Immuno-Regulatory Dynamics of Non-Structural Protein 1 of Avian Influenza A Viruses

IFN-β, NF-κB and AP-1 Perspectives

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Cover: *Illustration of influenza virus replication in a host cell*. The influenza virus particles attach to the host cell receptors via their hemagglutinin protein and enter into the cells by receptor-mediated endocytosis (top right). Acidification of the endosome releases the viral RNAs, which translocate to the nucleus and undergo transcription and replication. The ribonucleoprotein is exported to the cytoplasm for assembly. The hemagglutinin, neuraminidase and matrix protein 2 arrive at the plasma membrane after processing in the Golgi apparatus (bottom left), where formation of the viral particles occurs. The newly forming virus particles bud from the cell membrane and are released to infect other cells.

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Abstract

The type I interferon (IFN-α/β) system represents the first line of defense against influenza A viruses, and is mainly mediated by the interferon regulatory factors 3/7 (IRF3/7), nuclear factor kappa B (NF-κB) and activator protein 1 (AP-1) transcription factors. Therefore, the non-structural protein 1 (NS1) of influenza A viruses has intrinsic abilities to disarm the host cell IFN system, and as a consequence is deeply connected to influenza virus pathogenicity and virulence. Based on differences in the amino acid sequence of the NS1 genes, the influenza A viruses are divided into two distinct gene pools, named allele A and B. This division is strictly based on genetic characterization, and their functional consequences are largely unknown.

The results in this thesis provide novel structural and functional insights, indicating that allele A NS1 proteins of various avian influenza A viruses have a stronger capacity to inhibit the activation of IFN-β production than allele B proteins from corresponding subtypes. This claim was further confirmed by measurement of IFN stimulatory response element (ISRE) promoter activation, IFN-β mRNA transcription and IFN-β protein expression. Intriguingly, the allele-specific levels of inhibition correlated with the nature of the disease, where for example allele A influenza virus appeared to be more pathogenic than allele B viruses in minks (*Mustela vison*). Further investigations demonstrated that allele B NS1 protein resembles to its IFN antagonistic nature in exerting similar effect in preventing double stranded RNA (dsRNA) induced NF-κB and AP-1 promoter activation, which subsequently results in an overall inhibition of IFN-β production. In an effort to understand the structural basis of IFN inhibition, it was revealed that inhibitory activities for ISRE, NF-κB and AP-1 promoters and subsequent inhibition of IFN-β were mapped to the C-terminal effector domain of the NS1 protein.

Genetic characterization of a reassortant H9N2 influenza virus revealed that the virus acquired the NS segment from a H5N1 of genotype Z, clade 2.2. Screening the IFN antagonist abilities of both NS1 proteins (H5N1 and H9N2) demonstrated a high degree of functional homology at IRF3, ISRE, NF-κB and AP-1 levels, which resulted in strong inhibition of IFN-β production in cell culture system.

Taken together, these studies expand the information concerning the versatile nature of the NS1 protein and highlight its indispensible role in reassortant influenza A virus pathogenesis.

Keywords: Allele A and B, avian influenza, IFN-β inhibition, NS1 protein

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Dedications

To my Parents, Wife
and other Family Members
for their
Tireless Support

يَٰٓأَيُّهَا ٱلَّذِينَ ءَامَنُوٓاْ إِذَا قِيلَ لَكُمۡ تَفَسَّحُواْ فِي ٱلۡمَجَٰلِسِ فَٱفۡسَحُواْ يَفۡسَحِ ٱللَّهُ لَكُمۡۖ وَإِذَا
قِيلَ ٱنشُزُواْ فَٱنشُزُواْ يَرۡفَعِ ٱللَّهُ ٱلَّذِينَ ءَامَنُواْ مِنكُمۡ وَٱلَّذِينَ أُوتُواْ ٱلۡعِلۡمَ دَرَجَٰتٖۚ وَٱللَّهُ بِمَا
تَعۡمَلُونَ خَبِيرٞ ١١

"Believers, when you are told to make room in a meeting for others, do so. Allah will then make room for you. When you are told to disperse, do so. Allah will raise the position of the believers and of those who have received knowledge. Allah is well aware of what you do" Al-Quran, Sūrah-al-Mujādilah (Chapter 58, Verse 11).

Contents

List of publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Zohari, S., **Munir, M.,** Metreveli, G., Belák, S., and Berg, M. (2010). Differences in the ability to suppress interferon beta production between allele A and allele B NS1 proteins from H10 influenza A viruses. *Virology Journal* 7(1), 376.

II **Munir, M.,** Zohari, S., Metreveli, G., Baule, C., Belák, S., and Berg, M. (2011). Alleles A and B of non-structural protein 1 of avian influenza A viruses differentially inhibit beta interferon production in human and mink lung cells. *Journal of General Virology* 92(9), 2111–2121.

III **Munir, M.,** Zohari, S., and Berg, M. (2011). Non-structural protein 1 of avian influenza A viruses differentially inhibit NF-**κ**B promoter activation. *Virology Journal* 8(1), 383.

IV **Munir, M.,** Zohari, S., Belák, S., and Berg, M. (2011). Double stranded RNA induced activation of activating protein-1 promoter is differentially regulated by non-structural protein 1 of avian influenza A viruses. *Viral Immunology* (*accepted*).

V **Munir, M.,** Zohari, S., Abbas, M., Iqbal, M., Perez, R.D., Belák, B., and Berg, M. (2011). The NS gene segment of H9N2 influenza virus isolated from backyard poultry in Pakistan revealed strong genetic and functional similarities to the NS gene of highly pathogenic H5N1 (*manuscript*).

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Abbreviations

2′-5′-OAS	2′-5′-oligoadenylate synthetase
AIV	Avian influenza viruses
AP-1	Activator protein 1
ATP6V0D1	V-type proton ATPase subunit D 1
CPSF30	Cleavage and polyadenylation specificity factor 30 kDa subunit
cRNA	Complementary RNA
DIVA	Differentiation of infected and vaccinated animals
dsRNA	Double-stranded RNA
ED	Effector domain
eIF2-α	Eukaryotic translation initiation factor 2-α
eIF4GI	Eukaryotic translation initiation factor 4 GI
ER	Endoplasmic reticulum
ESCRT	Endosomal sorting complex transport
HA	Hemagglutinin
HPAIV	Highly pathogenic avian influenza viruses
IFNAR	IFN α/β receptor subunit 1
IKKε	Inhibitor of NF-κ kinase ε
IRF3	Interferon regulatory factor 3
ISGF3	Interferon stimulated gene factor 3
ISGs	Interferon stimulated genes
ISRE	Interferon stimulated response elements
JAK	Janus kinase
LPAIV	Low pathogenic avian influenza viruses
M1	Matrix protein 1
M2	Matrix protein 2
MAVS	Mitochondrial antiviral signalling protein
MDA5	Melanoma differentiation-associated gene 5
MDCK	Madin Darby Canine Kidney

mRNAs	Messenger RNAs
NA	Neuraminidase
NEP	Nuclear export protein
NES	Nuclear export signal
NF-κB	Nuclear factor kappa B
NLS	Nuclear localization signal
NoLS	Nucleolar localization signals
NP	Nucleoprotein
NS1	Non-structural protein 1
NSP	Negative sense protein
OIE	Office international des epizooties/World organization for animal health
ORF	Open reading frame
P13K	Phosphoinositide 3-kinase
PA	Polymerase acidic subunit of RNA-dependent RNA polymerase
PABPI	Poly(A)-binding protein I
PABPII	Poly(A)-binding protein II
PACT	PKR-associated activator
PB1	Polymerase basic 1 subunit of RNA-dependent RNA polymerase
PB2	Polymerase basic 2 subunit of RNA-dependent RNA polymerase
PKR	DsRNA-dependent serine/threonine protein kinase R
Poly I:C	Polyinosinic:polycytidylic acid
PRDs	Positive regulatory domains
RBD	DsRNA-binding domain
RIG-I	Retinoic-acid-inducible gene I
SOCS	Suppressor of cytokine signaling
STAT	Signal transducer and activator of transcription
TBK1	TANK binding kinase 1
TLRs	Toll like receptors
TRAF	TNF receptor-associated factor
TRIF	TIR domain-containing adaptor inducing IFN-β
TRIM25	Tripartite motif containing protein 25
UTR	Untranslated region
vRNA	Viral RNA
vRNP	Viral ribonucleoprotein
WHO	World Health Organization

1 Introduction

1.1 Background of avian influenza viruses

Avian influenza viruses (AIV) have emerged as devastating pathogens not only for poultry industries, where they infect millions of birds (over 200 million from 1999-2004) but also have human health implications, especially due to recent increase in spread of Asian H5N1 viruses (Capua & Alexander, 2008). Although wild aquatic birds, such as waterfowl and shorebirds, are primary natural hosts for avian influenza viruses, interspecies transmission has been recorded (Webster *et al.*, 1992). In wild aquatic birds, influenza viruses usually infect the lower intestinal tract and transmit via the fecal-oral route. Intriguingly, the genome of influenza viruses has been detected in at least 105 avian species (Olsen *et al.*, 2006). However, among all the subtypes of influenza A viruses, only H1N1, H2N2, H3N2, H5N1, H7N7, H7N2, H7N3 and H9N2 have been reported to cause illness in humans, as reviewed in Cheung & Poon (2007). This selective adaptation could be a result of steady evolution of AIV to several species of animals, and ultimately terminating in human pandemics several times in the last 100 years (Taubenberger & Morens, 2009) (discussed below in detail).

The highly pathogenic avian influenza virus (HPAIV) H5N1 is now widely prevalent in most of the countries of Southeast Asia, the Middle East, Europe, Africa and Asia. Although, transmission of AIVs to humans may require an intermediate host such as swine, there are indications of direct transmission of H5, H7 and H9 to humans (Fouchier *et al.*, 2004; Bridges *et al.*, 2002; Cameron *et al.,* 2000). The low pathogenic avian influenza viruses (LPAIV) such as H9N2 have shown increased infection levels in recent years, especially in the Middle East, Asia and Europe (Capua & Alexander, 2008). Provided that H9N2 viruses have potential to infect humans, monitoring of such cases would be of importance to ascertain their genetic variability and ability to switch

hosts (Capua & Alexander, 2008). Each of the human influenza pandemics occurs with a different approach and has different characters, which are primarily contributed by a complex and polygenic mechanism of host-switch (Taubenberger & Kash, 2010). This makes the understanding of AIV replication and transmission complicated. Taken together, future progress is required for identification of the molecular mechanisms of AIV host adaptation, pathogenesis and multifactorial host-pathogen interactions.

1.2 Biology of influenza viruses

1.2.1 Overview

Influenza viruses are members of the family *Orthomyxoviridae*. The virus particles are enveloped, containing a segmented, single-stranded, negative-sense RNA genome (i.e. the genome is complementary to mRNA, which by convention is termed positive-sense) (Klenk *et al.*, 2004). The genera in this family include influenza A virus, influenza B virus, influenza C virus, thogotovirus and isavirus (infectious salmon anemia virus) (Palese & Shaw, 2007). The three influenza viruses (A, B and C) differ in host range and pathogenicity. Based on their surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA), the influenza A viruses are further subdivided into 16 subtypes of HA protein (1-16) and 9 subtypes of NA (1-9) protein (Fouchier *et al.*, 2005; Laver *et al.*, 1984). Among 144 (16x9) theoretically possible combination of HA and NA influenza subtypes, at least 116 have been reported in birds. However, only the H5 and H7 subtypes are identified as HPAI in susceptible bird populations (Webster *et al.*, 1992).

1.2.2 Structure of influenza virus particles

The virions of influenza A and B genera are structurally identical, but influenza C virus particles are more divergent. Influenza A virus particles are pleomorphic in shape, which varies from small spherical (80-120 nm) to long filamentous (300 nm). Although morphologic characteristics are a genetic trait, the viral strain and cell-type used for propagation may significantly determine the shape of influenza viruses. The whole virion is composed of 1% RNA, 70% protein, 20% lipid and 5-8% carbohydrates (reviewed in Lamb & Krug, 2001).

The influenza A virions consist of eight single-stranded RNA segments, which encode for 11 (or up to 13) viral proteins during infection (reviewed in Garcia-Sastre, 2005; Lamb & Krug, 2001) (Figure 1 A). Additionally, a putative open reading frame (ORF) in the positive-sense of segment 8 has recently been identified, which encodes for a hypothetical negative sense

protein (NSP) of ~25 kDa (Zhirnov *et al.*, 2007). Recently, another protein encoded in segment 2 (in addition to PB1 and PB1-F2) has been identified, termed N40 (Wise *et al.*, 2009). Morphologically, influenza A virus particles are lined with a layer of approximately 500 spikes (each of 10-14 nm), protruded from a lipid envelope, which is derived from the host plasma membrane. These spikes appear as either rod-shaped (HA) or mushroom-shaped (NA) (Figure 1 B). The ratio of HA to NA generally varies from 4:1 to 5:1. The high density of HA is probably to enhance the chances for viral attachment. Additionally, the matrix protein 2 (M2) transverses the lipid envelope and overlays a matrix of M1 protein. The ratio between M2 and HA is usually about 1:10 to 1:100 (Zebedee & Lamb, 1988). The matrix protein 1 (M1) is the most abundant viral protein and underlies the lipid bilayer, and is associated with the vRNP core of the virion to structurally support the particle.

