplasm. In addition, the localization of NS1 in the nucleus is consistent with the literature.

TRBP and PACT play a regulatory role in PKR activation, and both are required for optimal RNA silencing by endogenous miRNA (Li *et al.*, 2006a, Daniels and Gatignol, 2012). Given the interactions between NS1, TRBP and PACT, future experiments will be directed towards understanding how NS1 influences PKR regulation and RNA silencing activity carried out by TRBP and PACT. As PKR is a well-known antiviral protein, and miRNA functions in post-transcriptional regulation of gene expression, elucidation of the biological relevance of interactions between NS1, TRBP and PACT will provide insights into better understanding of influenza virus life cycle.

Chapter 5: NS1 inhibits the host miRNA processing machinery by interacting with TRBP and PACT

5.1 Introduction

Following transcription by RNA polymerase II, pri-miRNA undergoes sequential processing prior to maturing into ~22nt miRNA. After the pre-miRNAs, ~60 nucleotide hairpin RNA, are produced from transcription products by the nuclear RNaseIII endonuclease Drosha and DGCR8, they are exported out of the nucleus to the cytoplasm by Exportin-5 and Ran-GTP for further cleavage into miRNA/miRNA* duplexes by the endoribonuclease Dicer (Ha and Kim, 2014). In humans, TRBP and PACT form heterodimers with Dicer and are required for optimal processing of pre-siRNAs and pre-miRNAs. Both TRBP and PACT have been shown to interact with influenza A virus NS1 protein in Chapter 4. Lee *et al* reported that knockdown of TRBP exerts more influence on siRNA-initiated gene silencing than knockdown of PACT, however using the same approach, conflicting results by Kok *et al* indicated a similar effect for the knockdown of either TRBP or PACT (Lee *et al.*, 2013, Kok *et al.*, 2007), whereas Noland *et al* suggested complexes containing PACT enhance strand selection for some miRNAs but not by TRBP-containing complexes (Noland and Doudna, 2013). Moreover, mRNA targeted by miRNAs overall is favoured for complexes containing PACT but not TRBP (Noland and Doudna, 2013). Although no exact consensus of roles TRBP and PACT has been reached, these data demonstrate the importance of TRBP and PACT in RNA processing by Dicer.

Duplexes of miRNA/miRNA* are loaded to Ago2 after dicing, which is the core catalytic component of the RNA-induced silencing complex (RISC). Loaded to RISC, miRNA/miRNA* duplexes are unwound and cleaved by Ago2 with the aid of endoribonuclease protein complex C3PO (Ye *et al.*, 2011, Ha and Kim, 2014). One

strand, named the passenger strand, is then degraded while the remaining RNA strand, named the guide strand, recognises specific sequences which are typically located in the 3' untranslated regions of target mRNA and complementarity of miRNA seed sequence. The binding of the miRNA-containing silencing complex to the target mRNA results in Ago2-mediated cleavage or translational repression (Ha and Kim, 2014).

Increasing evidence for involvement of miRNA in influenza virus infection has been obtained over the past few years. Study by Buggele *et al* identified that in A549 cells several endogenous miRNAs accumulate specifically following infection of Udorn and WSN strains (Buggele *et al.*, 2012). These miRNA, including miR-7, miR-132, miR-146a, miR-187, miR-200c, and miR-1275, are not able to be induced sufficiently by antiviral response pathways like TLR, RIG-I, interferon or cytokine stimulation, target components of innate immune signalling systems like IRAK1 and MAPK3 (Buggele *et al.*, 2012). Li *et al* also observed differential expression of miRNA upon influenza virus infection: the two strains used in the study (1918 pandemic strain and A/Texas/36/91) elicited distinct profiles of miRNA expression induction (Li *et al.*, 2010). Moreover, a 3' UTR reporter analysis by Song *et al* revealed that miR-323, miR-491 and miR-654 bind to PB1 of WSN strain and inhibit virus replication, even though the binding of three miRNAs to PB1 was not 'perfect-match' (Song *et al.*, 2010). By using miRNA microarrays, PITA and miRanda database screening, Ma *et al* found that miR-let-7c in A549 cells, which is perfectly complementary to 3' UTR of positive sense strand of viral M1 cRNA, is up-regulated after influenza virus infection, suggesting a role of miR-let-7c in host defence (Ma *et al.*, 2012). These findings demonstrate the involvement of miRNA in influenza virus infection.

Interestingly, the multifunctional protein NS1 of influenza A virus has been shown to suppress RNA interference in plants and *Drosophila* sp. (Hale *et al.*, 2008). Study by Kok *et al* showed that NS1 of WSN strain is an antagonist of interferon induction, but not RNAi-mediated silencing of different reporters (Kok and Jin, 2006). In

contrast, work by de Vries *et al* found NS1 of WSN strain is most potent in antagonizing gene silencing mediated by short hairpin RNA, and furthermore, the capability of NS1 to antagonize RNA-mediated gene silencing varies among different strains, which suggests a potential contribution to the differences in virulence (de Vries *et al.*, 2009).

Since preceding data in this thesis have shown that NS1 from six different influenza virus strains have been demonstrated to interact with TRBP and PACT, which play important roles in RNA processing by Dicer, this chapter will address the following questions: (i) is NS1 involved in any step of host RNA-mediated gene silencing processes (ii) do interactions between NS1, TRBP and PACT account for any such involvement. Thus the aim of this chapter is to elucidate the biological mechanism and functions of interactions between NS1, TRBP and PACT in host RNA interference during influenza virus infection.

5.2 Results

5.2.1 Association of NS1 with the RISC core component Ago2 protein

TRBP and PACT are known partners of Dicer, and required for optimal Dicer-mediated processing (Lee *et al.*, 2006, Kok *et al.*, 2007). The complex containing Dicer, TRBP and PACT load processed miRNA to the RISC; therefore, to determine whether NS1 is present in the Ago2-containing RISC along with the loading complex, an Ago2 pull-down assay was performed. HEK293T cells that stably express Ago2 were transfected with N-terminal MYC-tagged bait plasmids expressing Ud/NS1 and PACT, N-terminal HA-tagged prey plasmids expressing TRBP and three domains of Dicer (PAZ, RI and RII). Since the plasmids are constructed with a T7 promoter, the cells were infected with Modified Vaccinia Ankara expressing T7 RNA polymerase to drive the transcription from T7 promoter prior to the transfection. The cell lysates treated without (Figure 5.1a) or with RNase

A (Figure 5.1b) were immunoprecipitated with the anti-Ago2 antibody, and analysed by western blotting using anti-HA or anti-Myc antibodies. The bands are indicated with black arrows.

With NS1 alone or NS1 with Dicer over-expressed in HEK293T-Ago2 cells, NS1 was able to be pulled down, which indicates NS1 is associated with Ago2 protein, either by direct interaction, or via other endogenous components in the RISC. Although in the blot without RNase treatment pulldown of TRBP could not be detected which could be due to low expression level of TRBP in the assay, the presence of all miRNA loading players TRBP and PACT along with Dicer in the RISC were able to be confirmed in RNase treated blot. Since Dicer components have overlapping bands with heavy chain Ab, a different tag could possibly be used for Dicer to clarify the association of Dicer in the complex. As the cell lysates were treated with RNase A for 1 hour before the precipitation step, the association of NS1 with Ago2 via RNA bridge could be excluded.

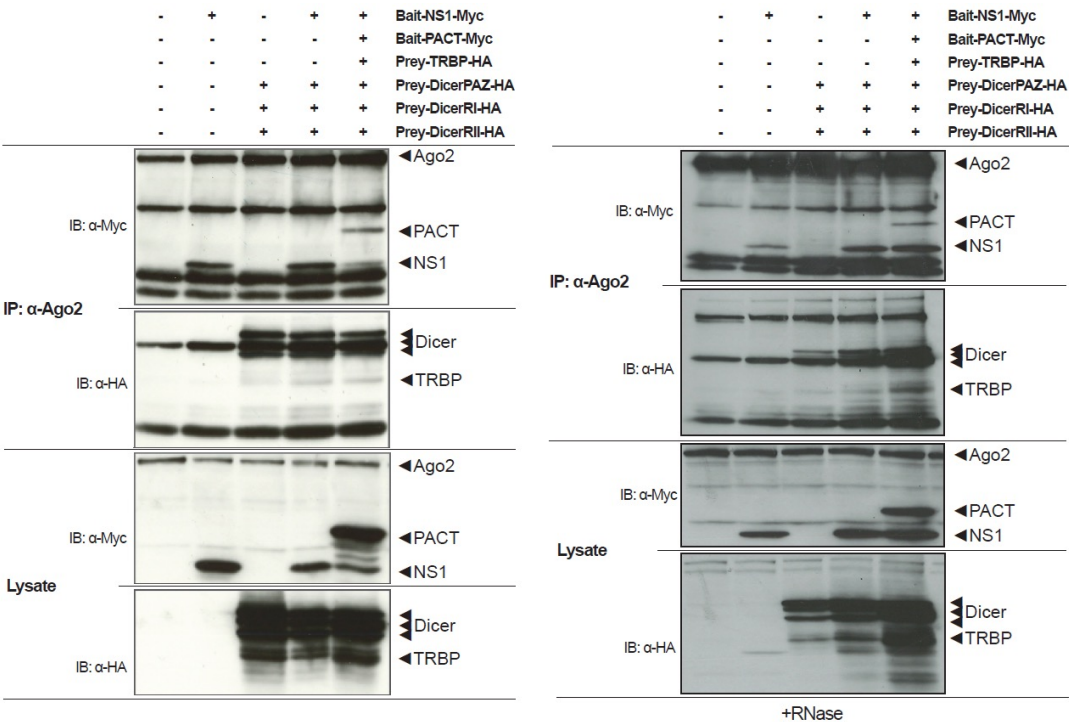


Figure 5.1 Association of NS1 with RISC core component Ago2 protein by Ago2 pull-down assay. Myc-Ud/NS1 and PACT were co-

transfected with HA-TRBP, Dicer (domains PAZ, RI and RII) into HEK293T-Ago2 cells. Cell lysates treated without/with RNase and immunoprecipitated with anti-Ago2 Ab were separated on 12% polyacrylamide gels and immunoblotted with either anti-HA or anti-Myc antibody. Samples in gel a) were not treated with RNase while samples in gel B) were treated with RNase. Specific bands corresponding to Ud/NS1, TRBP, PACT and domains of Dicer are indicated with black arrows. NS1, TRBP, PACT and Dicer were all associated with Ago2 protein.

5.2.2 Inhibition of the cellular miRNA processing machinery by NS1

There are several controversial reports on the ability of NS1 to suppress RNAi-mediated RNA silencing in mammalian cells (Kok and Jin, 2006, de Vries *et al.*, 2009). To confirm whether NS1 inhibits cellular RNA silencing in our system, a 3' UTR luciferase assay was designed as shown in Figure 5.2.