1.2.3 Viral ribonucleoprotein (vRNP)

The vRNP is the stable lipid-free core within the virion. The vRNP appears as flexible rods in thin sectioning of the virus. Structurally, vRNPs look like a twin-stranded helix, in which vRNPs present loops at one end and a bend at other. The strand seems to be folded back on itself and then coiled on itself, to form a type of twin-stranded helix (Figure 1 C) (reviewed in Lamb & Krug, 2001).

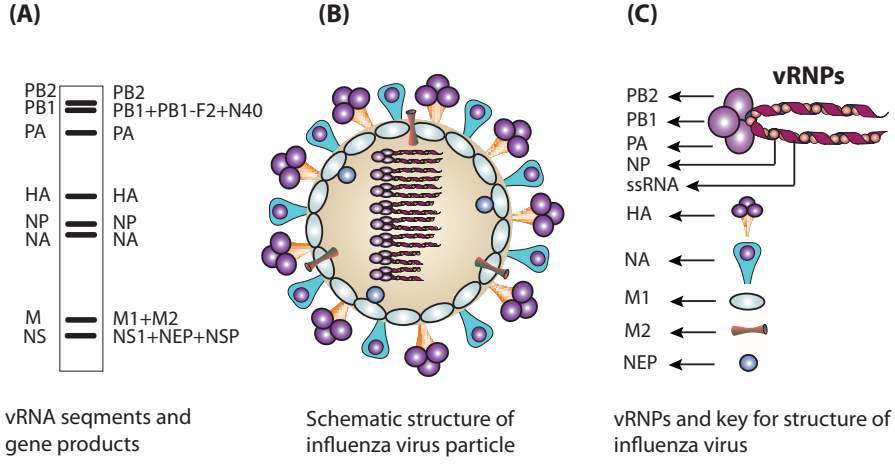

Figure 1. A schematic diagram of the influenza A virus particle and its components. **(A)** Eight gene segments of influenza A virus encode for 11-13 different proteins of variable sizes. **(B)** An influenza virus particle carrying all the structural components. **(C)** Composition of vRNPs, and a key for the influenza virus proteins.

Four viral proteins along with the viral RNA constitute the vRNP (Figure 1 C). Among these proteins, NP is the predominant protein, and it coats the viral RNA (vRNA). Associated with the vRNPs is the heterotrimeric RNA-dependent RNA polymerase complex, consisting of the two "polymerase basic" (PB1 and PB2) and one "polymerase acidic" (PA) subunits. These are present at only 30 to 60 copies per virion (Lamb & Krug, 2001; Inglis *et al.*, 1976; Lamb & Choppin, 1976).

The varied roles of these proteins are briefly outlined below in the context of the influenza virus replication cycle, and concisely summarized in Figure 2.

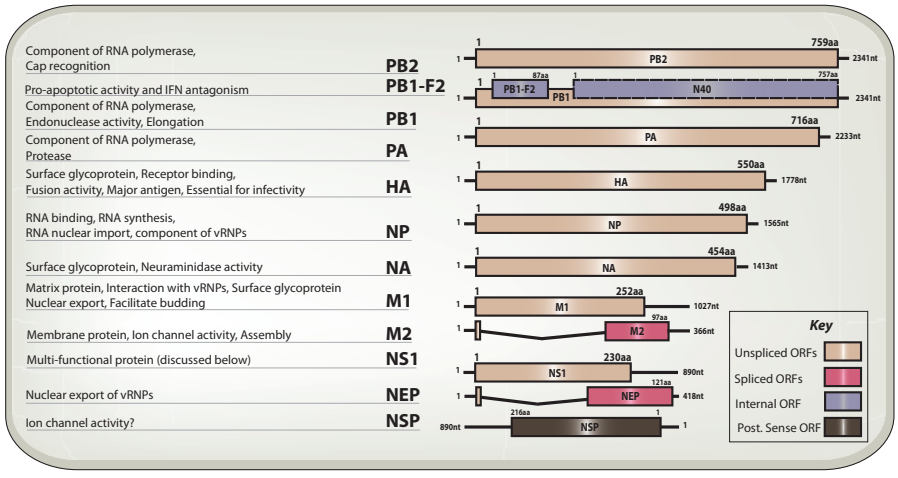

Figure 2. A schematic genome organization of the influenza A virus. The complete RNA segments are shown in nucleotides whereas their encoded proteins are presented in amino acids. The lines at the 5´and 3´ termini represent the noncoding regions. The PB1 segment contains a second ORF in the +1 frame resulting in the PB1-F2 protein. Another ORF encodes for N40, which is identical to that of maternal PB1 except for truncated 39 amino acids in the N-terminus, is shown in dotted lines. The M2 protein and the nuclear export protein (NEP) are encoded by spliced mRNAs (the introns are indicated by the V-shaped lines). A putative ORF for a hypothetical negative sense protein (NSP) within segment eight is shown.

1.3 Zoonotic potential and influenza pandemics

Influenza A viruses have a certain level of host specificity, but breaching host species barriers is not uncommon. Therefore, it is critical to understand the zoonotic potential of non-human (avian, swine and equine) influenza viruses, and to ascertain the contribution of these species in the generation of novel variants that could lead to future influenza pandemics. It has been demonstrated that reassortment of genomic fragments, involving those coding for glycoproteins (HA and NA), usually results in emergence of new pandemic strains of influenza viruses in humans. However, major epizootics have

repeatedly been reported from poultry, horses, seals, camels and pigs (Hayden & Palese, 1997; Yamnikova *et al.*, 1993).

Influenza pandemics are global outbreaks of the disease that cause serious illness and spread easily from human to human. In the case of influenza, such conditions happen when unusual new variants appear from the combination of unique HA and NA strains from different viruses, by antigenic drift and shift (reassortment) (discussed below). Although, the history of influenza A viruses dates back to Ancient Greek in about 413 BC (Potter, 1988), the first confirmed pandemic was the Russian flu of 1889-1892 (Potter, 2001; Kuszewski & Brydak, 2000). In the 20th century, a devastating pandemic (Spanish flu) appeared in 1918 and lasted into 1919 (Figure 3). Since the first isolation of influenza A viruses in 1933, it has been speculated based on serology that the HA protein in this pandemic was of avian origin. Viruses of this subtype circulated until the Asian flu in 1957, when a new virus of another HA and NA (H2N2) was isolated (reviewed in Webster *et al.*, 1992). It was confirmed that H2N2 virions are composed of PB1, NA and HA genes of an avian origin, and PB2, PA, nucleoprotein (NP), M and non-structural (NS) genes were acquired from previously circulating H1N1 viruses (Scholtissek *et al.*, 1978). These viruses circulated for 11 years when a new strain caused a new pandemic in 1968 with the introduction of a novel HA variant (H3). Besides the HA gene, these viruses acquired a PB1 gene segment from an avian virus, whereas the rest of the genes were from human influenza viruses (Kawaoka *et al.*, 1989; Scholtissek *et al.*, 1978). Although controversial, H1N1 reemerged in 1976 and were reported as Russian influenza (H1N1) (Dowdle, 1997). Interestingly, the Asian flu, Hong Kong flu and Russian flu pandemics have several factors in common, such as their appearances were sudden, all were first reported in China, all were antigenically different from already circulating viruses in human, and belonged to either H1, H2 or H3 (reviewed in Webster *et al.*, 1992). Provided that both pandemics (1957 and 1968) originated by genetic reassortment between avian and human influenza viruses, it was concluded that a highly pathogenic avian influenza virus H5N1 evaded the host-specific restriction and caused infection to humans in 1997-98.

After 41 years, in 2009, a swine origin flu hit the human population and spread to several countries around the globe (MacKenzie, 2009). It was determined that the swine flu virus (H1N1) was a mixture of genes from four different viruses: North American swine influenza, North American avian influenza, human influenza, and Eurasian swine influenza viruses (Figure 3). Taken together, these pandemics have originated from close contact among humans, avian and swine. The magnitude and spread of these influenza pandemics are determined by environment, socio-economical behaviors and

the geographical location of the influenza hot spot (reviewed in Kuiken *et al.,* 2006). Therefore, it is crucial to understand the general principle of these factors, which trigger the avian influenza viruses to breach the species barriers.

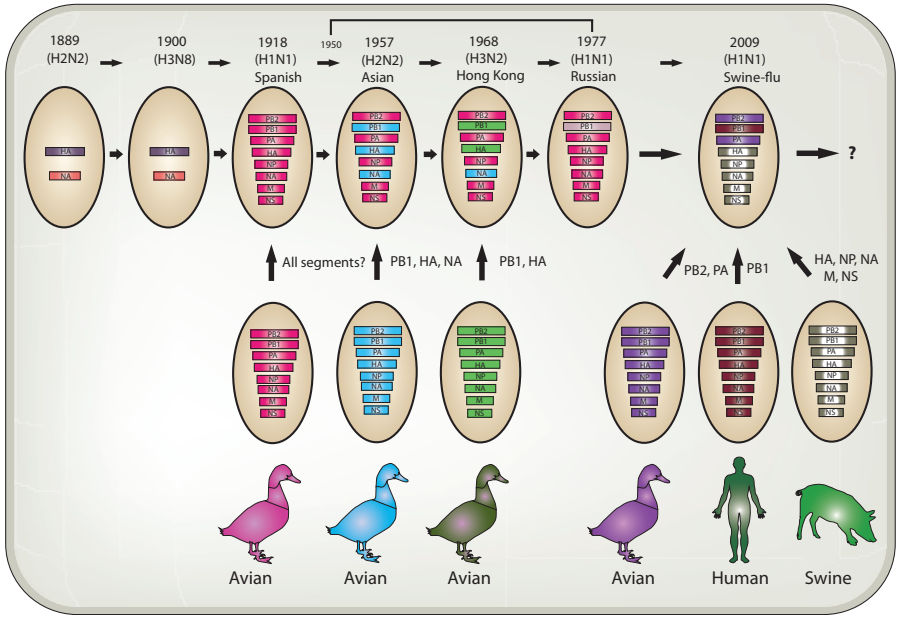

Figure 3. Evolution of influenza A viruses and their circulation in humans.

1.4 Molecular determinants of the host range restriction and pathogenicity

The majority of the influenza A viruses are host-specific, but owing to their capacity to infect a wide range of animals, this specificity is partial (Webster *et al.*, 1992). There is a great wealth of reports providing evidence for occurrence of interspecies virus transmission. Direct indications of the potential for adaptation are the transmission of avian H1N1 and human H3N2 to swine (Scholtissek *et al.*, 1983; Ottis *et al.*, 1982), swine H1N1 to humans (Rota *et al.*, 1989), an avian H3N8 to equines (Guo *et al.*, 1992), avian influenza viruses to seals (Webster *et al.*, 1981), to whales (Hinshaw *et al.*, 1986) and to minks (Klingeborn *et al.*, 1985). It has been experimentally proven that avian influenza viruses cannot replicate efficiently in humans nor do human influenza viruses have the ability to propagate in avian species, despite direct and close contact between the two species (reviewed in Ito & Kawaoka, 2000). In contrast, there are indications that avian influenza viruses can transmit and cause disease in humans in natural infections (Subbarao *et al.*, 1998; Claas *et*

al., 1994). In this complex scenario, and in the context of the recent swine influenza pandemic, it is clear that several viruses can readily evade host restriction barriers. Although, the complete list of factors that determine the host restriction is not fully illustrated, there have been significant advancements in this respect.

The hemagglutinin protein, due to its essential role in receptor recognition and attachment, is considered to be a primary determinant of host-range. The receptor specificity of influenza A viruses depends on its species of isolation (Webster *et al.*, 1992). Human seasonal viruses almost exclusively bind to N-acetylneuraminic (sialic) acid receptors **α**-2,6 linkage (NeuAc **α**-2,6), whereas avian influenza viruses predominantly bind to NeuAc **α**-2,3 linkage. Owing to having both NeuAc **α**-2,3 and NeuAc **α**-2,6 linkages, pigs and several avian species (pheasants, turkeys, quails) may act as mixing vessels and can generate reassortant viruses (reviewed in Medina & Garcia-Sastre, 2011) (Figure 4).