Firefly luciferase reporter psiCHECK-2 with 3' UTR of miRK4 and a eukaryotic expressing vector pcDNA KSHV miRNA miRK10/12 were kindly given by Dr Georg Malterer. In this vector, the sequence spanning the ten intronic miRNAs miR-K12-1 to 9 and miR-K12-11 of KSHV were subcloned into the pcDNA vector (Suffert *et al.*, 2011), thus generating a vector which expresses pre-miRNAs which need to be processed further by Dicer. The 3' UTR sequence of KSHV miRK10/12 target candidates were obtained from the Ensembl genome database and two 3' UTR sequences were created, one as a perfect match to the miRNA seed sequence and the other as an imperfect match. If KSHV miRNAs were successfully processed into mature miRNA, no firefly luciferase activity would be observed by co-transfection of psiCHECK-2 reporter, however, if any step of miRNA processing was disrupted, expression of luciferase would not be inhibited. A number of KSHV miRNAs rather than individual miRNA were used in this system to mimick the complexity of cellular miRNA expression.

To characterize whether NS1 inhibits cellular RNA silencing and whether NS1s from different strains used in Y2H screen vary in their ability to suppress miRNA-

mediated gene silencing, plasmids expressing Ud/NS1, WSN/NS1, H5N1/NS1, H7N1HP/NS1, H7N1LP/NS1 and pH1N1/NS1 were co-transfected with perfect or imperfect-match psiCHECK-2-miRK4 and pCDNA-miRK10/12 into HEK293T cells. Cells were lysed 24 hours post transfection for luciferase activity analysis. Inhibition level of luciferase by miRNA was normalized internally to Renilla luciferase activity then normalized to pCR3 empty plasmid transfected only. Renilla fluorescence intensities of all samples from Figure 5.3 (NS1s) and Figure 5.4 (Titration) are shown in Appendix Figure 1. As internal control, Renilla luciferase expression was expressed well in general. Figure 5.3 shows that a difference was observed across the six strains in their ability to inhibit luciferase activity, although all the NS1s were capable of suppression in imperfect match (Figure 5.3a), only Ud/NS1 and H7N1LP/NS1 delivered suppression effectively in perfect match (Figure 5.3b). Ud/NS1 was most potent in both perfect and imperfect match.

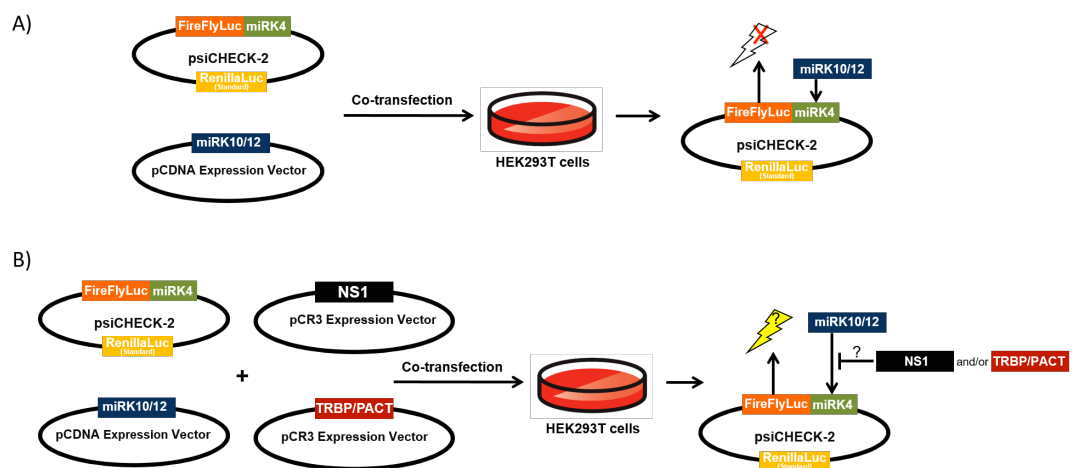


Figure 5.2 Scheme diagram of 3' UTR luciferase assay to investigate miRNA processing. psiCHECK2 is a Firefly luciferase reporter with 3' UTR constructed with KSHV miRNA binding sequence (miRK4) (Suffert *et al.*, 2011). psiCHECK2 also contains a Renilla luciferase gene as an internal control which is controlled by SV40 promoter and constitutively expressed. As shown in A), when HEK293T cells are transfected with psiCHECK-2-miRK4 plasmid together with plasmids expressing KSHV pre-miRNA, if KSHV miRNAs are successfully processed, no luciferase activity will be observed, however, as shown in B) if miRNA processing is disrupted by NS1, expression of luciferase is not inhibited.

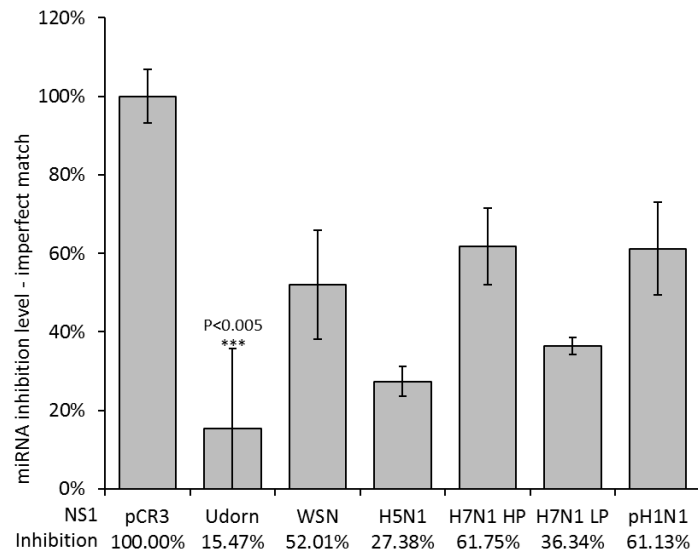
Using the same approach, a different amount of plasmid expressing Ud/NS1 was co-transfected with perfect or imperfect-match psiCHECK-2-miRK4 and pCDNA-

miRK10/12 into HEK293T cells. Cells were lysed 24 hours post transfection for luciferase activity analysis. In both perfect and imperfect match reporter assays, inhibition level of luciferase by miRNA which was normalized to pCR3 empty plasmid transfected, was decreased by Ud/NS1 in a dose-dependent manner. Interestingly, it was observed that Ud/NS1 in the imperfect match (Figure 5.4a) setup was more potent than perfect match (Figure 5.4b) in suppressing RNA silencing activity. In summary, these data suggest that NS1 is able to suppress cellular RNA silencing machinery to a variable extent.

5.2.3 De-repression of NS1-mediated RNA silencing by TRBP and PACT

Since NS1 is associated with the RISC core component Ago2, and the only members of miRNA processing machinery known to interact with NS1 are TRBP and PACT, a 3' UTR luciferase assay was performed to check whether input of TRBP and PACT restores NS1-inhibited host RNAi. TRBP only, PACT only or TRBP and PACT were overexpressed in HEK293T cells, which were co-transfected with plasmid expressing Ud/NS1, miRK10/12 and reporter psiCHECK-2-miRK4 (imperfect-match). Cells were lysed 24 hours post transfection, and luciferase activities were analysed. Inhibition levels were first normalized to Renilla luciferase activity and subsequently to the empty pCR3 that was used as a negative control.

a)



b)

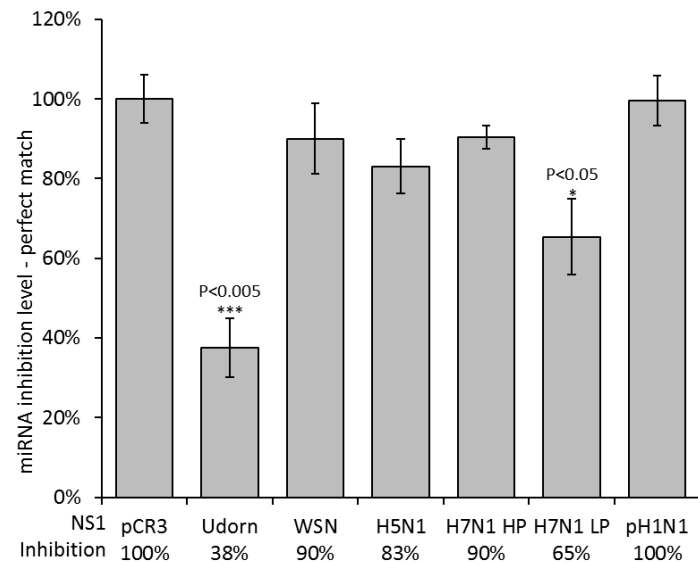
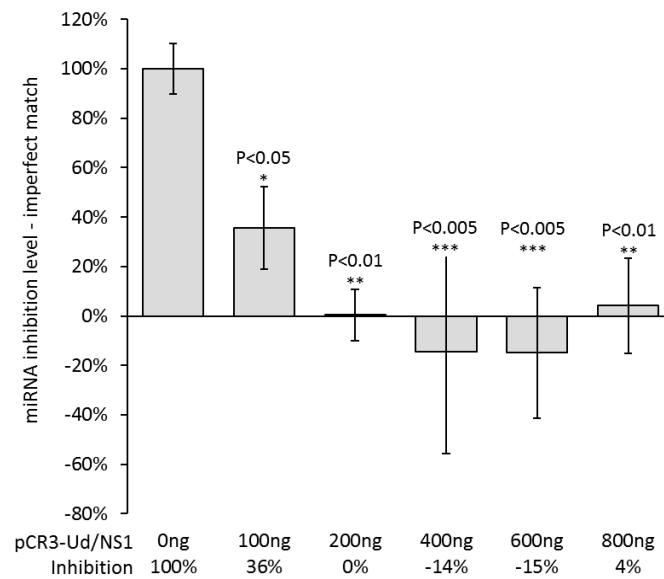


Figure 5.3 Differential RNA silencing inhibition levels by NS1 proteins of different Influenza strains. Plasmids expressing KSHV miR10/12, NS1 of six different strains were co-transfected with psiCHECK luciferase reporter constructed with imperfect (a) or perfect-match (b) KSHV MiR4 sequence into HEK293T cells. Inhibition level by miRNA was determined by measurement of Firefly luciferase activity 24 hr post-transfection, and normalized to Renilla luciferase and pCR3 empty plasmid transfected cells. Inhibition level (%) was indicated, and error bars represent standard deviation of triplicates in one representative experiment. Statistical significance is indicated with p-value calculated against pCR3 control. In both perfect and imperfect match reporter assays, Ud/NS1 showed highest inhibition level of RNA silencing activity. In perfect match, all the inhibition level of RNA silencing activity by NS1 were lower than in imperfect match.

a)



b)

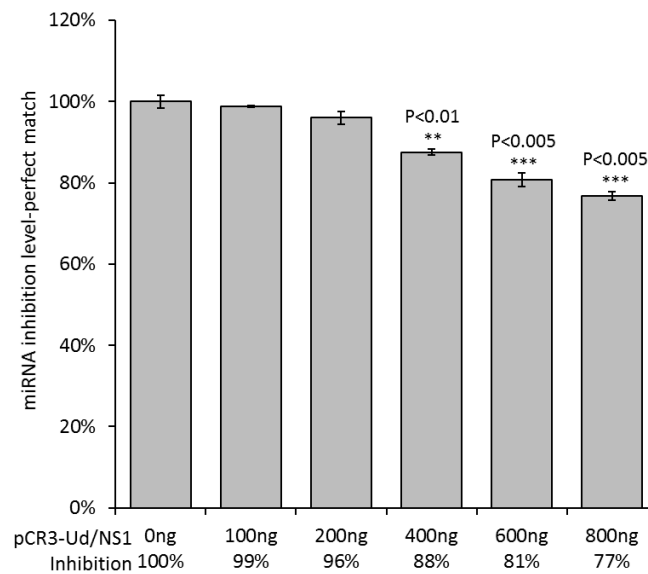


Figure 5.4 Dose-dependent inhibition of RNA silencing activity by NS1. Plasmids expressing KSHV miR10/12, Ud/NS1 of different concentrations were co-transfected with psiCHECK luciferase reporter constructed with imperfect (a) or perfect-match (b) KSHV MiR4 sequence into HEK293T cells. Inhibition level by miRNA was determined by measurement of Firefly luciferase activity 24 hr post-transfection, and normalized to Renilla luciferase and pCR3 empty plasmid transfected cells. Concentrations of Ud/NS1 (ng) and inhibition level (%) were indicated, and error bars represent standard deviation of triplicates in one representative experiment. Statistical significance is indicated with p-value

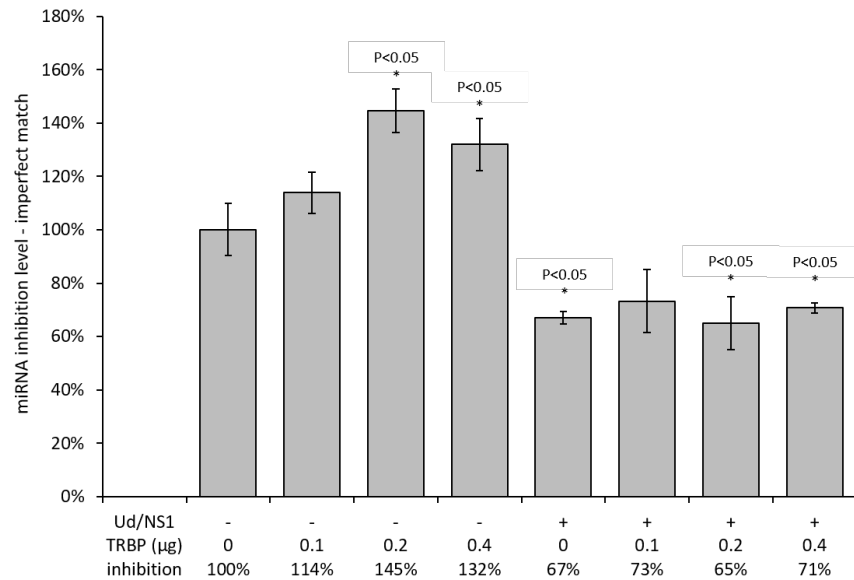
calculated against pCR3 control. In both perfect and imperfect match reporter assays, inhibition level of luciferase by miRNA was decreased by NS1 in a dose-dependent manner.

As shown in Figure 5.5 and Figure 5.6, over-expression of TRBP only, PACT only or both TRBP and PACT together increased miRNA inhibition level in a dose dependent fashion; this indicates TRBP and PACT are able to enhance miRNA processing. However, with/without TRBP only (Figure 5.5a) or with/without PACT only (Figure 5.5b) overexpressed in the cell, Ud/NS1 still suppressed RNA silencing activity, which suggests TRBP or PACT alone is unable to completely restore the suppression of RNA silencing by NS1. By over-expression of both TRBP and PACT as shown in Figure 5.6, miRNA inhibition level increased by ~40%, suggesting suppression of RNA silencing by NS1 was rescued.

5.2.4 PACT is required for association of NS1 with RISC-loading complex

To confirm whether NS1 is still associated with Ago2 in influenza virus infected cells, HEK293T cells stably expressing myc-tagged Ago2 protein were infected with MOI=3 Udorn strain and lysed 12 hours post infection. Cell lysates treated with RNase A were immunoprecipitated with anti-Ago2 antibody and analysed by western blotting. As shown in Figure 5.7, a weak band of Ud/NS1 indicated with a red arrow was detected after Ago2 pull down. It confirmed that in Udorn virus infected HEK293T cells, NS1 is present in the RISC-loading complex. The weakness of the band could be due to the relatively low MOI used in the infection.

a)



b)

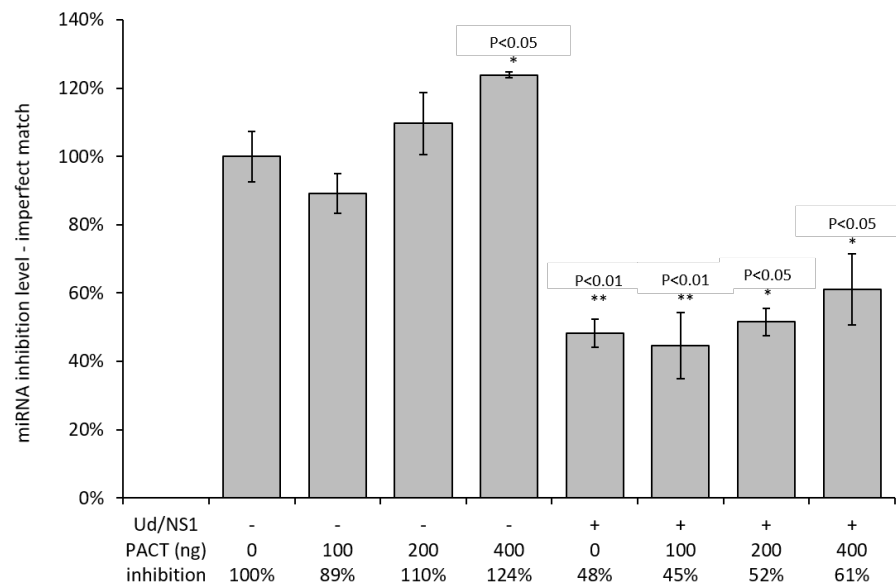


Figure 5.5 TRBP and PACT alone unable to restore RNA silencing activity inhibited by NS1. Plasmids expressing KSHV miR10/12, Ud/NS1 and TRBP or PACT were co-transfected with psiCHECK luciferase reporter constructed with imperfect-match KSHV MiR4 sequence into HEK293T cells. Inhibition level by miRNA was determined by measurement of Firefly luciferase activity 24 hr post-transfection, and normalized to Renilla luciferase and pCR3 empty plasmid transfected cells. Inhibition level (%) was indicated, and error bars represent standard deviation of triplicates in one representative experiment. Statistical significance is indicated with p-value calculated against pCR3 control. Either increasing input of TRBP (A) or PACT (B) were unable to restore RNA silencing activity inhibited by Ud/NS1.

To exclude the possibility that the association of NS1 with Ago2 is mediated by other members of RISC or the RISC loading complex, knockdown of TRBP and PACT followed by Ago2 pull-down assay was performed. HEK293T cells stably expressing myc-tagged protein were transfected with siRNAs against TRBP or/and PACT, NP siRNA was used as positive control for siRNA knockdown. Knockdown levels of both TRBP and PACT are shown in figure 6.1a. Cells were infected with Udorn strain (at MOI=3) 48 hours post transfection and lysed 8 hours post infection. Cell lysates treated with RNase A were immunoprecipitated with anti-Ago2 antibody and analysed by western blotting. As shown in Figure 5.8, knockdown of NP lead to a reduction of NS1 expression, thus NS1 was unable to be pulled down. Interestingly, no band of NS1 was observed during depletion of PACT, however in the absence of TRBP, NS1 was still associated Ago2. In addition, knockdown of both TRBP and PACT showed the same effects as knockdown of PACT. Taken together, these results suggest NS1 associates with Ago2, the key component of RISC, by interacting with PACT.

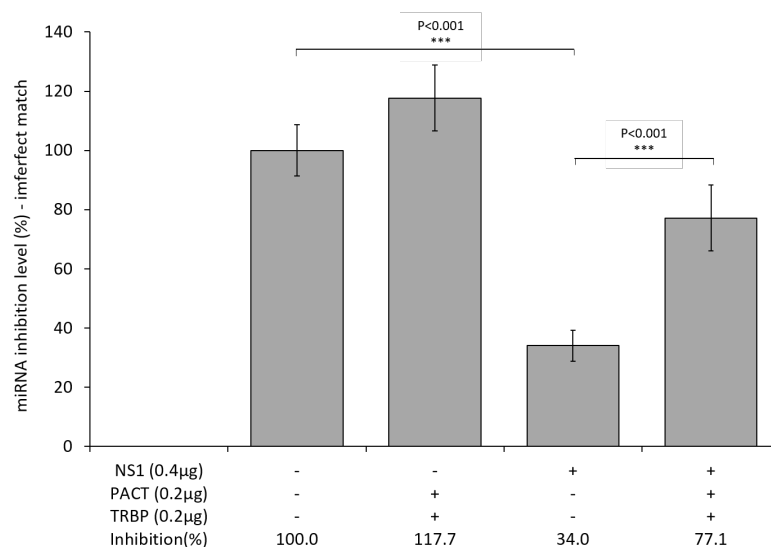


Figure 5.6 Derepression of NS1-mediated inhibition of RNA silencing by TRBP and PACT together. Plasmids expressing KSHV miR10/12, Ud/NS1 and TRBP and PACT were co-transfected with psiCHECK luciferase reporter constructed with imperfect-match KSHV MiR4 sequence into HEK293T cells. Inhibition level by miRNA was determined by measurement of Firefly luciferase activity 24 hr post-transfection, and normalized to Renilla luciferase and pCR3 empty plasmid transfected cells. Inhibition level (%) was indicated, and error

bars represent standard deviation of three independent experiments. Statistical significance is indicated with p-value calculated against pCR3 control. Input of TRBP and PACT restored RNA silencing activity inhibited by Ud/NS1.

5.2.5 NS1 competes with TRBP and PACT which facilitate RISC-loading complex

Since TRBP and PACT are required for optimal miRNA processing, the insufficient binding of TRBP and PACT to Ago2 could lead to ineffective miRNA loading (Lee *et al.*, 2006, Kok *et al.*, 2007, Lee *et al.*, 2013). To characterize whether interactions between NS1, TRBP and PACT result in insufficient binding of TRBP and PACT to Ago2, HEK293T cells stably expressing myc-tagged Ago2 were transfected with plasmids overexpressing HA-tagged TRBP, PACT and GFP-tagged Ud/NS1, cells were lysed 24 hours post transfection. In addition, cells were transfected with plasmids overexpressing HA-tagged TRBP and PACT then infected with MOI=3 Udorn strain, cells were lysed 8 hours post infection. Cell lysates treated with RNase A were immunoprecipitated with anti-Ago2 antibody and analysed by western blot.