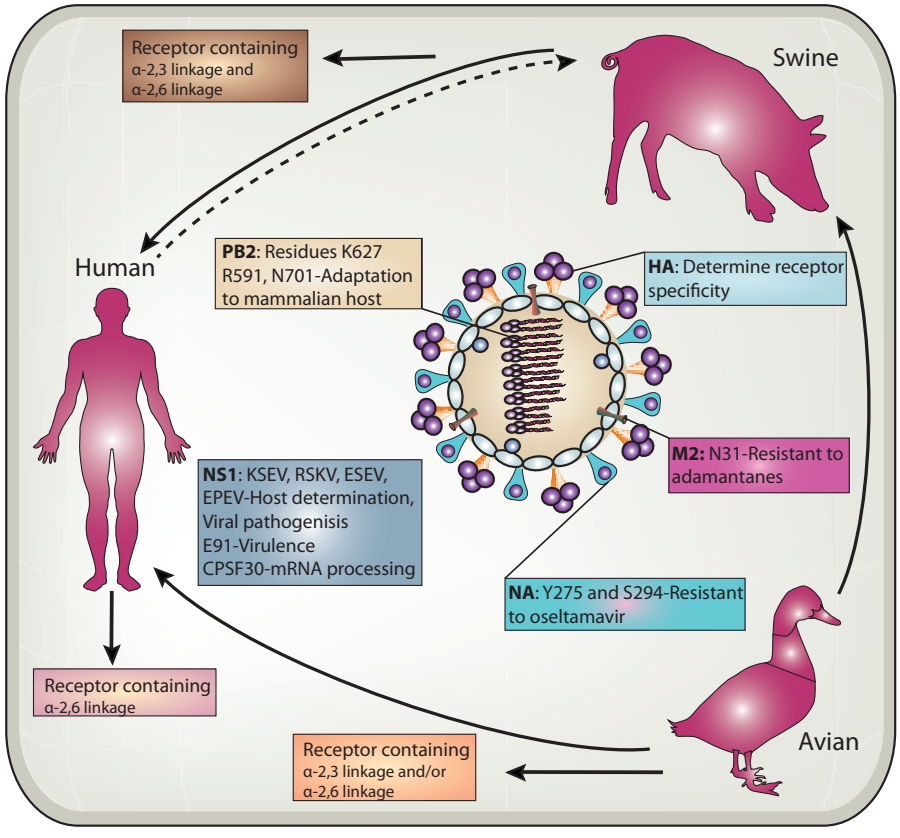

Figure 4. Factors responsible for influenza A virus host adaptation and pathogenesis.

For efficient infection and replication of influenza A viruses in the host, receptor binding is not enough. There is increasing evidence about the role of RNA-dependent RNA polymerase basic subunit 2 (PB2) in the pathogenicity of influenza viruses. Interestingly, it has been shown that all human influenza A viruses carrying K267 amino acid are highly pathogenic in mice, whereas K267G substitutions can determine the low pathogenicity of avian viruses in mammalian models (Hatta *et al.*, 2001; Subbarao *et al.*, 1993). Fatal infections of HPAI H7N7 and HPAI H5N1 in humans were also associated with K267 substitution within PB2 (de Wit *et al.*, 2010; Fouchier *et al.*, 2004). Further, it has been established that D701N substitution in PB2 is crucial for the adaptation of avian origin viruses to mammalian species (de Jong *et al.*, 2006; Gabriel *et al.*, 2005). Interestingly, PB2 protein of the 2009 pandemic H1N1 lacked both of these substitutions, but it still replicated in mammals. It was concluded that another residue at position 591 (R591) could compensate both of the previously characterized substitutions (Yamada *et al.*, 2010; Mehle & Doudna, 2009).

Apart from HA and PB2, other components of viral ribonucleoprotein (PA, PB1, NP), and internal genes (M2, NS1, NA and PB1-F2) can also play a role in host discrimination, and are markedly associated with the pathogenicity and virulence of influenza A viruses. The various roles of these proteins in virus tropism and pathogenicity are briefly summarized in Figure 4.

Recently, using genome-wide approaches it has been highlighted that not only viral elements but host specific ones also contribute to successful replication and virulence of influenza infection (reviewed in Watanabe *et al.*, 2010). These factors contribute to all of the steps in influenza viral replication. Therefore, these are discussed in the context of viral replication (see below). Briefly, it has been demonstrated that cellular V-type proton ATPase subunit D 1 (ATP6V0D1) leads to decreased viral replication if silenced with small interfering RNA (Hao *et al.*, 2008). ATPase ATP6V0D1 mediates the viral endocytosis process. It has further been shown that a cellular protein, CAMK2B, also facilitates the replication of influenza A viruses (Karlas *et al.*, 2010; Konig *et al.*, 2010). Recently, it has been revealed that human CLE/C14orf166 protein (hCLE) interacts with the influenza A virus polymerase complex. This interaction was found to be important for induced viral polymerase activity, viral RNA transcription and replication, virus titer, and viral particle production, which in turn results in increased viral replication (Rodriguez *et al.*, 2011). A molecular understanding of these cellular regulators of influenza infection may help to resolve many concerns of host-pathogen interactions, and may also help to develop novel therapeutic agents to combat this deadly disease (Medina & Garcia-Sastre, 2011).

1.5 Replication of influenza A viruses

Replication of influenza A viruses is based on the following fundamental steps, as illustrated in Figure 5.

1.5.1 Receptor binding

The viral epical glycoprotein HA binds to N-acetylneuraminic (sialic) acid receptors on the host cell surface (Skehel & Wiley, 2000) (Figure 5). Influenza viruses isolated from humans and swine show high affinity to one kind of sialic acid (**α**-2,6 linkage) receptors, whereas avian and equine viruses primarily recognize sialic acid of another kind (**α**-2,3 linkage) (Daly *et al.*, 2011). In humans, both kind of these receptors exist but **α**-2,6 linkage is predominant (Matrosovich *et al.*, 2004). On the other hand, **α**-2,3 linkage is more common in duck. Pigs are more susceptible to the disease due to having both forms of sialic acid receptors (Ito *et al.*, 1998).

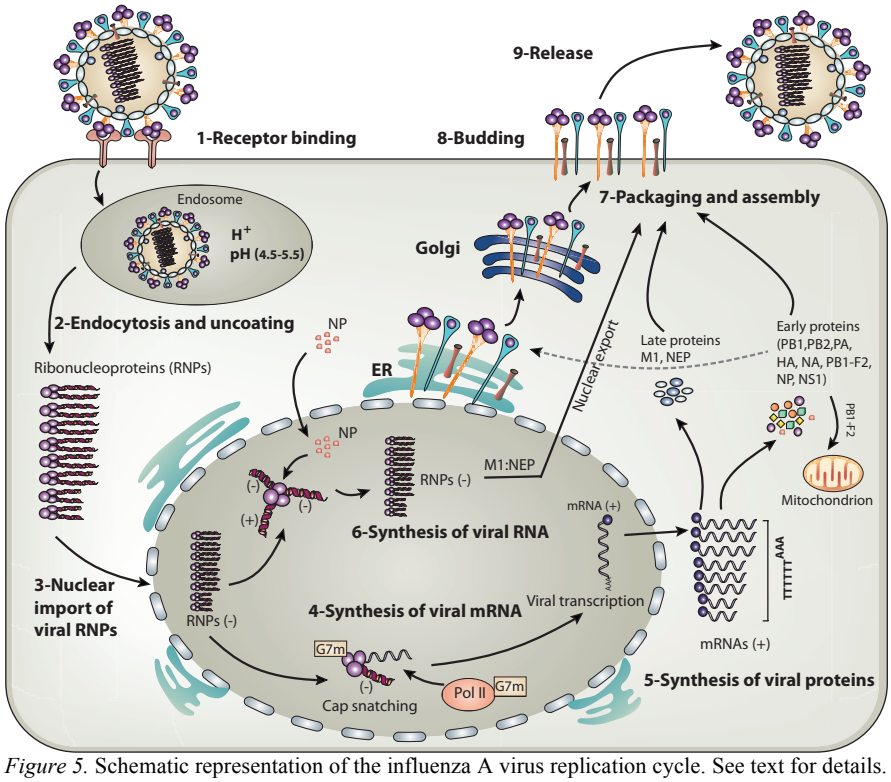

Figure 5. Schematic representation of the influenza A virus replication cycle. See text for details.

1.5.2 Endocytosis and uncoating

After binding to receptors, the whole virus particle is transported into the cell through receptor-mediated endocytosis in the form of pre-lysosomal, low pH vesicles (Whittaker & Digard, 2006). The involvement of host GTPase (Roy *et al.*, 2000) and rearrangements of cellular actin (Lakadamyali *et al.*, 2003) are essential to facilitate such internalization. Two cellular kinases further accompany this process: protein kinase C (Sieczkarski *et al.*, 2003) and phosphoinositide 3-kinase (Ehrhardt *et al.*, 2006).

The acidity (pH 4.5-5.5) of the endosomal compartment is crucial for the uncoating and subsequent release of viral RNPs, which is completed in two steps (Whittaker & Digard, 2006; Lamb & Krug, 2001). The low pH triggers a conformational change in the HA protein, and cellular proteases cleave it into two subunits: HA1 and HA2. The HA2 mediates the fusion of viral and endosomal membranes. Although essential for viral infectivity, this event alone is insufficient to deliver the individual viral genomic segments into the cytoplasm. Therefore, the low pH also triggers the flow of protons into the virus via M2 ion channels, causing dis-association of vRNPs, which are otherwise attached to M1 (Lamb & Pinto, 2006; Bui *et al.*, 1996).

1.5.3 Nuclear import of viral RNPs

Following release, the vRNPs are transported into the nucleus via interaction with the cellular importin-α/β (Whittaker & Digard, 2006; O'Neill *et al.*, 1995; O'Neill & Palese, 1995), which is mediated by multiple nuclear localization signals (NLSs) of the nucleoprotein. Apart from nucleoprotein, all of the other components of RNPs also carry the NLSs (Nieto *et al.*, 1992; Mukaigawa & Nayak, 1991; Nath & Nayak, 1990). Upon nuclear translocation, the vRNP strongly interacts with insoluble “nuclear matrix” or “chromatin components” (Whittaker & Digard, 2006; Bui *et al.*, 2000).

Unlike most other negative strand RNA viruses, replication and transcription of influenza A viruses occur in the nuclei of the infected cells (Jackson *et al.*, 1982; Herz *et al.*, 1981). This is possibly due to two transcriptional requirements: initiation of viral mRNA synthesis requiring the capped 5´-ends of host messenger RNAs (mRNAs) to act as primers (Plotch *et al.*, 1981), and two viral mRNAs requiring splicing (reviewed in Lamb & Krug, 2001). However, two other families (*Bunyaviridae* and *Arenaviridae*) require 5´cap structures for mRNA transcription, yet these replicate in the cytoplasm (reviewed in Buchmeier *et al.*, 2001). On the other hand, a member of *Bornaviridae* family (Borna disease virus) requires splicing and therefore replicates in the cell nucleus (de la Torre, 2001). This indicates that the

replication and transcription of influenza A viruses in the nucleus is most likely due to the requirement of splicing.

1.5.4 Synthesis of viral mRNA

The viral mRNA synthesis has to occur in the nuclei of the infected cells, and requires a full RNP complex: NP, PA, PB1 and PB2 (see Figure 1 C) (Portela & Digard, 2002; Huang *et al.*, 1990). Since influenza viruses use the translation machinery of host cells, the mRNA need to be capped [addition of 7-methylguanylate (m^7G) at the 5′-end] and polyadenylated [addition of poly(A) tail at the 3′-end] for efficient translation into protein(s). For polyadenylation, influenza A viruses encode a stretch of 5 to 7 uracils (U) in the negative sense vRNA. The viral polymerase transcribes this stretch into positive sense as a string of adenosines (A), which make the poly(A) tail of mRNA (Robertson, 1979). For capping, the PB2 subunit binds to the 5′cap of the host pre-mRNAs and the PB1 subunits of the viral polymerase cleave the 5′capped primer (m^7GpppXm cap structure of 10-13 nucleotides) from host pol II-mRNA transcript (cap snatching) owing to an endonuclease activity (Plotch *et al.*, 1981), which acts as a primer to initiate viral mRNA synthesis (reviewed in Krug, 1981).

As a consequence of post-transcriptional modifications, three types of viral mRNA translocate to the cytoplasm: (i) six viral mRNAs that lack introns (PA, PB1, PB2, NP, HA, NA), (ii) two viral mRNAs that contain introns but non-spliceable (M1, NS1), and (iii) two mRNAs (M2, NEP) which are generated by the splicing of M1 and NS1 mRNAs, respectively (Krug *et al.*, 1989). The viral polymerase and cellular splicing factors (serine/arginine-rich splicing factor SF2/ASF) regulate the splicing of M1 mRNA (Shih & Krug, 1996; Shih *et al.*, 1995), whereas the splicing of NS1 mRNA was previously speculated to be controlled by the NS1 protein itself (Garaigorta & Ortin, 2007). However, recently it was revealed that the NS1 protein neither auto-regulates the splicing of its own viral NS1 mRNA nor affects the accumulation of NS2 mRNA (Robb *et al.*, 2010).

1.5.5 Cytoplasmic export and synthesis of viral proteins

Once the viral mRNAs are polyadenylated and capped, these are exported into the cytoplasm to be translated. The viral proteins are translated into two phases: early and late. The viral surface glycoproteins such as HA, NA and M2 are processed in the endoplasmic reticulum (ER) and glycosylated in the Golgi apparatus (Golgi). All these proteins carry apical sorting signals that direct them to the plasma membrane for virus assembly (Lamb & Krug, 2001). The other viral proteins, such as NP and M1, are transported to the nucleus to bind

with vRNA for the composition of vRNPs, while NS1 are partially transported to the nucleus to regulate antiviral mRNA maturation (discussed below in detail) (Hale *et al.*, 2008c). PB1-F2 localizes on mitochondrial antiviral signaling (MAVS) protein to block downstream IFN signaling (Varga *et al.*, 2011).