Both in overexpression (Figure 5.9a) and infection (Figure 5.9b) assays, band sizes of TRBP reduced while NS1 was overexpressed and infected. Although bands of PACT in overexpression setup were not detected due to the low expression level, in infection setup a reduced band size of PACT can be confirmed. These data suggest interactions of NS1 with TRBP and PACT lead to insufficient binding of TRBP and PACT to RISC-loading complex. Thus these competitive bindings allow NS1 to block miRNA maturation.

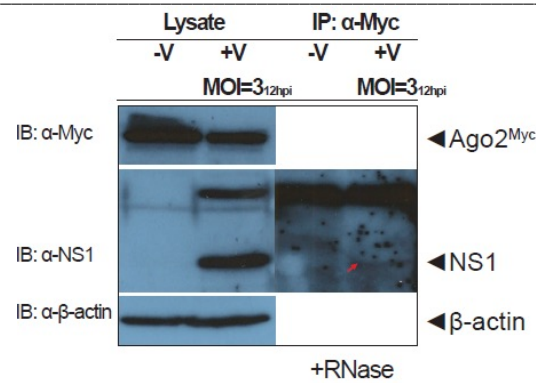


Figure 5.7 Association of NS1 with RISC core component Ago2 protein in infected cells. HEK293T cells stably expressing myc-tagged Ago2 protein were infected with MOI=3 Ud strain and lysed 12 hours post infection. Cell lysates treated with RNase and immunoprecipitated with anti-Myc antibody were boiled with 4X Laemmli buffer, separated on 12% polyacrylamide gels and immunoblotted with anti-NS1 or anti-Myc antibodies. Upon influenza virus infection, NS1 is associated with RISC core component Ago2 protein.

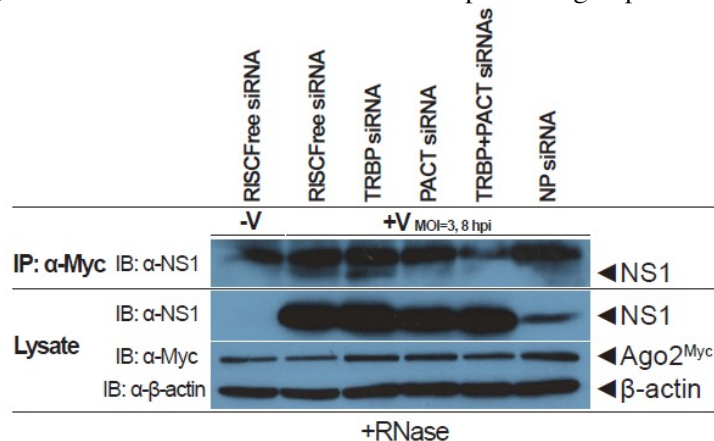
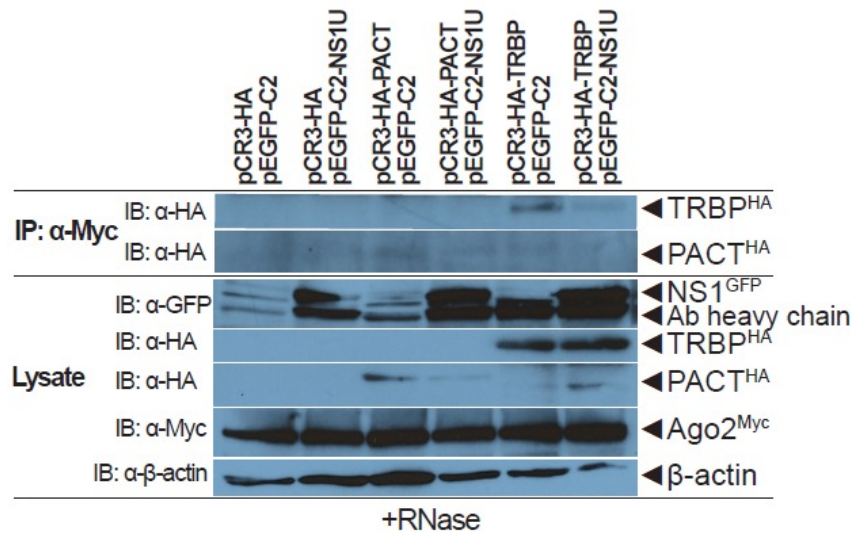


Figure 5.8 Knockdown of PACT abolishes the association of NS1 with Ago2. HEK293T cells stably expressing myc-tagged protein were transfected with siRNAs against TRBP or/and PACT, 48 hours post transfection cells were infected with MOI=3 Ud strain and lysed 8 hours post infection. Cell lysates treated with RNase and immunoprecipitated with anti-Ago2 antibody were boiled with 4X Laemmli buffer, separated on 12% polyacrylamide gels and immunoblotted with anti-NS1 or anti-Myc antibodies. NS1 is unable to associate with Ago2 in the absence of PACT, but not TRBP.

a)



b)

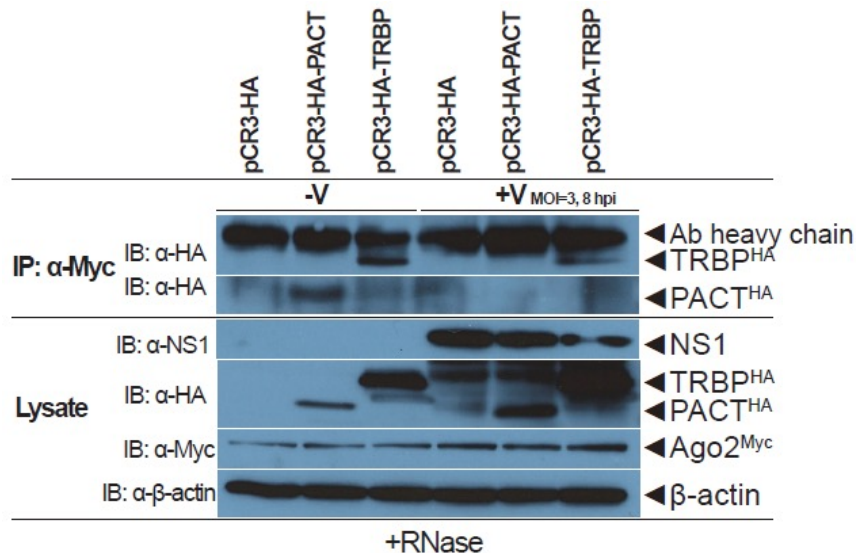


Figure 5.9 Competitive binding of NS1 to Ago2-associated TRBP and PACT. A) HEK293T cells stably expressing myc-tagged protein were transfected with plasmids overexpressing HA-tagged TRBP, PACT and GFP-tagged Ud/NS1, cells were lysed 24 hours post transfection. B) Or cells were transfected with plasmids overexpressing HA-tagged TRBP and PACT then infected with MOI=3 Udorn strain, cells were lysed 8 hours post infection. Cell lysates treated with RNase and immunoprecipitated with anti-Ago2 antibody were boiled with 4X Laemmli buffer, separated on 12% polyacrylamide gels and immunoblotted with anti-NS1, anti-HA, anti-Myc or anti-GFP antibodies. TRBP and PACT are unable to associate with Ago2 with the input of NS1 in both overexpression system and infection setup.

5.3 Discussion

In this chapter, influenza A virus NS1 protein has been demonstrated to inhibit miRNA-mediated RNA silencing activity, and to associate with RISC core component Ago2 protein by interacting with PACT. For the first time, the mechanism of this inhibition by NS1 is characterized. By binding with TRBP and PACT which form a heterodimer and facilitate Dicer-mediated RNA processing, NS1 prevents TRBP and PACT functioning in the RISC-loading complex, resulting in ineffective miRNA maturation and thus suppression of RNA silencing activity.

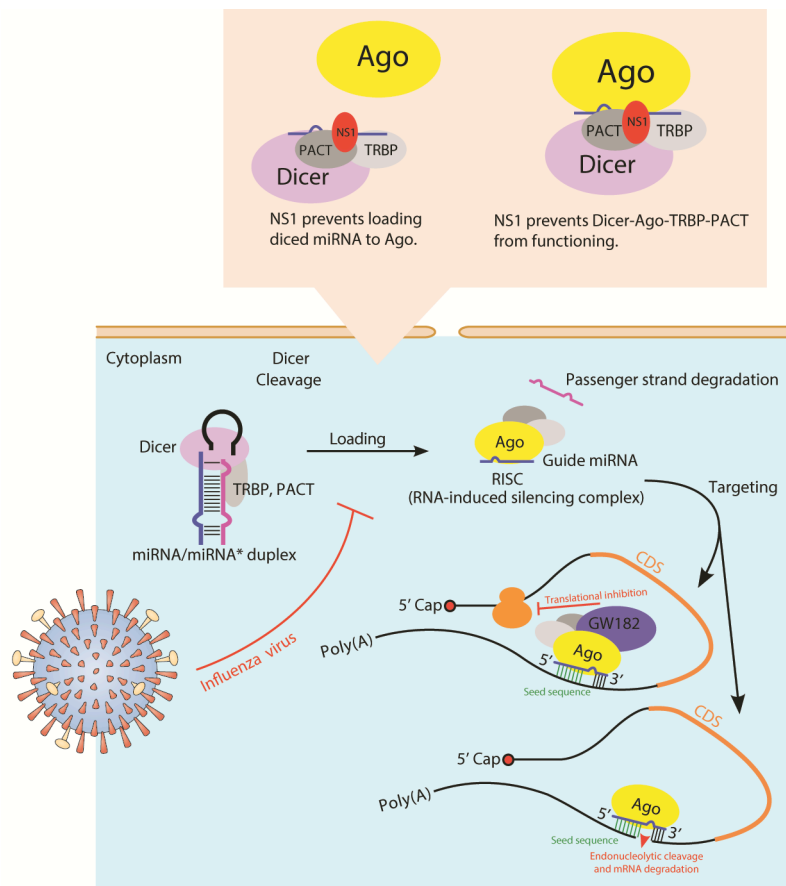


Figure 5.10 Schematic diagram of the inhibitory role of NS1 in Dicer-mediated RNA processing. Schematic representation showing that NS1 interacts with TRBP and PACT to prevent loading dicer-processed miRNA to RISC. Through formation of complex containing NS1, TRBP, PACT and Dicer, NS1 is able to inhibit the loading of Dicer-processed miRNA from miRNA/miRNA* duplex to Ago2-contained RNA-induced silencing complex, thereafter inhibit the RNA silencing activity. Known events in the miRNA pathways are shown in black arrows, events showing inhibition effects are shown in red arrows.

In this study, firefly luciferase reporter psiCHECK-2 with 3' UTR of miRK4 and pCDNA expressing KSHV miRNA miRK10/12 were used to measure RNA silencing activity. Given the expression vector is inserted with sequence spanning the ten intronic miRNAs miR-K12-1 to 9 and miR-K12-11 of KSHV, compared to existing studies, it mimicked the complexity of cellular RNAi by miRNA. Difference of ability of NS1 across six strains in suppressing host RNAi was observed. Interestingly, in this 3' UTR assay, although all the NS1s were capable of suppressing in imperfect match with seed sequence of KSHV miRNAs, only Ud/NS1 and H7N1LP/NS1 showed suppression effectively in perfect match. A loss in NS1's suppression in perfect match could be simply due to processed miRNA working more efficiently in RNA silencing with its seed sequence fully complementary to the target. Ud/NS1 was the most potent in both perfect and imperfect match, hence was chosen for further investigation for the mechanism. In contrast to the data by de Vries *et al* that found WSN/NS1 was most potent in suppressing host RNAi, WNS/NS1 in this study was unable to suppress host RNAi effectively, especially in the perfect match setup (de Vries *et al.*, 2009). Although both of our studies were carried out in HEK293T cells, the difference in assay systems could contribute to this. As Kok *et al* reported in HeLa cells, WSN/NS1 did not inhibit shRNA-mediated silencing and cell types could also play a role (Kok and Jin, 2006). Although only Ud/NS1 here was demonstrated mechanistically to inhibit RNA silencing by interacting with TRBP and PACT, since NS1s from six strains used in the Y2H screen all interact with TRBP and PACT, the mechanism across strains could be conserved. The ability of inhibition of RNA silencing by NS1 could be dependent on binding affinity between NS1, TRBP and PACT, which should be addressed in the future.

The mechanism proposed here suggests NS1 inhibits host cellular miRNA processing through binding with TRBP and PACT, as shown in Figure 5.10. TRBP and PACT form a heterodimer and associate with Dicer to facilitate Dicer-mediated RNA processing. It has been reported that Dicer, TRBP and Ago2 can form a trimer

as RISC-loading complex even without a trigger of dsRNA. RISC-loading complex subsequently binds to pre-miRNA, which was diced into miRNA/miRNA* duplex. The guide miRNA strand selected from the duplex was loaded into Ago2 to generate functional RISC, and the passenger strand was discarded. The association of NS1 with TRBP and PACT prevents RISC-loading complex from functioning effectively to form RISC, thus miRNA maturation and RNA silencing are inhibited. Surprisingly, here it identified interaction of NS1 with PACT but not TRBP that mediates the association of NS1 with RISC-loading complex, depletion of PACT leads to the absence of NS1 from Ago2. Considering that in the 3' UTR assay the restoration of inhibition of RNA silencing required both TRBP and PACT, it could be due to NS1 attaching to the heterodimer of TRBP and PACT, via PACT. This is supported by co-localization of NS1, TRBP and PACT in the cytoplasm obtained in Chapter 4. Although Dicer, TRBP and Ago2 can form a triple complex without an RNA bridge, and RNase A was used in all Ago2 pull-down assays, it cannot be completely excluded that RNA could be protected inside the large complex which constitutes NS1 and RISC loading components Dicer, TRBP, PACT and Ago2. Further binding site mapping of interactions between NS1, TRBP and PACT is needed to understand the mechanism in more details, as well as the structure of this large complex. With the mutant of NS1 that does not bind to TRBP and PACT, mutant recombinant influenza virus could be generated to determine the binding feature of NS1 in RISC-loading complex. Attempt of binding site mapping was actually made in this study by using different Ud/NS1 deletion mutants in Y2H system against TRBP and PACT, but it did not provide valuable information. Y2H system may not be the ideal assay to map the binding sites for NS1, particularly since the deletion mutants might not be folded properly in the yeast cells. Alternatively, alanine scanning can be used in future. Recombinant viruses containing mutants of NS1 that do not bind to TRBP and PACT can be made to verify whether mutant NS1 can still be associated with Ago2 complex following mutant virus infection.

Comparison studies of miRNA profiling between uninfected and influenza A virus infected cells have revealed that the virus induces a change in the miRNA landscape of the host cell, which illustrates the involvement of miRNA in the influenza virus life cycle. While many miRNA were identified as antiviral miRNAs, such as miR-323, miR-491, miR-654, miR-3145 and miR-let-7c that directly bind to viral RNA transcripts, some miRNAs were rather identified as negative regulators of antiviral innate immune signalling pathways, such as miR-29c, miR-132, miR-146a, or miR-1275 (Song *et al.*, 2010, Ma *et al.*, 2012, Guan *et al.*, 2012, Buggele *et al.*, 2012). However, in these studies observation of antiviral effects or downregulation of host defence was based on transfection of additional miRNAs or their mimics into the cells. Although cellular functions and virus replication are changed accordingly, it may not reflect what is really happening in the cell during infection. Experiments investigating depletion of expression of these miRNA are needed to verify the necessity of these miRNA for virus replication. A study conducted by Bogerd *et al.*, using deep sequencing, demonstrated that infection by DENV or WNV does not result in production of virus-induced miRNA (Bogerd *et al.*, 2014). In addition, replication of a range of viruses, for example, influenza A virus, DENV, WNV, YFV, measles virus, HIV-1, etc. was not affected in the cells lacking functional Dicer (Bogerd *et al.*, 2014). During infection, RNAi antagonists encoded by the viruses probably already have an impact on RNAi processing, it might not be surprising to observe no significant change in miRNA landscape with or without functional Dicer. Mutant viruses lacking RNAi antagonists, for example, influenza virus NS1-deletion mutant should be used to determine whether overall miRNA level in the cell is influenced by virus infection. It is rare that virus replication is dependent on a cellular miRNA, such is the case for hepatitis C virus and miR-122 (Luna *et al.*, 2015). Thus in the study here, it is hypothesized that human viruses, especially RNA viruses, to facilitate their productive replication have evolved mechanisms to suppress the RNA silencing activity carried out by endogenous miRNA. The mechanism, at least in the step of Dicer-mediated RNA processing, could be due to interactions with TRBP and PACT by a viral factor; in the case of influenza A virus,

NS1. Concerning those miRNAs that aid influenza virus replication, the differentiated role of TRBP and PACT in guiding miRNA strand selection might explain why some miRNAs could eventually be processed but most cellular miRNAs remain unprocessed due to inhibition by NS1, which should be characterized in future. Interestingly, although mechanisms have not been investigated, evidence of Ebola virus VP35 and HIV-1 Tat suppressing host RNA silencing activity have been published (Haasnoot *et al.*, 2007, Unwalla and Rossi, 2008). As VP35 and Tat also share similar role with influenza virus NS1 in inhibiting PKR activation, this mechanism of dual role of viral factor in RNAi and PKR regulation could be conserved among RNA viruses.

Chapter 6: Depletion of TRBP and PACT affects influenza virus replication and the host immune response

6.1 Introduction

In addition to important roles in Dicer-mediated RNA processing, TRBP and PACT are regulators of PKR activation. Stresses such as oxidation and serum starvation induce phosphorylation of PACT, which leads to association of PACT with PKR and subsequently PKR activation (Patel *et al.*, 2000, Singh *et al.*, 2011, Singh and Patel, 2012). Unlike PACT, a positive regulator of PKR, TRBP negatively regulates the activation of PKR. Without stress, PACT is inactive and forms heterodimers with TRBP. Under conditions of stress, phosphorylation of PACT results in a weakened interaction between TRBP and PACT, and significantly increases the ability of PACT to activate PKR (Singh *et al.*, 2011, Singh and Patel, 2012). The RNA binding protein PKR plays a key role in antagonizing virus infection. Phosphorylated eIF2 α , the activated, PKR-phosphorylated α subunit of the eIF2 translation initiation factor, shuts off cellular and virus protein synthesis, thus inhibiting virus replication (Garcia *et al.*, 2006, Hale *et al.*, 2008, Ayllon and Garcia-Sastre, 2015). In addition, PKR activation and eIF2 α phosphorylation induced by foreign RNA decrease the level of the NF κ B signalling inhibitor I κ B. Enhanced activity of NF κ B amplifies the production of the antiviral agent IFN β . Active PKR is also required for optimal activation of kinases p38, JNKs, SAPKs, as well as IRF1, p53, STAT1, ATF, STAT3, and AP-1 which are the downstream transcription factors induced by these kinases (Garcia *et al.*, 2006, D'Acquisto and Ghosh, 2001).

NS1 has been reported to directly bind to and inhibit PKR that has been activated by either dsRNA or PACT (Li *et al.*, 2006a). The interaction between NS1 and PKR does not require the RNA binding domain of NS1 and the interaction can occur in the absence of dsRNA. The interaction is mediated by amino acids 123-127 in effector

domain of NS1 (Li *et al.*, 2006a). Tawaratsumida *et al* also showed that the interaction between NS1 and PACT prevents PACT from interacting with RIG-I, which leads to inefficient production of type 1 interferons (Tawaratsumida *et al.*, 2014). Since TRBP and PACT regulate PKR and IFN β induction (D'Acquisto and Ghosh, 2001), and it has been demonstrated in this study that NS1 from six different influenza virus strains all interact with TRBP and PACT, the following questions will be addressed: (i) how is IFN β production influenced by the interactions between NS1, TRBP and PACT upon influenza virus infection; and (ii) is influenza virus replication affected by these interactions. Thus the aim of this chapter is to elucidate the biological mechanism and functions of interactions between NS1, TRBP and PACT in immune response and influenza virus replication.

6.2 Results

6.2.1 Knockdown of TRBP and PACT inhibits influenza virus replication

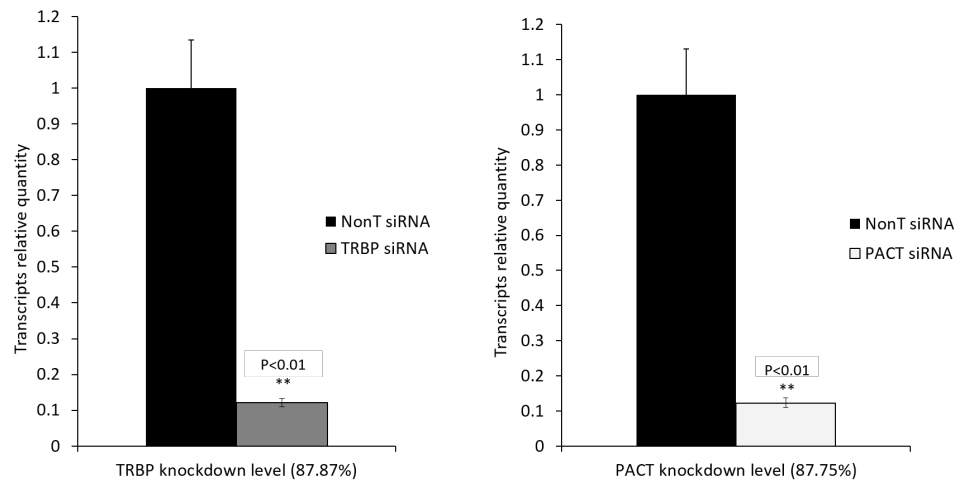
Although TRBP and PACT are involved in regulation of PKR and IFN β production, it is not known whether influenza virus replication is affected by TRBP and PACT (Li *et al.*, 2006a, Singh *et al.*, 2011, D'Acquisto and Ghosh, 2001). In order to characterize the role of TRBP and PACT in influenza virus replication, A549 cells were transfected with 50nM siRNA against TRBP or PACT for 48 hours and infected with MOI=0.02 Udorn virus. RNA was extracted for qPCR and supernatants were collected for plaque assay at difference time points post infection. Knockdown level of TRBP and PACT was analysed by real-time qPCR using RNA samples 48 hours post transfection. As shown in Figure 6.1a, normalized to non-targeting siRNA, transcription of TRBP and PACT was knocked down by siRNA by ~87%. Virus growth was measured by plaque assay and quantification of total NP synthesized in the cell. NP was selected to represent intracellular virus replication. In both plaque assay and qPCR, shown in Figure 6.1b, c and d, depletion of TRBP and PACT inhibited virus growth. Total NP RNA was ~10 fold less in TRBP depleted cells,

which produced 1 log less virus than the control. Surprisingly, depletion of PKR activator PACT in infected A549 cells showed similar effects as TRBP, but inhibition of virus growth was less, and virus titres slowly caught up with control at the end point of infection, however, total NP RNA remained ~5 fold less than control. These data suggest both TRBP and PACT play a role in influenza virus replication.