1.5.6 Synthesis of viral RNA

The mRNA transcription and subsequent synthesis of viral proteins occur earlier than the replication of vRNA, due to the need of these proteins in the upcoming processes (Nagata *et al.*, 1989; Hay *et al.*, 1977). Later during the infection, the switch from mRNA transcription to replication of complementary RNA (cRNA) and vRNAs occurs in two stages: the initial synthesis of cRNA (+) and copying of cRNA into new vRNA (-) (reviewed in Elton *et al.*, 2006; Lamb & Krug, 2001). In spite of the fact that both mRNAs and cRNAs are positive sense, mRNAs cannot be used to form new vRNAs due to having a 5´host-derived cap, whereas vRNAs cannot be used for protein translation due to not having a cap and being encapsidated by the NP protein (Elton *et al.*, 2006). The three polymerase subunits (PA, PB1, PB2) are essential for efficient cRNA synthesis (Nakagawa *et al.*, 1996). The polymerase is stabilized by the nuclear importation of newly synthesized NP, either by modifying the transcription template (Fodor *et al.*, 1994; Hsu *et al.*, 1987), or by directly modifying polymerase function (Elton *et al.*, 2006). On the other hand, only PA and NP are required for the synthesis of new vRNA from cRNA (Elton *et al.*, 2006; Shapiro *et al.*, 1987).

1.5.7 Packaging of RNA and assembly of virus particles

The newly synthesized vRNA is transported to the cytoplasm, mediated by the M1-NEP complex that remains attached to the vRNPs and this is a tightly regulated process (reviewed in Whittaker & Digard, 2006). Additionally, NEP interacts with the human CRM1 protein that is responsible for nuclear export of vRNPs (Neumann *et al.*, 2000). Once exported to the cytoplasm, all of the vRNA segments that carry discrete packaging signals are incorporated into a virus particle (reviewed in Bouvier & Palese, 2008). The assembly of viral RNAs and viral RNPs occurs only at the apical side of the cell (Boulan & Sabatini, 1978). This assembly is polarized, which is directed by the independent targeting of viral HA, NA, and M2 proteins to the apical cell membrane (Whittaker & Digard, 2006; Kundu *et al.*, 1996; Hughey *et al.*, 1992).

1.5.8 Budding and release

The budding is a complex phenomenon and may involve multiple steps to be completed. The initiation of the budding process starts with the deformation of the plasma membrane due to clustering of HA and NA (reviewed in Rossman & Lamb, 2011). The M1 protein then not only binds to the cytoplasmic tail of HA and NA to cause docking, but also recruits the M2 protein at the site of budding. It has recently been shown that M2 localizes to the neck of newly budding virions, which results in membrane scission and virion release (Rossman *et al.*, 2010). This process was found to be independent of the endosomal sorting complex transport (ESCRT) machinery, which is a well-characterized mechanism for budding of other viruses. The NA further catalyzes the removal of sialic acid from the infected cells as well as from HA and NA molecules. This subsequently results in virus release and prevents self-aggregation of progeny virions (Lamb & Krug, 2001; Palese *et al.*, 1974). Moreover, it is believed that NA also facilitates the transportation of virions through the layers of the respiratory tract (Lamb & Krug, 2001).

1.6 Reassortment of the influenza A virus gene segments

Genetic variations within influenza A viruses lead to possible changes in future epidemiological behavior and may result in human pandemics. There are at least two kind of genetic variations identified: antigenic drift and antigenic shift. Antigenic drift refers to minor changes in the antigenic determinant of surface glycoproteins (HA and NA) over the years. These changes are minor (0.8% substitutions per year) and have additive effect on the pathogenicity of influenza viruses (Robertson *et al.*, 1987). Mechanistically, these modifications occur due to the error prone replication of viral RNA polymerase, evolutionary pressure, the novel environment of the host, immune pressure, or antiviral drug pressure (Landolt & Olsen, 2007; Smith *et al.*, 2004; Domingo *et al.*, 1998). Although these changes may not lead to pandemics, antigenic drift over a period of time can make a strain considerably different from the original pandemic virus. Such a scenario has been observed in viruses causing pandemics in human history: 1918 to 1956, 1957 to 1967 and 1968 to 1990.

On the other hand, it is possible that two different subtypes of influenza virus, when replicating in the same cell, may lead to generation of novel viruses, termed antigenic shift or reassortment. These major changes could lead to emergence of new pandemics, as has been recently reported (swine flu) (Figure 6). Due to having both kinds of receptors (α-2,3 and α-2,6 linkage), pigs can act as a "mixing vessel", and this situation is further aggravated due to their susceptibility to avian influenza viruses. The emergence of the Asian

(H2N2) 1957 and Hong Kong (H3N2) 1968 pandemics was due to reassortment of three and two viral segments, respectively, whereas the rest of the segments were from the preceding strains (reviewed in Kuiken *et al.*, 2006). To conclude, it is obvious that reassortment is the fundamental requirement for influenza A viruses to cause pandemics.

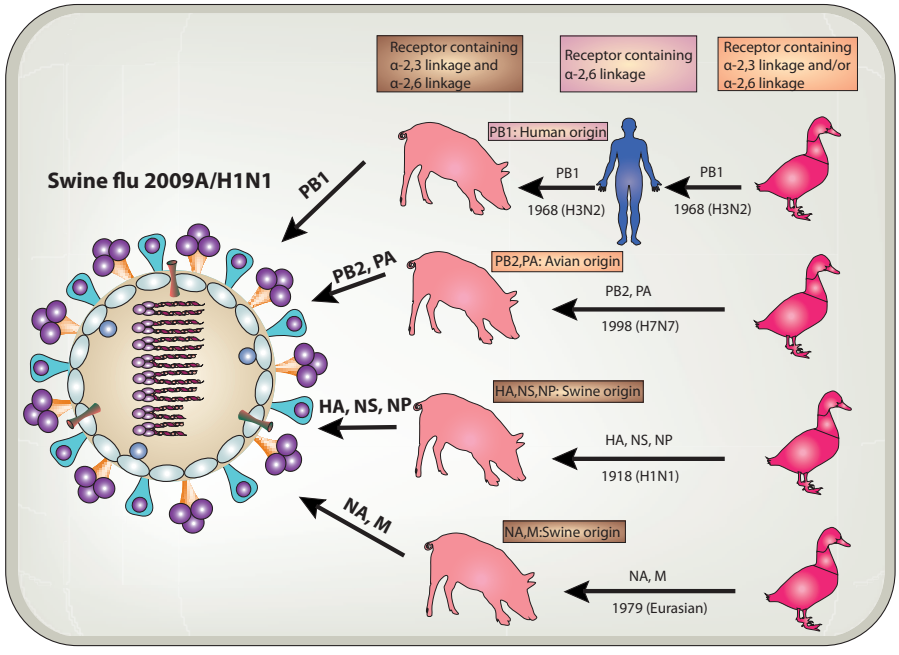

Figure 6. Reassortment of gene segments of influenza A viruses. The genetic characterization of recent swine flu virus (2009A/H1N1) indicated that it is a quadruple re-assortment of four different viruses: North American swine influenza (HA, NS, NP), North American avian influenza (PB2, PA), human influenza (PB1), and Eurasian swine influenza viruses (NA, M).

1.7 Immune response against influenza A viruses, with special reference to IFN induction

One role of eukaryotic cells is to limit viral replication and spread. For this reason, viruses have to cross different layers of the immune system to be able to replicate (reviewed in Randall & Goodbourn, 2008). As a part of the humoral immunity, neutralizing antibodies can reduce the viral titer by a significant level by binding to viral capsid proteins. Additionally, the complement system can cause lysis of the virus or promote phagocytosis by coating the virions (Lachmann & Davies, 1997). Upon failure of these barriers, the virus gets the chance to enter into the cells, and this event leads to activation of different signaling pathways. The one prominent part of this

cascade is the stimulation of type I interferons (IFN-α/β). The induced IFNs then restrict the virus propagation at various levels.

Different immune cells (such as plasmacytoid dendritic cells) are capable of producing type I IFNs to respond quickly to invading pathogens and to prepare the neighboring cells (Figure 7). The way that IFN-β expression is induced depends upon the mode of entry and the replication of the viruses. The viral activation of cellular helicases, retinoic-acid-inducible gene I (RIG-I) and melanoma differentiation-associated gene 5 (MDA5) and/or toll like receptors (TLRs), mediate recruitment of downstream signaling proteins, which terminate at activated IFN regulatory factor 3 (IRF3) and nuclear factor kappa B (NF-κB) transcription factors. These are minimal events to initiate the IFN-β mRNA transcription.

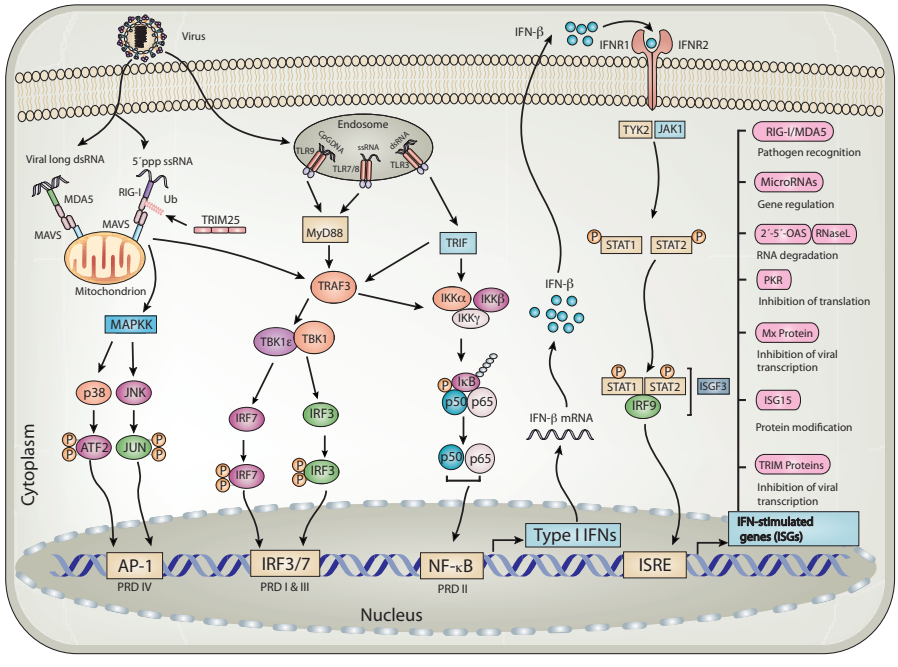

Figure 7. Induction of IFNs and the antiviral state in eukaryotic cells. The dsRNA, detected by either RIG-I/MDA5 or TLRs, initiates downstream signaling from MAVS, TRIF and TRAF3. These adaptors then activate the IRF3/IRF7, NF-κB and AP-1 transcription factors by orchestrating the assembly of multi-protein complexes. Once activated, IRF3/7, NF-κB and AP-1 translocate to the nucleus where they stimulate transcription of IFN-β, among others. Binding of IFN-β to IFNAR through auto- and para-crine manners activates the JAK/STAT pathway, and terminates at the activated ISGF3 transcription factor. The ISGF3 then translocate to the nucleus and initiate transcription of more than 300 genes characterized in ISRE promoters, which subsequently establish the antiviral state against the invading pathogens. See text for abbreviations.

In case of activation of the helicases, double-stranded RNA (dsRNA) of more than 30 bp length, produced during viral infection, interacts with RIG-I and MDA5 through their N-terminal caspase recruitment domain (CARD) with the mitochondrial antiviral signalling protein (MAVS, also known as IPS1, VISA, or CARDIF), thereby leading to the activation of two kinase complexes: TANK binding kinase 1-inhibitor of NF-κ kinase ε (TBK1-IKKε) and IKK-α/β /γ. TBK1 or IKKε, in turn, phosphorylates IRF3 and IRF7, while the IKK-α/β/γ complex phosphorylates the IκB protein, which leads to its ubiquitination and proteasomal degradation, resulting in NF-κB activation. The tripartite motif containing protein 25 (TRIM25) has been shown to ubiquitinate and activate RIG-I (reviewed in Munir, 2010). The optimal induction of IFNs also requires activation of c-jun/ATF-2 heterodimer (reviewed in Randall & Goodbourn, 2008).

In the case of TLRs signaling, dsRNA also interacts with TLR3 (Akira & Takeda, 2004), which activates NF-κB and IRF3 transcription factors via the TRIF (TIR domain-containing adaptor inducing IFN-β) adaptor protein and members of the TNF receptor-associated factor (TRAF) family (Yamamoto *et al.*, 2002).

While being inactive, NF-κB, IRF3/IRF7 and c-jun/ATF-2 (AP-1) remain in the cytoplasm. The activation signals lead to phosphorylation of the C-terminus of IRF3/7. The changes caused by this modification lead to dimerization and exposure of the nuclear localization signal (NLS) in IRF3/7. This NLS mediates the nuclear translocation of IRF3/7. The inhibitor of NF-κB (IκB) keeps the NF-κB in the cytoplasm, which upon receipt of appropriate signal undergoes ubiquitination and proteasomal degradation. Removal of IκB makes the NLS accessible, which leads to nuclear translocation of NF-κB (reviewed in Hayden & Ghosh, 2004). Phosphorylation of c-jun and ATF-2, two heterodimeric components of AP-1, also causes nuclear translocation.