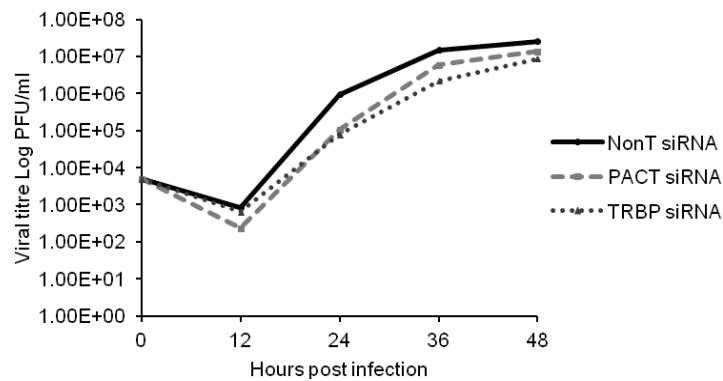
6.2.2 Cell viability post infection improves with knockdown of TRBP

Using the same approach as 6.2.1, A549 cells were transfected with 50nM siRNA for 48 hours and infected with MOI=0.02 Udorn strain. Cell morphology was checked at different time points post infection. As shown in Figure 6.2, cell viability improved by knockdown of TRBP, which is consistent with 6.2.1 that found knockdown of TRBP leads to a reduction in virus titre and less RNA synthesis. To quantify cell viability, an independent assay was performed by infecting TRBP and PACT-depleted cells. Cell viability was quantified by measuring fluorescence emitted by CellTiter-Blue which was added 60 hpi. In non-infected cells, cell viability was not affected by depletion of TRBP and PACT. Compared to RISC-free siRNA transfected cells, depletion of TRBP increased cell viability 60 hpi, while depletion of PACT decreased cell viability 60 hpi. These data also indicate TRBP could play a pro-viral role in influenza virus infection.

a)



b)



c)

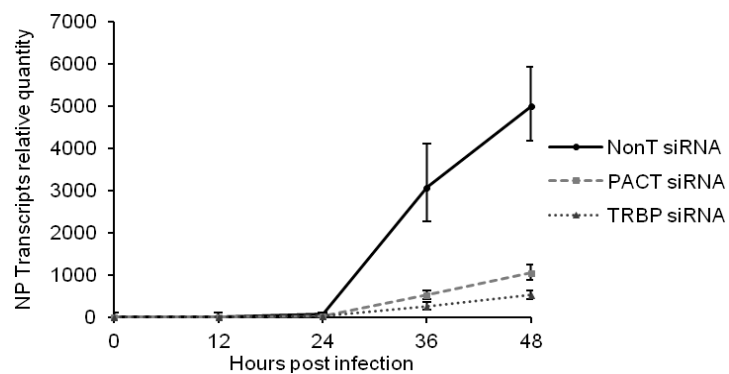


Figure 6.1 Inhibition of influenza virus replication by knockdown of TRBP or PACT. A549 cells were transfected with 50nM siRNA for 48 hours and infected with MOI=0.02 Ud strain. a) RNA was extracted 48 hours post transfection, TRBP and PACT transcripts were analysed by qPCR 48 hours post transfection. TRBP and PACT siRNA transfected were normalized to NonTargeting siRNA transfected cells. Transcription of TRBP and PACT was knocked down by siRNA by ~87%. b) and c) RNA was extracted for qPCR and

supernatants were collected for plaque assay at difference time points post infection. TRBP and PACT siRNA transfected were normalized to NonTargeting siRNA transfected cells at 12 hours post infection in qPCR, error bars represent standard deviation of triplicates in one representative experiment of three. Plaque assay was done by titrating supernatant on MDCK cells. After knockdown of TRBP and PACT, virus titre decreased by 1 log during the early hours of infection (b) and NP transcripts decreased by 5 folds (c).

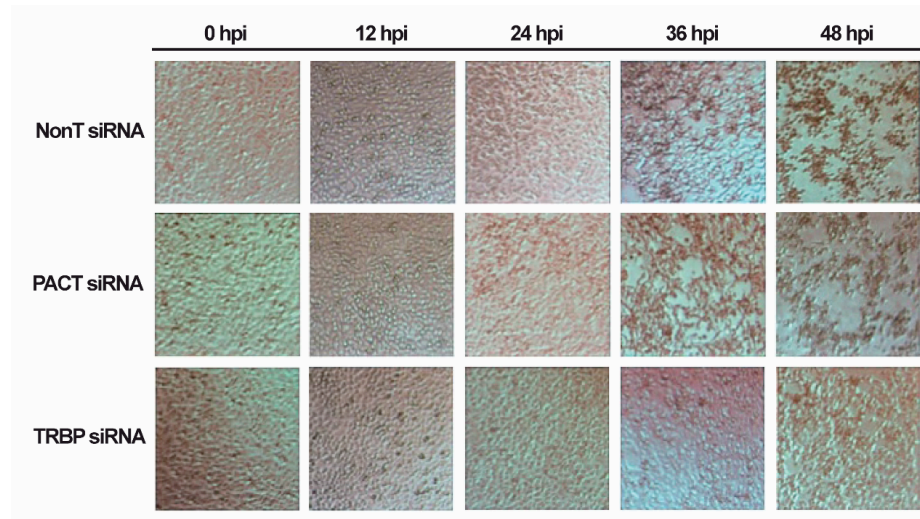


Figure 6.2 Morphology of cells transfected with siRNAs and infected with influenza virus. A549 cells were transfected with 50nM siRNA for 48 hours and infected with MOI=0.02 Udorn strain. Cell morphology was taken by light microscope at different time points post infection. TRBP knocked-down cells showed better viability after influenza virus infection.

6.2.3 Knockdown of TRBP inhibits IFN β production in influenza virus infected cells

To characterize whether IFN β production is affected by TRBP and PACT, quantification of IFN β mRNA was performed in TRBP and PACT-depleted and infected cells. A549 cells were transfected with 50nM siRNA for 48 hours and infected with MOI=0.02 Udorn virus. RNA was extracted at different time points post infection, and IFN β mRNA was analysed by qPCR. Strikingly, Figure 6.4a shows that IFN β production in TRBP knocked down cells was ~5 fold less at 36 and 48 hours post infection compared to control, while no difference was observed for control depletion of PACT. Unchanged expression of IFN β in PACT-depleted cells

could be due to cells overcoming the absence of PACT-induced IFN β by other pathways.

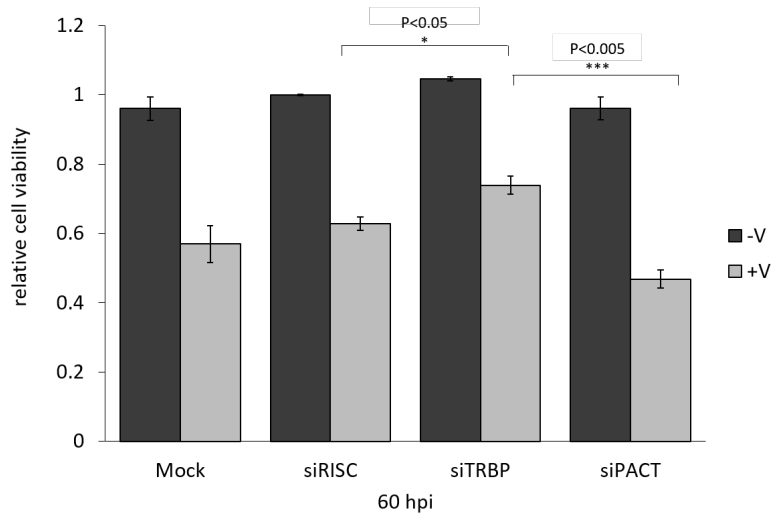
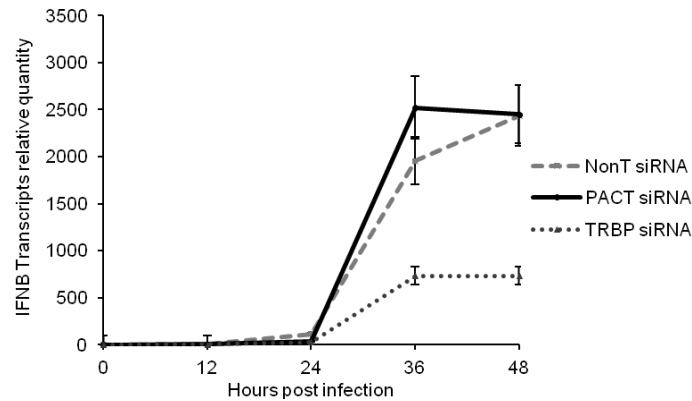


Figure 6.3 Improved cell viability in TRBP-depleted A549 cells post infection. A549 cells were transfected or mock transfected with siRNA for 48 hours, and infected by MOI=0.02 Udorn strain. CellTiter-Blue was added to cells at 60 hours post infection and fluorescence representing cell viability was measured. Statistical significance is indicated with p-value. Depletion of TRBP shows better viability compared to control.

To further investigate the role of TRBP in IFN β induction, A549 cells were transfected with siRNA for 48 hours. A higher MOI of 1 or 4 of Udorn virus was used to infect transfected cells. RNA was harvested at 4 or 9 hours post infection to check whether there was a difference during the early hours of infection. IFN β mRNA level was analysed by qPCR and normalized to GFP siRNA transfected and uninfected cells at 4 hours post infection. As shown in Figure 6.4b depletion of TRBP increased IFN β production in uninfected cells by ~8 fold (both 4 hpi and 9 hpi). However, at 9 hpi, IFN β levels in TRBP-depleted cells which were infected with MOI=1 or MOI=4 virus were 2.5 or 4 fold less than GFP siRNA control. Taking together, these data may suggest knockdown of TRBP that activates PKR leads to increased IFN β production and induces anti-viral state of the cell, which may explain why knockdown of TRBP inhibits influenza virus replication. Less viral RNA could also result in decreased IFN β production in TRBP-depleted cells.

a)



b)

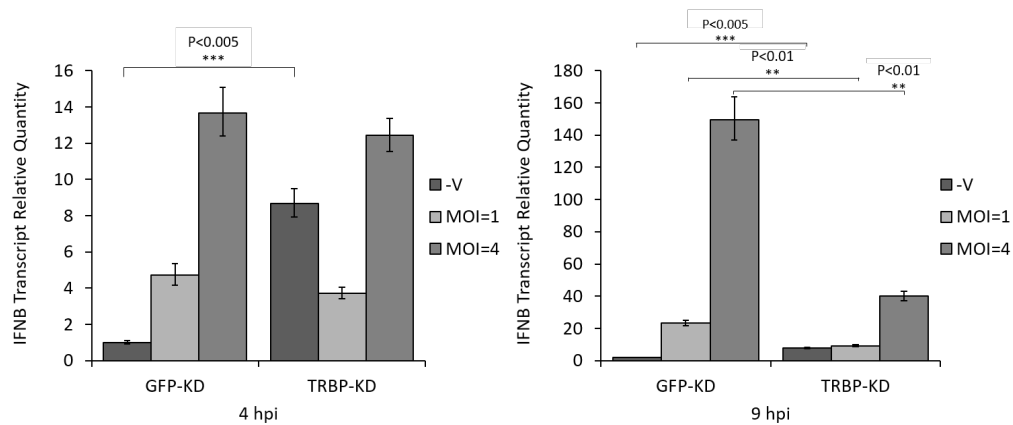


Figure 6.4 Block of IFN β production by knockdown of TRBP in infected cells. a) A549 cells were transfected with 50nM siRNA for 48 hours and infected with MOI=0.02 Udorn strain. RNA was extracted for at difference time points post infection, and analysed by qPCR. TRBP and PACT siRNA transfected were normalized to NonTargeting siRNA transfected cells at 0 hour post infection. IFN β production in TRBP knocked down cells decreased by 5 folds at 36 and 48 hours post infection compared to control. b) A549 cells were transfected with siRNA for 48 hours before infected with MOI=1 or 4 Udorn virus, RNA was harvested at 4 or 9 hours post infection. IFN β level was analysed by qPCR and normalized to GFP siRNA-transfected and uninfected cells. Depletion of TRBP increased IFN β production in uninfected cells, but decreased FN β production in infected cells. Error bars represent standard deviation of triplicates in one representative experiment of three. Statistical significance is indicated with p-value.

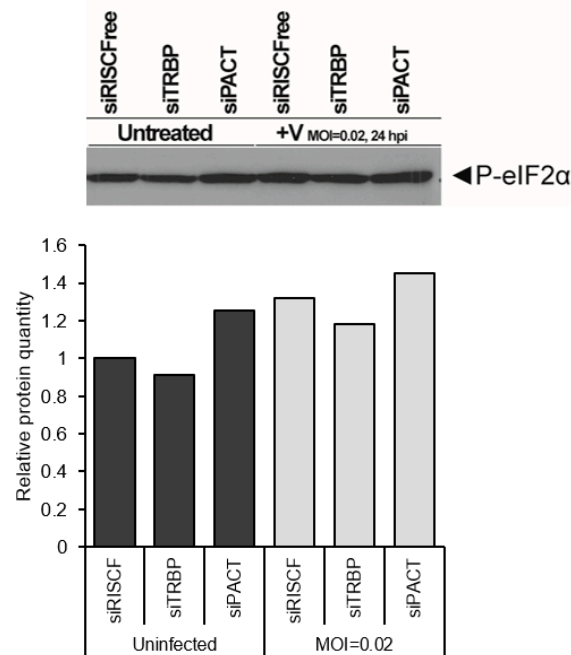


Figure 6.5 No change of phospho-eIF2 α by knockdown of TRBP and PACT in infected cells. HEK293T cells were transfected with 50nM siRNA and infected with MOI=0.02 Udorn strain 48 hours post transfection. Cells were lysed 24 hours post treatment and cell lysates were boiled with 4X Laemmli buffer, separated on 12% polyacrylamide gels and immunoblotted with anti- phospho-eIF2 α antibody. Protein content was quantified by ImageJ, and normalized to NonTargeting siRNA uninfected.

6.2.4 No change in phosphorylation of EIF2 α in infected cells by depletion of TRBP and PACT

Activated PKR phosphorylates eIF2 α to shut off cellular and virus protein synthesis, thereby inhibiting virus replication (Garcia *et al.*, 2006). NS1 is known to inhibit PKR by direct binding, as PKR regulators TRBP and PACT were identified to interact with NS1 here in this study, there could be potentially multiple levels of PKR inhibition. By binding to TRBP and PACT, NS1 could inhibit activation of PKR through PACT. Thus to test this hypothesis, phosphorylation of eIF2 α was checked by infecting TRBP and PACT-depleted cells. HEK293T cells were transfected with 50nM siRNA, and infected with MOI=0.02 Udorn virus 48 hours post transfection. Cells were lysed 24 hours post treatment and cell lysates were analysed by western

blot. As shown in Figure 6.5, the level of eIF2 α phosphorylation was not changed by influenza virus infection. Knockdown of TRBP and PACT failed to have an effect on eIF2 α phosphorylation either. This could be due to the low MOI used in the assay, such that there was not enough cells infected to allow detection of activation of PKR and consequent eIF2 α phosphorylation in this assay. There were not enough viruses to activate PKR. Future experiment must be done with a much higher MOI to activate PKR, furthermore, phosphorylation of eIF2 α and PKR should be both checked. This experiment is crucial which examines whether NS1 is able to inhibit PKR at multiple levels.

6.3 Discussion

The results presented in this chapter show that depletion of TRBP and PACT blocks viral RNA synthesis of influenza virus, and inhibits influenza virus replication. In addition, cell viability improves by knockdown of TRBP but not PACT post infection, suggesting TRBP is required for influenza virus replication. Furthermore, IFN β induction during infection is not changed in PACT depleted cells, suggesting cells may overcome the absence of PACT to induce IFN β by other pathways during infection. However, surprisingly, knockdown of TRBP in infected cells significantly limits IFN β production following virus infection. This could be due to that fact that knockdown of TRBP results in increased expression of IFN β thus producing an antiviral state which limits virus infection in these cells. Characterization of eIF2 α phosphorylation fails to show effect of knockdown of TRBP and PACT on PKR activation. Further experiments to characterise phosphorylation PKR and eIF2 α with higher MOI are required to examine whether NS1 is able to inhibit PKR in multiple levels (Figure 6.6). The difficulty in determining exact role of TRBP and PACT in IFN β induction and PKR regulation is that the complex involves four proteins, NS1, PKR, TRBP and PACT are all able to interact with each other respectively. It is difficult to dissect role of individual protein independent of the other. In addition the assays presented in this chapter reply on siRNA knockdown,

which however may have off-target effects, eg other components in innate response could be altered by using these siRNAs.

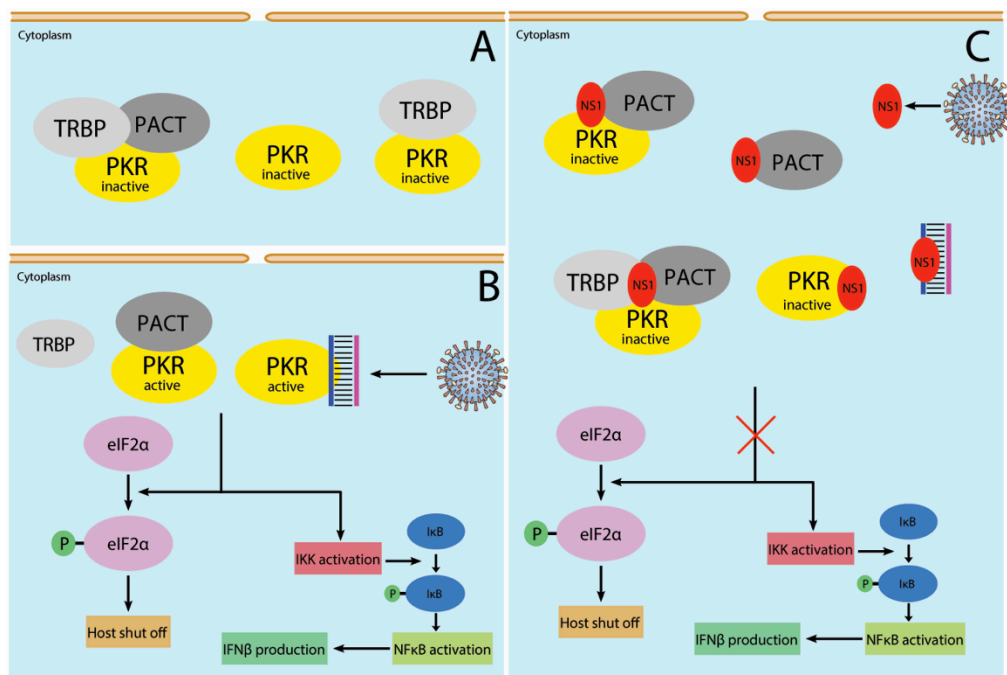


Figure 6.6 Schematic diagram of possible inhibition of PKR by NS1 at multiple levels. A) Without stress or infection, PKR is inactive, TRBP is able to keep PACT and PKR in inactive form. B) Upon infection or stress, TRBP no longer binds to PKR or PACT, PKR can be activated by PACT or by dsRNA. Activated PKR phosphorylates eIF2α to induce host shut off and also induces IFNβ production. C) Once NS1 is expressed, it inhibits PKR by direct binding. NS1 can also bind to dsRNA and PACT to prevent PKR activation at multiple levels. As a result, phosphorylation of eIF2α and IFNβ production are inhibited.

TRBP is a strong inhibitor of PKR and during conditions of no stress, it forms heterodimer with PACT, to prevent PACT activation. Phosphorylation of PACT weakens the interactions between TRBP and PACT, thereby PKR can be activated (Singh *et al.*, 2011, Singh and Patel, 2012). Active PKR blocks IκB which is an inhibitor of NFκB signalling. Activation of NFκB signalling subsequently leads to production of IFNβ (Garcia *et al.*, 2006, D'Acquisto and Ghosh, 2001). Thus it is not surprising to observe a pro-viral role of TRBP, whereby knockdown of TRBP inhibits influenza virus growth. In contrast to TRBP, PACT is presumed to be an anti-viral factor, and it has been reported that PACT binds to RIG-I to enhance IFNβ

promoter activity (Tawaratsumida *et al.*, 2014). Strikingly, a similar effect of PACT compared to TRBP in infected cells was observed: depletion of PACT also inhibited virus growth. In a study by Clerzius *et al* on HIV-1, it was found that PACT reversed phosphorylation of PKR and eIF2 α induced by HIV-1, resulting in rescue of PKR-mediated inhibition of host shut off (Clerzius *et al.*, 2013). It appears that the role of PACT is reversed in influenza virus infection, particularly since RNA synthesis by influenza virus is strongly inhibited in the absence of TRBP and PACT, interactions between NS1, TRBP and PACT could mediate regulation of viral RNA synthesis. Therefore, binding site mapping of interactions between NS1, TRBP and PACT is needed to understand whether disruption of these protein-protein interactions results in the same effects as depletion of TRBP and PACT.