In the nucleus, these three transcription factors assemble in a cooperative manner to make an enhansosome, which binds to its respective positive regulatory domains (PRDs). The IRF3/IRF7, NF-κB and AP-1 bind to PRD I/III, PRD II and PRD IV, respectively, where they induce the transcription of IFN-α, IFN-β and pro-inflammatory cytokines (TNF, IL6, IL1β, etc). Following production, IFN-α and IFN-β initiate a positive feedback-production loop by binding to the IFN-α/β receptor (IFNAR) in an auto- and para-crine manner. Receptor-mediated activation of Janus kinase-signal transducer and activator of transcription (JAK-STAT) signaling molecules results in the recruitment and phosphorylation of IRF9 into the STAT1/STAT2 heterodimer, to make interferon-stimulated gene factor 3 (ISGF3) (Mukaigawa & Nayak,

1991). Several protein phosphatases and the suppressors of cytokine signaling (SOCS), such as SOCS1 and SOCS3, are found to be involved in negative regulation of STATs (Yasukawa *et al.*, 1999). Upon nuclear localization of ISGF3, it binds to interferon-stimulated response elements (ISREs) (Kessler *et al.*, 1990), which consequently leads to the transcription of hundreds of IFN-stimulated genes (ISGs). These ISGs play fundamental roles in a wide range of cellular activities, including transcriptional and translational regulation of immune responses (de Veer *et al.*, 2001). The collective actions of these ISGs make the cell immune to invading pathogens, and provide an antagonistic environment to limit virus propagation and spread.

1.8 Non-structural protein 1 (NS1)

In spite of having multiple layers of eukaryotic strong immune responses against influenza A virus, the virus still gets chance to replicate in the host cell. In this acquisition, NS1 protein plays an exceptional role. The NS1 protein will be discussed comprehensively in the following section.

1.8.1 Synthesis of NS1 protein

The eighth and shortest segment (890 nucleotides) of the influenza A virus encodes for two mRNAs in infected cells (Figure 8). The translation of collinear primary mRNA results in synthesis of the 26 kDa NS1 protein, whereas translation of a spliced mRNA give rise to 14 kDa nuclear export protein (NEP) (Inglis *et al.*, 1979; Lamb & Choppin, 1979). Both of these proteins share 10 amino acids at the N-terminus, which are translated from a common 56 nucleotides at 5´ end. After sharing 10 initial residues, a deletion of 473 nucleotides occurs by splicing, and translation continues in the +1 ORF which again results in sharing approximately ~70 residues (Lamb & Lai, 1980). Since influenza A viruses encode proteins in both spliced and unspliced mRNAs, only 10% of the NS gene segment is spliced (Lamb *et al.*, 1981; Lamb *et al.*, 1980). Until recently, it was believed that the NS1 protein regulates the production of its own viral NS1 mRNA (Smith & Inglis, 1985; Lamb *et al.*, 1978; Skehel, 1972). In contrast, work by Robb *et al.* (2010) pointed out that the NS1 protein neither auto-regulates the splicing of its own viral NS1 mRNA nor affects the accumulation of NEP mRNA (Robb *et al.*, 2010).

1.8.2 Structure of the NS1 protein

The length of NS1 proteins is strain-specific and varies from 202 aa to 237 aa (reviewed in Dundon & Capua, 2009). The truncation of ~15 to 30 residues at

the C-terminus has not biologically been shown to be associated with altered viral pathogenicity or transmission. However, removal of interacting sites in the C-terminus (PDZ ligand, NLS and PABII) will confer an advantage or disadvantage on the virus. Recently, it has been shown that an 11 aa truncation in the C-terminus of the NS1 protein did not alter the intracellular localization in both transfected and infected cells, indicating the nucleolar localization signals (NoLS) are probably not crucial for nucleolus localization of NS1 protein. Although the 2009 pandemic virus with an 11 aa truncation in its NS1 protein was unable to block host gene expression, due to its lack of ability to bind with PABII (mapped in these 11 aa truncations), it increased the viral pathogenicity in mice (Tu *et al.*, 2011).

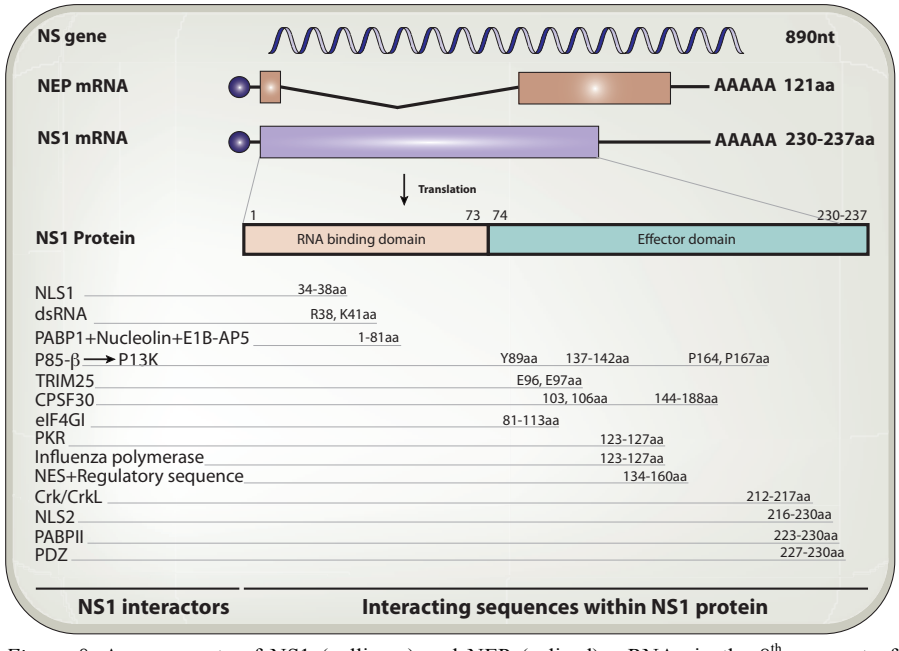

Figure 8. Arrangements of NS1 (collinear) and NEP (spliced) mRNAs in the 8th segment of influenza A viruses, and a schematic structural diagram of the NS1 protein. The previously characterized residues identified as responsible for particular functions are denoted.

Structurally, the NS1 protein of influenza A viruses has been divided into two distinct functional domains. The dsRNA-binding domain (RBD) comprises of the first 73 residues at the N-terminus, and the effector domain (ED) is located at the C-terminus (74-230/237). Both domains are characterized as being essential for the multi-functionality of the NS1 protein (reviewed in Hale *et al.*, 2008c; Lin *et al.*, 2007).

The RBD exists in a unique symmetric head-to-tail homodimer structure with each monomer consisting of three **α**-helices: residues Asn4-Asp24 (**α**1), Pro31-Leu50 (**α**2) and Ille54-Lys70 (**α**3) (**α**1´, **α**2´, **α**3´ are the corresponding residues in the antiparallel monomer) (Figure 9 A) (Chien *et al.*, 2004; Chien *et al.*, 1997; Liu *et al.*, 1997). The primary role of RBD is to bind to several species of RNAs, including dsRNA (Figure 9 A), mRNAs containing poly(A) (Qiu & Krug, 1994) and U6 snRNA (Qiu *et al.*, 1995), but it is unable to bind with dsDNA or dsRNA-DNA hybrid (Lin *et al.*, 2007), in a sequence independent manner (Chien *et al.*, 2004; Qian *et al.*, 1995; Hatada & Fukuda, 1992). It has been proposed that dimerization is essential for the functionality of RBD (Yin *et al.*, 2007; Wang *et al.*, 1999).

Biophysical studies have proposed that the ED of the NS1 protein also form a homodimer, and these dimers either exist in strand-strand (Bornholdt & Prasad, 2006) or helix-helix conformation (Xia *et al.*, 2009; Hale *et al.*, 2008a). In any case, each monomer consists of three **α**-helices and seven **β**-strands. Strand-strand conformation proposes that the dimer interface forms by the interaction between the first N-terminal **β**-strand of each monomer, whereas helix-helix conformation suggests that this interface is formed by the interaction between long **α**-helices, where Try-187 plays a crucial role in the dimerization of EDs (Figure 9 B). The primary function of ED is to mediate host-cell protein interactions, and to stabilize the RBD (Hale *et al.*, 2008c).

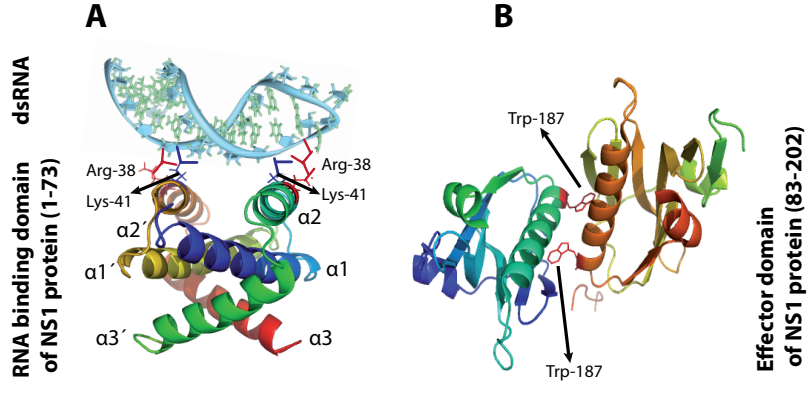

Figure 9. Structure of the influenza A virus NS1 protein (RBD and ED). **(A)** Model representation of a homodimer of the RBD (PDB file: 1NS1). A hypothetical model is shown where dsRNA interacts with RBD, primarily mediated by Arg-38 (red) and Lys-41 (blue). **(B)** Model representation of a homodimer of the ED (PDB file: 3D6R), where a residue (Try-187) crucial for ED dimerization is labeled. These images were prepared using MacPyMol.

The crystal structure of the full-length NS1 protein (A/Vietnam/1203/2004) recently revealed considerable discrepancies when compared to the individual RBD and ED structures (Bornholdt & Prasad, 2008). The authors noted that the individual domains (RBD and ED) interact with their respective domains in the neighboring NS1 protein, instead of dimerization and formation of a single NS1 protein. This leads to the formation of a chain of NS1 molecules with altered RBD and ED dimers. It was further proposed that the NS1 chain wraps around long dsRNA to form a tubular structure. The dimerization interface for ED was different from both models proposed before (strand-strand and helix-helix). However, it is noteworthy that the structure of this full length NS1 of A/Vietnam/1203/2004 exhibits substitutions in nine amino acids, including mutations at R38A and K41A, to eliminate its dsRNA binding activity.

1.8.3 Structural insights into expression pattern of NS1 protein

The NS1 protein is an essential virulent factor for influenza A viruses, and it targets some of its action in the nucleus, including inhibition of the processing of host cell pre-mRNA. For efficient action, each domain of the NS1 protein carries a specific sequence element that mediates active nuclear import known as NLS (Greenspan *et al.*, 1988). The binding of importin-α/β to NLS containing proteins leads to nuclear translocation via a nuclear pore complex. The NLS in RBD (NLS1) is a monopartite (a stretch of four to six arginines or lysines) and is mapped in highly conserved residues from 35-41, where residues at 35, 38 and 41 are essential for importin-α binding and subsequent nuclear translocation; the same residues are also important for interacting with dsRNA (Melen *et al.*, 2007; Yin *et al.*, 2007) (Figure 10 A). Due to this identical sequence motif in the dsRNA binding and NLS, it was suggested that RBD is dynamic in function and may import dsRNA to the nucleus (Melen *et al.*, 2007). On the other hand, NLS2 is a bipartite (two stretches of basic amino acids are separated by a spacer 10-12 amino acids long) and is located at the C-terminus of some NS1 proteins. Specifically, Lys-219, Arg-220, Arg-231 and Arg-232 constitute NLS2, and with the involvement of Arg-224 and Arg-229 can also act as NoLS (Melen *et al.*, 2007). The nucleo-cytoplasmic transport is mediated by a nuclear export signal (NES) located within the 138-147 residues (Li *et al.*, 1998) (Figure 10 B). It has recently been shown that NS1 interact with nucleolin (Murayama *et al.*, 2007) and nuclear dot 10 (ND10) structures in the nucleus (Sato *et al.*, 2003). The amino acid at position 221 is found to be crucial for NLS and NoLS (Han *et al.*, 2010). At this position, allele A of both H6N8 and H4N6 strains has lysine (K), while allele B has tyrosine (Y), apart from other differences in both NES and NLS (see Figure 15, in results and discussion section). Moreover, it has also been suggested that the function of

the NS1 protein is likely to be influenced by the nuclear localization pattern (Volmer *et al.*, 2010).