As discussed in Chapter 5, NS1 binds to PACT to prevent the heterodimer of TRBP and PACT functioning effectively in the RISC-loading complex. Given that inhibition of RNA silencing is in favour of virus replication, the hypothesis here is that the role of interactions between NS1, TRBP and PACT in host RNAi inhibition is independent of role of TRBP and PACT in PKR regulation and IFN β induction. Upon NS1 expression, NS1 binds to TRBP and PACT, which already form a triple complex with Dicer (Kok *et al.*, 2007). Moreover, besides inhibiting PKR by direct binding, NS1 also binds to TRBP and PACT molecules that are not working in miRNA processing duties to prevent activation of PKR by PACT.

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Chapter 7: Conclusions and outlook

Seasonal epidemics and occasional pandemics caused by influenza A viruses are a threat to public health and global economy. The very last influenza pandemic that started in early 2009 by H1N1 virus caused an estimated 60.8 million cases of infections worldwide (Webster and Govorkova, 2014). The host species barrier into humans has been overcome several times in the past by influenza virus, most recently by avian influenza A H7N9 viruses in China, which resulted in a high mortality in humans (Gao *et al.*, 2013, Li *et al.*, 2014). Moreover, it has been reported that influenza viruses are becoming resistant to antiviral drug amantadine and oseltamivir, which raised a huge concern (Hayden and de Jong, 2011). Like all viruses, influenza viruses rely on the host cellular machinery for life cycle. Accordingly, it is of great interest and essential to identify host factors involved in virus replication to better understand the mechanisms of the virus life cycle and to search for potential targets for the development of novel diagnostics and antiviral therapeutics. One of the ways of achieving this is to investigate protein-protein interactions between host and virus. Availability of genome-wide approaches, such as Y2H, RNA interference and proteomics has shed new light on host factors involved in virus replication (Watanabe *et al.*, 2010).

The work presented in this thesis was to investigate the interactions between host and influenza A virus in two contexts. Thus, this thesis is divided into two separate sections which explore the interactions first from a genome-wide screen, then went in to detailed functional analysis of specific interactions. The genome-scale yeast two-hybrid protein interaction screen performed using 6 different influenza A virus strains and analysis are discussed in detail in Chapter 3. As discussed in chapter 3, in our study we found 114 out of 127 high-confidence interactions identified in the screen between the host and influenza virus are novel. Overall, 38% (18/47) of high-confidence interactions were validated for at least one influenza strain in LUMIER assays. GO-term analysis of our Y2H dataset with two existing public datasets, Y2H

dataset by Shapira and a combined data set from the five siRNA screen-based studies highlights 4 classes of enriched terms, which are intracellular transport, RNA processing, protein folding, cell cycle and signal transduction (Hao *et al.*, 2008, Brass *et al.*, 2009, Karlas *et al.*, 2010, König *et al.*, 2010, Shapira *et al.*, 2009). In our screen ARCN1 was shown to interact with M1, which is also discussed by König in endosome transport vehicle formation required by the virus. KPNB1, CAMK2B were found to interact with NP here also were reported to play a role in viral RNA transcription and nuclear accumulation of viral protein. Interesting interactions also include NP with PAICS, an enzyme involved in purine biogenesis. Investigation of these individual interactions and the elucidation of their biological relevance will provide valuable insight into the virus life cycle.

Among the interactions identified in our Y2H screen, two interesting interactions - NS1 from all six influenza strains was found to interact with TRBP and PACT - were further investigated in details from chapter 4 to chapter 6. In chapter 4, the two interactions were first confirmed in Y2H direct mating assay, in addition, direct mating Y2H data did not show any difference in affinity of interaction of NS1 with TRBP and PACT. With the treatment of RNase, both Ud/NS1 and WSN/NS1 were confirmed to interact with TRBP and PACT by CoIP. It was that first time TRBP was confirmed to interact with NS1 in a biochemical binding assay. Although a possibility that RNA is protected in the complex from the access of RNase cannot be completely ruled out, an in vitro binding assay is needed in the future. The colocalization of NS1 with TRBP and PACT was observed in the cytoplasm. Confirmation of the affinities between NS1s, TRBP and PACT might reveal differences between the human and avian strains. It would also be interesting to clarify whether pathogenicity is influenced by the difference in binding affinity.

TRBP and PACT play an important role in Dicer-mediated RNA silencing (Chapter 5) (Lee *et al.*, 2006, Kok *et al.*, 2007, Lee *et al.*, 2013). The work presented in this thesis revealed that NS1 forms a heterodimer with TRBP and PACT, both of which facilitate Dicer-mediated RNA processing, and thus limits their role in the RISC-

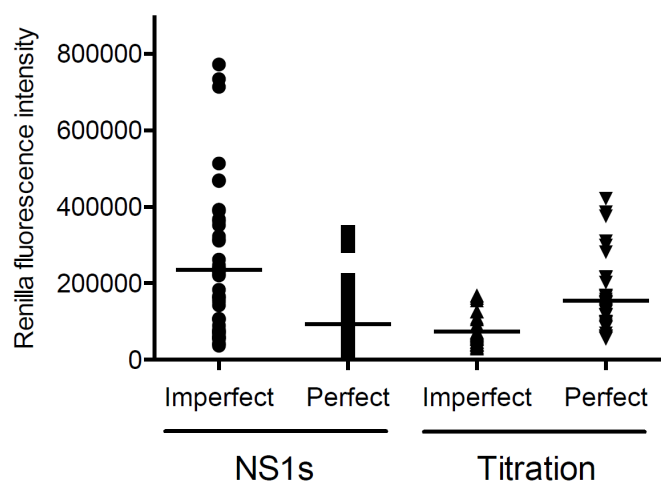
loading complex, which leads to an inhibition of miRNA maturation and suppression of RNA silencing. The binding of NS1 to PACT and TRBP disrupts the binding of the RISC-associated core component Ago2. A more detailed mapping of the binding site of NS1 to TRBP and PACT will help to better understand the structure and the mechanics of this large complex. A NS1 mutant deficient in TRBP and PACT binding will allow to generate a recombinant influenza virus that can be used to determine the effect of the NS1-mediated inhibition of RNA silencing in infection assays in cell culture and in animal models.

As TRBP and PACT are also regulator of antiviral protein PKR, implicated to take part in interferon pathway, Chapter 6 show that depletion of TRBP and PACT blocks RNA synthesis of influenza virus, and inhibits influenza virus growth (Patel *et al.*, 2000, Li *et al.*, 2006a, Singh *et al.*, 2011, Singh and Patel, 2012). In addition, cell viability improves by knockdown of TRBP but not PACT post infection, indicating TRBP is required for influenza virus replication. Furthermore, IFN β induction during the infection is not changed in PACT depleted cells, suggesting cells overcome the absence of PACT to induce IFN β by other pathways during the infection. However, knockdown of TRBP in infected cells limits IFN β production. Given that inhibition of RNA silencing could be in favour of virus replication as discussed in chapter 5, the role of interactions between NS1, TRBP and PACT in host RNAi inhibition is independent of role of TRBP and PACT in PKR regulation. When NS1 is expressed, NS1 binds to TRBP and PACT, which already form triple complex with Dicer. Besides inhibition of PKR by direct binding, NS1 could also inhibits activation of PKR by binding to PACT. In other viruses such as Ebola virus and HIV, their components VP35 and Tat also share similar role with influenza virus NS1 in inhibiting PKR activation (Haasnoot *et al.*, 2007, Unwalla and Rossi, 2008), this mechanism of dual role of viral factor in RNAi and PKR regulation could be conserved among RNA viruses. Further work can be directed to verify the hypothesis of multiple levels of PKR inhibition by NS1, and clarify how TRBP could enhance virus replication by controlling IFN β production. To achieve this, cells can

be overexpressed with mutant TRBP that does not bind NS1 and infected with virus, and comparison can be made between TRBP and mutant TRBP.

In summary, this study first aimed at providing a broad picture of interactions of host with influenza A virus across different strains, as well as elucidating role of specific interactions in virus life cycle. Although it is mission impossible to map out and investigate in detail the every interaction within the interactome in this study, it still has added to the existing knowledge on how the influenza A virus interplays with the host, especially the mechanisms how the virus exerts influence on important host functions – host RNA silencing and regulation of viral interferon induction. The ultimate aim is that these interactions identified in this screen could be employed to develop novel diagnostics and antiviral therapeutics against influenza A viruses.

Appendices



Appendix Figure 1 Renilla fluorescence intensity of relevant assays. Plasmids expressing KSHV miR10/12 and the psiCHECK luciferase reporter (either an imperfect or perfect-match with miR4) were co-transfected with plasmids expressing NS1, PACT or TRPB into HEK293T cells. Firefly and Renilla luciferase activity was measured 24 hr post-transfection. “NS1s” represent data in Figure 5.3, “Titration” represents data in figure 5.4. As internal control, Renilla luciferase was expressed well in general.

	UDOR	WSN	UDOR	WSN	UDOR	WSN	H5N1	H7N1-	H7N1-	UDOR	WSN	H7N1-	SWINE	H7N1-	UDOR	WSN	UDOR	WSN	UDOR	WSN	H5N1	H7N1-	UDOR	WSN	
Prey gene symbol	M2	M2	M1	M1	NA	NA	NP	NP	NP	NP	NP	NS1	NS1	NS1	NS1	NS1	NS2	PB1	PB1	PB2	PB1.F2	PB1.F2	PB1.F2	PB1.F2	
TARBP2												24	28	73	59	53									237
HOXA1										67	149														216
ZMAT4												44	23	51	1	17									136
PAICS							34	16		46	38														134
PRKRA												43	12	26	13	33									127
MTIF3	1	87																							88
ELAVL3									58																58
CRK												8	2	40											50
TP53							3	4		11	11														29
ZMAT3															7	16									23
PSME2								3		4	15														22
USP14												6	7	7											20
PSMC3																							20	20	
MRPL36								9		3	6														18
SHMT2		15																							15
ACOT13					1		4	5		1	4														15
C9orf30							3	2		3	5														13
FHL3																							13	13	
HSD17B12															7	6									13
PIK3R3															12	1									13
PABPN1							4	3		1	4														12
RBM45							4	8																	12
KPNA3							1	8			1														10
SYCE1															10										10
TCEAL8											1		9												10
Kpna6								2		6	1														9
CCDC56																	8								8
TCAP															8										8
SON												1	2	2	3										8
DCTD							5	2																	7
ZNF19																					7				7
STAU1												1	2	3											6
ZNF346												4		2											6
CMPK1				2	4																				6
FTH1							1			4	1														6
NIF3L1							1	1		1	3														6
SFRS3											2						4								6
SNRNP25										6															6
TOM1L1			6																						6
PRR19												1	2	2											5
CAMK2B							3	2																	5
GIPC2										3	2														5
IMPDH2							2	1		1	1														5
RHBDD1										3	2														5
RRM2	2	3																							5
TNFAIP1											5														5
MMADHC																						2	3	5	5

[illegible]

Appendix Table 1 List of 127 high confidence hits identified in Y2H screen.

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