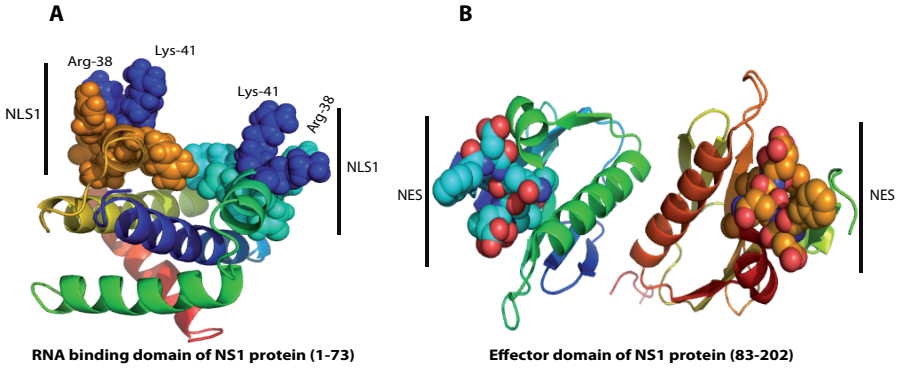

Figure 10. The nuclear localization signal 1 (NLS1) in RBD and nuclear export signal (NES) in the ED of the NS1 protein. **(A)** Model representation of the RBD homodimer (PDB file: 1NS1). Residues comprising the NLS1 are highlighted in spheres. **(B)** Model representations of the ED homodimer (PDB file: 3D6R). Residues comprising the NES are highlighted in spheres. Images were prepared using MacPyMol.

During infection, the NS1 protein primarily localizes in the nucleus, especially at late time points of infection (Newby *et al.*, 2007; Greenspan *et al.*, 1988), despite the fact it is considered to be an early protein, as it is expressed at high levels in the early course of the infection (Krug & Etkind, 1973). Since the NS1 protein contains both NLS and NES, a recent study showed that NS1 protein was predominantly localized in the cytoplasm during viral infection, whereas it was localized in the nucleus under transfection of NS1 constructs (Wang *et al.*, 2010). However, the nuclear localization pattern strikingly varies between different influenza strains, and may depend upon different factors independent of NLSs and NES (reviewed in Hale *et al.*, 2008c).

1.8.4 Characterization of the NS1 protein: allele A and allele B

Based on phylogenetic analysis of the nucleotide sequences, the NS gene of influenza A viruses can be divided into two gene pools, initially named allele A and allele B (Treanor *et al.*, 1989) (Figure 11). Historically, it was established that the NS segment of human, most swine and equine, and many avian influenza viruses belongs to allele A, whereas allele B exclusively contains the strains from avian influenza viruses, with two exceptions (Ludwig et al., 1991). Despite being a mammalian virus, the NS gene of A/equine/Jilin/1/1989/H3N8 was found to belong to allele B (Guo *et al.*, 1992). Recently, another virus, originating from swine

(A/Swine/Saskatchewan/18789/2002/H1N1), was also placed in the allele B category of influenza A viruses (Zohari *et al.*, 2008a). The former isolate caused severe disease and was highly transmissible in equines, whereas the later isolate did not appear to be transmitted to other farms. Due to its high identity to avian influenza A viruses, it was speculated that isolates in these outbreaks originated from an avian source. Nearly all subtypes of avian influenza A viruses with a combination of any HA and NA carry both the A and B NS alleles. Furthermore, there has been no evidence of intermediate divergence of lineages. This suggests that selection of either of the alleles may be a result of a balancing act between influenza A viruses and their hosts (Dugan *et al.*, 2008).

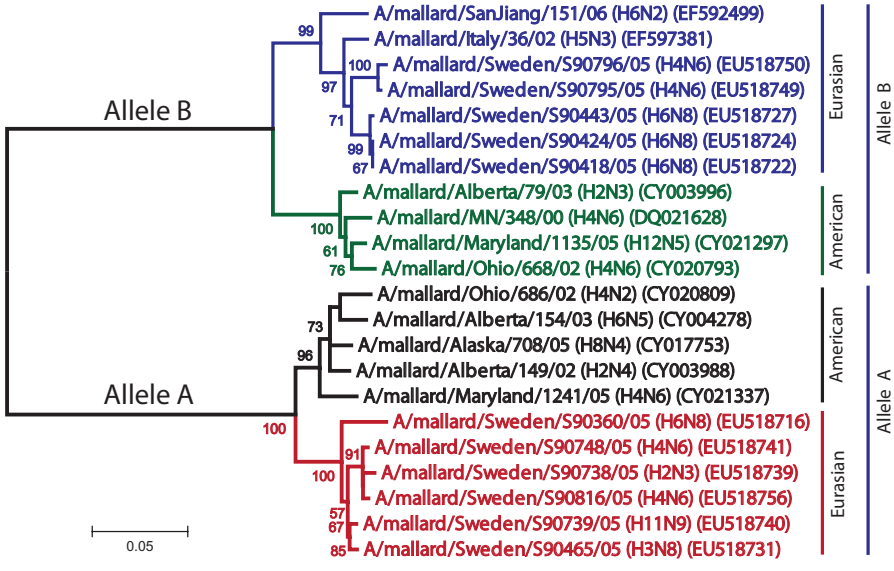

Figure 11. Phylogentic relationship of allele A and B NS1 genes. The tree was constructed using the complete open reading frame for NS1 gene, in MEGA 5.0 with the neighbor-joining method. The numbers on key nodes indicate the percentage of bootstrap values of 2000 replicates (only values more than 50% are shown).

The contribution of allele A and B NS1 protein in virulence and pathogenicity is not entirely clear. However, it was speculated that the high rate of nonsynonymous to synonymous substitution per site (ratio dN/dS) in NS genes is a direct indication of NS1 protein adaptive nature in both avian and human (Chen & Holmes, 2006). In this regard, it has been shown that a reassortant highly pathogenic avian influenza virus subtype H7N1 (A/FPV/Rostock/34), when carrying allele B NS gene segment, undergoes attenuation in squirrel

monkeys (Treanor *et al.*, 1989). In contrast, recently it was demonstrated that if the NS gene segment of A/FPV/Rostock/34 is replaced with the NS segment of allele B of A/Goose/Guangdong/1/96 (H5N1) it becomes more virulent and can produce infection in mice (Ma *et al.*, 2010). It was further concluded that the NS gene segment of H5N1 is crucial to change cell-tropism, host range and virus replication. Later, a report from the same group further explained the role of allele B in the virus replication (Wang *et al.*, 2010). They demonstrated that changing the NS gene segment under the same background alters the antiviral response, apoptosis, production of viral RNA and viral transcription. However, these alterations were found to be independent of *allele-type* since the presence of allele B NS segment increased the replication in one recombinant isolate whereas it did not affect the replication of others, while keeping the rest of the seven segments constant (Wang *et al.*, 2010). This discrepancy may likely be due to multiple roles of the NS1 protein, at both the viral and host ends.

In short, it was demonstrated that the presence of the allele B NS gene segment facilitates the replication of influenza viruses in accordance with an earlier report (Treanor *et al.,* 1989), and to some extent with later reports (Ma *et al.,* 2010; Wang *et al.,* 2010). Currently, van Wielink *et al.* (2011) have shown that Madin Darby Canine Kidney (MDCK) cells expressing allele B NS1 protein increase the delNS1 influenza virus (influenza viruses without an NS1 protein) replication to a significantly higher titer and thus established the basis for commercial vaccine production, with the added advantage of differentiation of infected and vaccinated animals (DIVA) strategies (van Wielink *et al.,* 2011).

It has been observed that the genetic variability between allele A and B can be as high as 38%. However, both alleles are highly conserved among themselves, because homology within alleles is found to be 93-100%. Since the allele B NS1 protein is under continuous selection pressure, it has been proposed that upon entry of allele B influenza viruses into human they might undergo high selection pressure and give rise to allele A NS1s (Hale *et al.*, 2008c). If this is the case, it can be used as an evolutionary marker, and it would require *in silico* analysis to resolve this concern. In accordance to this, it has recently been shown that human influenza A viruses are closer to allele A than allele B (Chen *et al.*, 2006), and the majority of highly pathogenic avian influenza A viruses with the potential to infect humans contain an allele A NS1 protein (Zohari *et al.*, 2008b).

An X-ray crystallographic structure of the ED of allele B NS1 protein (A/Duck/Albany/76) has recently been presented (Hale *et al.*, 2008a). Structural comparison of allele A and allele B (A/Puerto Rico/8/34) (Bornholdt & Prasad, 2006) EDs indicated several structural differences. The orientation

of the β-hairpin was found to be different between the two alleles. Although the biological function of this region is still to be identified, it is considered that the residues in this loop are associated with the cytoplasmic export of the NS1 protein. Although the biological function remained unaffected, the orientation of Tyr-89, that is essential for the interaction with phosphoinositide 3-kinase (P13K) (Hale *et al.*, 2006), was different between allele A and B. The orientation of Trp-187, a residue identified as important for interaction with the cleavage and polyadenylation specificity factor 30kDa subunit (CPSF30), was also not identical. Previously, it had been shown that this site is biologically functional in the NS1 protein of A/Duck/Albany/76. On the other hand, the NS1 protein from A/Puerto Rico/8/34 did not show any interaction with CPSF30 (Kochs *et al.*, 2007; Hayman *et al.*, 2006).

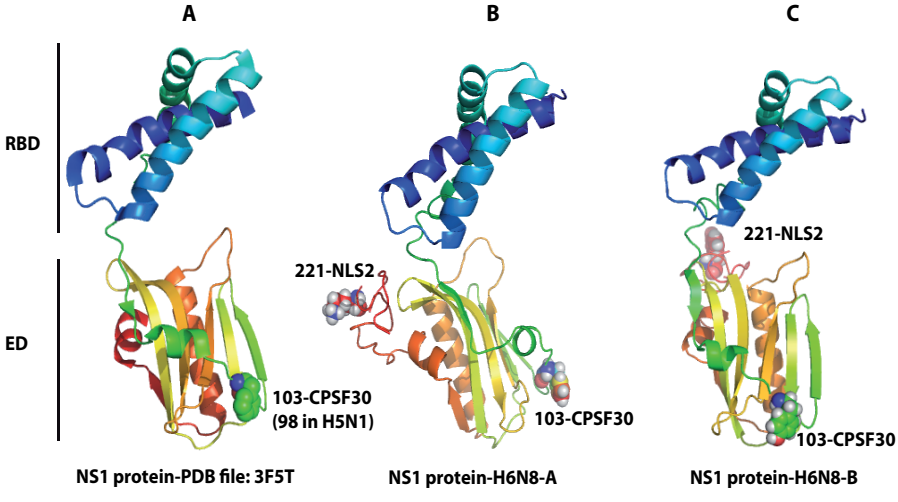

Figure 12. A model presentation of the amino acids responsible for interaction with CPSF30 and NLS2 in the effector domain. **(A)** A model structure of the NS1 protein (PDB file: 3F5T). **(B)** The structure of allele A NS1 protein of H6N8 subtype predicted using multiple threading alignments on the I-TASSER server (Roy *et al.*, 2010). **(C)** The model structure of the allele B NS1 protein of H6N8 subtype, using the I-TASSER server. The residues involved in mediating interaction between NS1 protein and CPSF30 are shown as spheres and labeled in all the structures. The residue at position 221 identified as essential for nuclear localization of the NS1 protein is also shown in spheres and labeled in both allele A and B NS1 proteins.

Recent resolution of the full-length protein structure of the NS1 protein leads to the conclusion that the structure of ED alone is considerably different from the structure of ED in the full length NS1 protein (Bornholdt & Prasad, 2008) (Figure 12 A). A comparison of the ED of A/Puerto Rico/8/34 and of the full-length protein of A/Vietnam/1203/2004 showed that the ED dimers of full-length NS1 are more twisted towards the center of each monomer. Although the contribution of a five-residue deletion in the NS1 linker region of A/Vietnam/1203/2004 cannot be ruled out, it was suggested that the presence of RBD might determine the orientation of these dimers.

Based on the level of sequence differences between allele A and B NS1 proteins, it is likely that the 3D structures of allele A and B differ from each other, as was indicated previously. Apparently, the *in silico* structure prediction of both alleles NS1 showed considerable differences in the overall orientation (Figure 12 B and C). The position of two residues that are characterized as functionally important was also found to be either more exposed (allele A, Figure 12 B) or more internalized (allele B, Figure 12 C). Based on these indications, it would be of particular interest to resolve the complete structure of the allele B NS1 protein, and to compare the functional and structural differences in parallel.

1.8.5 Mechanisms of NS1 protein in abrogating host IFN responses

The production of IFNs in response to influenza viruses is certainly a limiting factor for viral propagation (illustrated in Figure 7). Therefore, influenza A viruses have evolved intrinsic capacities, mainly mediated by the NS1 protein, to suppress IFN production, and this act is a prerequisite of influenza A viruses replication. The NS1 protein is a relatively small protein that both performs and regulates a plethora of cellular activities. The primary role of the NS1 protein is to counteract the host antiviral responses by participating in both protein–RNA and protein-protein interactions. A great wealth of reports has demonstrated the molecular mechanisms of the NS1 protein in production and downstream effects of IFN. By these reports, it is apparent that most of the functions of the NS1 protein are either strain dependent or host dependent (Kochs *et al.*, 2007; Hayman *et al.*, 2006; Geiss *et al.*, 2002). However, the well-characterized functions of the NS1 protein in antagonizing IFN production are briefly discussed below.

Double stranded RNA (dsRNA) sequestration

The inhibition of IFNs by the NS1 protein is primarily linked to dsRNA binding (Lu *et al.*, 1995) (label number 1 in Figure 13). It has been demonstrated that dsRNA produced during viral replication must be at least

30-80 bp to elicit type I interferons (IFN-α/β) responses (Wang & Carmichael, 2004). The NS1 protein has the ability to sequester variable lengths of dsRNAs and to prevent exposure of dsRNAs to any interactions for downstream signaling. It has been described that NS1 protein interacts with a wide range of RNAs including dsRNA (Wang *et al.*, 1999; Hatada & Fukuda, 1992), viral genomic RNA (Hatada *et al.*, 1997), viral mRNA (Marion *et al.*, 1997a; Park & Katze, 1995), poly(A)-containing RNAs (Qiu & Krug, 1994), and small nuclear RNAs (snRNAs) derived from the U6 promoter (Qiu *et al.*, 1995). Structural analysis has indicated that several highly conserved residues, including Thr-5, Pro-31, Aps-34, Arg-35, Arg-38, Lys-41, Gly-45, Arg-46 and Thr-49, are involved in NS1-dsRNA interaction either directly or by improving stability of this complex (Yin *et al.*, 2007; Wang *et al.*, 1999). Recently, the detailed structural arrangement of NS1-dsRNA has unambiguously shown that the shallow concave surface of RBD, which is a highly conserved region in the NS1 protein, recognizes the major groove of dsRNA (Cheng *et al.*, 2009). An influenza isolate mutated at Arg-38 (R38A) and double mutated at Arg-35 (R35A) and Arg-46 (R46A) completely abolished the ability of RBD to interact with dsRNA, indicating that these two positions are critical for this interaction. The mutations at Thr-49 and Ser-42 residues reduced this interaction 10-fold, demonstrating the essential role of these residues in stabilizing NS1-dsRNA interaction (Cheng *et al.*, 2009). However, several studies have shown that the R38A and K41A substitutions are enough to disrupt this interaction and to attenuate the virulence (Yin *et al.*, 2007; Wang *et al.*, 2002; Wang *et al.*, 1999).

Interestingly, all of the allele A and B NS1 proteins are conserved at all of these residues. However, both alleles carry 15 substitutions in this highly sensitive region (from position 5-50) for NS1-dsRNA interaction, including a stretch of eight consecutive residues (Figure 15, results and discussion section). The biological significance of this region in the virulence of influenza A viruses remains to be addressed.

NS1 interaction with RIG-I

Although it is unambiguously believed that the NS1 protein sequesters the dsRNA to keep it away from the cellular sensors such as RIG-I, no detectable level of dsRNA has been found in influenza A virus infected cells (Weber *et al.*, 2006). Further, it has been suggested that other molecular patterns (RNPs, single-stranded RNA) may be more dangerous signals for immune responses. Consistent with this, it was found that RIG-I directly recognizes the influenza A virus ssRNA (Pichlmair *et al.*, 2006). Several studies have demonstrated that Arg-38 and Lys-41 are also potentially involved in interaction between the

RIG-I and NS1 protein of PR8 (label number 2 in Figure 13) (Guo *et al.*, 2007; Mibayashi *et al.*, 2007; Opitz *et al.*, 2007; Pichlmair *et al.*, 2006). Although this phenomenon is not yet fully resolved, it is clear that 5´triphophosphorylated ssRNA stabilize the RIG-I-NS1 complex (Pichlmair *et al.*, 2006).

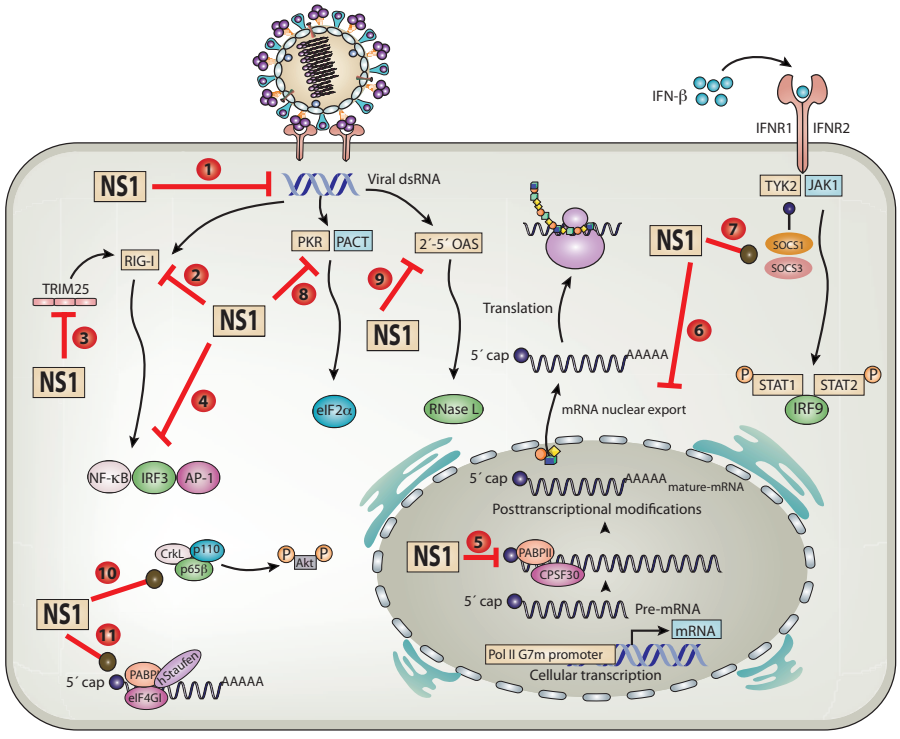

Figure 13. Schematic illustration of the multiple functions of the NS1 protein, with primary focus on the anti-IFN actions. The specific functions of the NS1 protein are labeled from 1-10, according to their sequence of description in the text. See text for detailed description of these functions.

NS1 interaction with TRIM25

TRIM25 ubiquitinates and subsequently activates the RIG-I, an essential element for downstream signaling. It has recently been described that the NS1 protein of influenza A viruses not only sequesters dsRNA to prevent it from binding to RIG-I but also targets TRIM25, and thus disables the production of type I IFNs (Gack *et al.*, 2009) (label number 3 in Figure 13). It has been observed that NS1 mediated inhibition of TRIM25-dependent signaling is not the sole action of the NS1 protein because complete inhibition of IFN induction in influenza virus-infected TRIM25$^{-/-}$ mouse embryonic fibroblasts

was not observed. Influenza viruses with a double mutation (E96A and E97A) abolish the ability of the NS1 protein to inhibit TRIM25, thereby eliminating the IFN-suppressive activity of NS1, which leads to efficient IFN production and the complete loss of virulence (reviewed in Munir, 2010). These two residues are highly conserved in all NS1 proteins. Both allele A and B NS1 proteins carry identical residues at position 96 and 97; however substitutions were observed in allele B NS1 at both ends of these sites (at 94, 95 and at 98). It would be interesting to observe the role of these residues in the stability and interaction of the NS1-TRIM25 complex.

Inhibition of IRF3, NF-κB, AP-1 transcription factors, key regulators of IFN production

The critical role of three transcription factors (IRF3, NF-κB, AP-1) in the regulation of IFN-β production is outlined in Figure 7. The NS1 proteins amazingly target all of these transcription factors to maximize the chances of countering the IFN production (label number 4 in Figure 13). Initially, a report from Adolfo García-Sastre's lab demonstrated that the NS1 proteins are general inhibitors of the interferon-signaling pathway, primarily by inhibiting the IRF3 phosphorylation, and suggested that this inhibition is an important virulence factor for influenza viruses (Talon *et al.*, 2000). In this report, they noticed that in the A/PR/8/34 (PR8)-infected human epithelial cells the NS1 protein could not completely prevent the activation of IRF3. This indicates that there could be alternative ways for the NS1 to target the innate immune response of the host. Consistent with this hypothesis, they further explained that the NS1 protein prevents the activation of the virus- or dsRNA-induced NF-κB pathway and subsequently antagonizes the IFN-β synthesis (Wang *et al.*, 2000).

The molecular mechanism behind the inhibition of these transcription factors is not entirely defined. However, it is likely that the NS1 protein based sequestration of dsRNA, a proximal inducer of these transcription factors, may lead to a general prevention of IFN system activation. It was plausible to hypothesize that the NS1 protein must also inhibit the activation of the AP-1 transcription factor. Two years later, a report from the same lab identified that the NS1 antagonizes both virus- and dsRNA-induced activation of the AP-1 transcription factor (Ludwig *et al.*, 2002). These findings made it clear that inhibition of the IFN system is one of the key functions of the NS1 protein, which plays a significant role in the replication and also the pathogenesis of influenza A viruses.

NS1 interaction with CPSF30 and PABPII

Post-transcriptional modification of newly transcribed eukaryotic mRNAs (including IFN-β mRNAs) is an essential event for their maturation and function. Cleavage and polyadenylation specificity factor 30kDa (CPSF30) is required for 3′-end cleavage and polyadenylation of host cellular mRNAs (Wahle & Keller, 1996). In addition to CPSF30, a poly(A)-binding protein II (PABII) is also required to process the elongation of a short sequence (~10 nucleotides) at the 3′-end of mRNAs, thereby facilitating rapid processing of poly(A) addition (Bienroth *et al.*, 1993). PABII is also an integral component of the nuclear export machinery for mRNAs.

As described above, the sequestration of dsRNA by the NS1 protein is not directly related to the level of inhibition of the IFN-β mRNA synthesis. Despite this, the production of IFN-β is observed to be decreased in influenza A virus-infected cells, which leads to the concern of additional viral factors responsible to limit the production of IFN-β. In this context, it has earlier been shown that the NS1 protein interacts and sequesters the CPSF30 (Nemeroff *et al.*, 1998) (label number 5 in Figure 13). As a consequence, IFN-β mRNA produced in influenza A virus-infected cells escape the 3′-end processing to form mature IFN-β, and therefore the IFN response along with other antiviral mRNAs succumbs (Das *et al.*, 2008; Twu *et al.*, 2006; Noah *et al.*, 2003; Kim *et al.*, 2002). The crystal structure of the NS1-CPSF30 complex revealed that the ED of NS1 requires substantial rearrangements in the original dimer form. In a tetrameric complex, two EDs of NS1 protein make a head-to-head conformation and then interact with second and third zinc fingers (F2F3) of CPSF30 (Das *et al.*, 2008). Although, this interaction of the NS1 is mediated by amino acid 144 and 184-188 (the 186 region) (Twu *et al.*, 2006; Li *et al.*, 2001), the F103 and M106 have been described as essential to stabilize this tetrameric complex (Das *et al.*, 2008). These sites (F103 and M106) are highly conserved, and only three viruses including PR8 show F103S substitution. Interestingly, PR8, due to having S103, did not bind to CPSF30 both *in vitro* and *in vivo* (Kochs *et al.*, 2007; Macken *et al.*, 2001). Provided that allele B NS1 protein also contains an F103Y substitution, it is valuable to investigate the role of 103Y in contrast to 103F and 103S in interaction with the CPFS30. On the other hand, a single substitution (G184R) can abolish the ability of the NS1 protein to inhibit IFN-β pre-mRNA processing, and is enough to attenuate the virus, indicating that NS1-CPSF30 interaction is essential for viral replication (Das *et al.*, 2008).

Furthermore, the NS1 protein also interacts with PABPII and blocks the elongation of the poly(A) tail of the pre-mRNA, resulting in interference with mRNA maturation (Chen *et al.*, 1999). Additionally, this interaction also leads

to nuclear retention of antiviral mRNAs, including IFN-β mRNA (label number 5 in Figure 13, nucleus). It has been demonstrated that amino acid positions 215-237 or 223-237 are essential for this NS1-PABPII interaction (Chen *et al.*, 1999). Being 230 amino acids long, the allele A NS1 protein differs from allele B by five or three amino acids respectively, according to the prediction.

Inhibition of mRNA nucleo-cytoplasmic transport by the NS1 protein

Upon transcription and completion of post-transcriptional modifications, the cellular mRNAs need to be exported to the nucleus for translation. The nucleo-cytoplasmic export is mediated by a set of adapters, such as NXF1, p15, Rae1, E1B-AP5, Nup98, hnRNP-like protein and Rae1/mrnp41/Gle2. In addition to NS1 interaction with CPSF30 and PABPII, it has recently been found that NS1 also interacts with essential components of the mRNA export machinery: NXF1, p15, Rae1, E1B-AP5 and Nup98 (Satterly *et al.*, 2007) (label number 6 in Figure 13). In this way, it directly inhibits the mRNA nuclear export with simultaneous retention of poly(A)-mRNA, which subsequently impairs the immune responses and enhances the viral virulence. Both domains (RBD and ED) were found to be involved in this interaction, but p15 interacted exclusively with the ED of the NS1 protein (Satterly *et al.*, 2007). Additionally, the NS1 protein also makes direct interaction with a novel ~70 kDa NS1-binding protein (NS1-PB) (Wolff *et al.*, 1998). Co-localization of NS1-PB with the SC35, a spliceosome assembly factor, suggests that NS1-BP is involved in the mRNA splicing machinery of eukaryotes (Wolff *et al.*, 1998).

Interestingly, the NS1 protein exclusively blocks the nuclear export of cellular mRNAs, and it does not inhibit the viral mRNA export. This suggests that viral mRNA export is independent of eukaryotic nuclear export machinery. However, there are indications that the NS1 protein also block self-mRNA export (Garaigorta & Ortin, 2007; Satterly *et al.*, 2007).

Inhibition of IFN signaling by NS1

The type I IFNs, soon after their secretion, bind to IFNARs and activate the JAK/STAT pathway. Until recently, it was not known that the NS1 protein interferes in IFN signaling, which left open an option to use type I IFN as a therapeutic agent. A recent study revealed that the NS1 protein of H5N1 interferes in the type I IFN signaling in three possible ways (Jia *et al.*, 2010). First, the NS1 protein down-regulates the IFN α/β receptor subunit IFNAR1 and to some extent IFNAR2 expression. Second, inhibition of STATs phosphorylation leads to abolition of the nuclear translocation of STATs

complex to initiate transcription of ISGs. Third, it has further been demonstrated that the expression of NS1 induces up-regulation of the SOCS1 and SOCS3, which are potent JAK/STAT inhibitors (Jia *et al.*, 2010) (label number 7 in Figure 13). Based on these observations, it can be concluded that the NS1 protein directly abrogates the type I IFN signaling and therefore facilitates viral replication and virulence.

Inhibition of IFN-inducible antiviral proteins by NS1 protein

Apart from multiple ways of IFN production (pre- and post-transcriptional) and IFN signaling inhibition, the NS1 protein also specifically blocks the activation of two cytoplasmic IFN-inducible antiviral proteins: dsRNA-dependent serine/threonine protein kinase R (PKR) (Min *et al.*, 2007) and 2′-5′-oligoadenylate synthetase (2′-5′-OAS) (Min & Krug, 2006) (Figure 13).

PKR is maintained as an inactive monomer under normal conditions and undergoes activation in response to dsRNA of viral or synthetic origin, such as polyinosinic:polycytidylic acid [poly(I:C)], or by another stimulus, such as PKR-associated activator (PACT). PKR activation by these stimuli is believed to disrupt the auto-inhibitory state of PKR, enabling its homo-dimerization and auto-phosphorylation, and thereby stimulate eukaryotic translation initiation factor 2-α (eIF2-α) substrate binding to block viral protein synthesis (reviewed in Garcia *et al.*, 2006). Additionally, PKR is also associated with the up-regulation of gene expression of different cytokines, including IFN-β, mediated through IRF3 and NF-κB responsive promoters (reviewed in Goodbourn *et al.*, 2000; Sen, 2000). Initially it was believed that the NS1 protein inhibits PKR activation by sequestration of dsRNA (Hatada *et al.*, 1999; Lu *et al.*, 1995). The efficient inhibition of PKR by dsRNA binding defective NS1 proved that it interacts in a dsRNA-independent manner, probably by direct interaction with PKR (Li *et al.*, 2006a) (label number 8 in Figure 13). Functional mapping studies indicated that residues 123-127 in the ED domain directly interact with the N-terminal 230-amino acid region of PKR (Min *et al.*, 2007; Li *et al.*, 2006a). Specifically, substitutions at 123/124 or 126/127 abolish the PKR inhibitory activities of NS1 and render the activation of PKR (Min *et al.*, 2007). Subsequently, the viral protein synthesis was dramatically inhibited due to phosphorylation and activation of eIF2-α. Provided that PKR is associated with IFN induction, inhibition of PKR is another role of NS1 protein in antagonism of IFN production.

A putative by-product of influenza virus replication, dsRNA, also activates the 2′-5′-OAS, which terminates at the activation of RNaseL pathways. The activated RNaseL inhibits viral replication (translation) by degrading single-stranded RNAs, such as mRNAs and ribosomal RNAs, into short fragments

(reviewed in Sen, 2000). It has been shown that the NS1 protein inhibits the 2′-5′-OAS pathway, and this function is mapped into the RBD (label number 9 in Figure 13). It is further suggested that NS1 has a higher binding affinity for dsRNA than 2′-5′-OAS. Given that nuclear fragments, produced as a result of 2′-5′-OAS/RNaseL activities, can also activate the IFN-β induction via binding to RIG-I/MDA-5 (Malathi *et al.*, 2007), it is likely that inhibition of 2′-5′-OAS activity by NS1 may also contribute to limiting IFN-β induction (Donelan *et al.*, 2003; Talon *et al.*, 2000).

Two cellular proteins, P13K and Crk adaptor proteins (Crk/CrkL), play an important role in the regulation of multiple host-cell processes, such as anti-apoptosis, cell growth, proliferation, cytokine production and cell signaling. It has been demonstrated that IFN activation of P13K counters many functions of STAT signaling. The NS1 protein of influenza A viruses regulates these host cell-signaling pathways in a way that would alter to optimize virus replication (Hale *et al.*, 2008b; Heikkinen *et al.*, 2008; Hale & Randall, 2007) (label number 10 in Figure 13).

Redundant roles of NS1 protein in IFN regulation

It has been observed that, unlike other influenza A virus infections, PR8 does not activate the IRF3, but instead results in the activation of large amount of IFN-β cytokine. There are discrepancies behind the molecular mechanism for lack of IRF3 activation under PR8 infection. The A/Udorn/72 influenza A virus, when carrying NS1 protein of PR8, activates the IRF3. This suggests that the mechanism for absence of IRF3 activation is independent of the PR8-NS1 protein, and other factors might be involved. Moreover, PR8 is reported to interact with RIG-I, which results in IRF3 activation (Mibayashi *et al.*, 2007; Pichlmair *et al.*, 2006). The ability of the NS1 protein to limit IFN-α and tumor necrosis factor-α induced host-cell gene expression has also been reported in several studies (Hale *et al.*, 2008c; Kochs *et al.*, 2007; Hayman *et al.*, 2006; Geiss *et al.*, 2002; Seo *et al.*, 2002). These facts lead to the hypothesis that the role of the NS1 protein is probably mutually redundant in regulating IFN response and there are anonymous roles of the NS1 protein in this regulation, which may depend upon several unidentified factors, from both the viral and the host ends. Moreover, NS1 seems to be an opportunist protein, which regulates and modifies its functions depending upon viral need in the course of pathogenesis.

1.8.6 NS1 protein and influenza A virus replication

The NS1 protein plays a significant role in enhancing influenza virus replication, primarily by both viral mRNA synthesis and viral mRNA

translation. It has been noted that a complex of NS1-CPSF30 joins the cellular RNA polymerase II to make a macromolecular complex, and contributes substantially in the regulation of viral RNA synthesis (Kuo & Krug, 2009; Engelhardt *et al.*, 2005). The time of production of influenza A viral proteins is either early (NS1 and NP) or late (other proteins, mainly HA and M1), as explained previously and outlined in Figure 5. The NS1 protein has been shown to temporally regulate the synthesis of viral mRNA and vRNA (Min *et al.*, 2007; Falcon *et al.*, 2004; Wolstenholme *et al.*, 1980). A double mutation at IIe-123 and Met-124 leads to up-regulation of viral proteins (especially the late proteins) early in the infection (Min *et al.*, 2007). Although the molecular mechanism is unclear, it is possible that the same substitutions (123 and 124) result in the activation of PKR (discussed previously). It is speculated that this viral mRNA regulation may be PKR-dependent. Alternatively, interaction of NS1 with the viral polymerase complex in infected cells has been suggested to play a crucial role in viral RNA synthesis (Min *et al.*, 2007; Falcon *et al.*, 2004; Marion *et al.*, 1997b).

Provided that NS1 interacts with the 5´UTR (un-translated region) of viral mRNAs (Park & Katze, 1995; Garfinkel & Katze, 1993), it is likely that the NS1 protein enhances the translation initiation of viral mRNAs (Marion *et al.*, 1997a; de la Luna *et al.*, 1995; Enami *et al.*, 1994). In addition to binding with viral mRNA, the N-terminal 81 amino acids of the NS1 protein have also shown to interact with poly(A)-binding protein I (PABPI) (Burgui *et al.*, 2003). Furthermore, the NS1 protein also interacts with eukaryotic translation initiation factor GI (eIF4GI) by its N-terminus 113 amino acids (Aragon *et al.*, 2000). Interaction with these factors leads to enhancement of the translation of viral mRNA, specifically by recruiting viral mRNA translation initiation complexes (label number 11 in Figure 13) (Burgui *et al.*, 2003).

An hStaufen is a dsRNA and tubulin binding protein, which is implicated in the transportation of RNAs via its interaction with microtubules, and subsequently enhancement of the translation. The C-terminal effector domain of the NS1 protein interacts with the dsRNA-binding domain of hStaufen (Falcon *et al.*, 2004). It has been suggested that this interaction may facilitate the viral mRNA location in the proper milieu for effective translation. Taken together, interaction of NS1 with cellular hStaufen, eIF4GI and PABPI complex specifically recruits viral mRNA and facilitates efficient translation (label number 11 in Figure 13).

In summary, the influenza virus research community has made several breakthroughs in the last few years. As a result of this, it has become clear that the NS1 protein evades host immune responses in multiple ways. Here, I have discussed quite a few of these functions of the NS1 protein of influenza A

viruses. It has been demonstrated that NS1 proteins play indispensible roles in tissue tropism, virulence and pathogenesis. Owing to their dynamic and versatile nature, it is essential to identify the most pressing scientific problems in understanding the precise molecular mechanisms underlying the multifunctional character of NS1 proteins, and to screen for additional functions of the NS1 protein. Understanding the biological implication of the NS1 protein would lead to clarification of its roles in determining AIV pathogenicity, virulence, host adaptation and multifactorial host-pathogen interaction.

2 Aims of the thesis

The overall objective of this work was to characterize the allele A and B NS1 proteins of avian influenza A viruses for their ability to circumvent host immune responses and to modulate different signaling pathways. A further focus was to map the structural, functional and species-specific mechanisms in which different NS1 proteins may contribute to the pathogenicity of influenza A viruses.

The specific aims were to:

- Investigate the roles of allele A and B NS1 proteins of influenza A viruses, and find whether these proteins have functional preferences over inhibition of IFN-β production (Paper I).

- Demonstrate the underlying molecular mechanism for the differential and allele specific inhibition of IFN-β (Paper II).

- Map and compare the structural and functional ability of allele A and B NS1 proteins to inhibit dsRNA-induced NF-κB promoter activation (Paper III).

- Address whether NS1 proteins of allele A and allele B influenza A viruses varied in their capability to confer dsRNA-induced AP-1 promoter activation (Paper IV).

- Characterize a reassortant H9N2 isolate and parallel comparison of H9N2-NS1 protein to a highly pathogenic H5N1 for their ability to regulate different signaling pathways (Paper V).

- Investigate the molecular mechanisms in the regulation of IFN-β production in human and avian cells by the NS1 proteins of H9N2 and H5N1 influenza A viruses (Paper V).

3 Materials and methods

3.1 Influenza A viruses

The influenza isolates (Table 1) used in the study were grown in the allantoic cavity of 9-day-old embryonated eggs and stored at -80 °C until use.

Table 1. *Characteristics of influenza A viruses used in this study.*

Name of the isolate	Subtype	Allele	Abbreviated name	Accession Numbers	Paper
A/mink/Sweden/3900/84	H10N4	A	pNS-mink/84	GQ176140	I
A/chicken/Germany/N/49	H10N7	B	pNS-chicken/49	GQ176132	I
A/mallard/Sw/412/05	H6N8	A	H6N8-A	EU518721	II, III, IV
A/mallard/Sw/418/05	H6N8	B	H6N8-B	EU518722	II, III, IV
A/mallard/Sw/818/05	H4N6	A	H4N6-A	EU518757	II, III, IV
A/mallard/Sw/795/05	H4N6	B	H4N6-B	EU518749	II, III, IV
A/Puerto Rico/8/34	H1N1	A	PR8-A	CY040174	II
A/mallard/Sw/754/05	H4N6	A	N/A[a]	EU518742	II
A/mallard/Sw/807/05	H4N6	A	N/A	EU518753	II
A/mallard/Sw/229/03	H10N8	A	N/A	N/A	II
A/mallard/Sw/825/05	H4N3	A	N/A	EU518759	II
A/mallard/Sw/465/05	H3N8	A	N/A	EU518731	II
A/mallard/Sw/424/05	H6N8	B	N/A	EU518724	II
A/mallard/Sw/443/05	H6N8	B	N/A	EU518727	II
A/mallard/Sw/796/05	H4N6	B	N/A	EU518750	II
A/Chicken/Pak/BYP/2010	H9N2	A	H9N2/NS1	N/A	V
A/tufted-duck/Sw/V789/06	H5N1	A	H5N1/NS1	EU122008	V

[a] not applicable

3.2 Cell cultures

A549, a type II alveolar epithelial cell line from human adenocarcinoma (ATCC, CCL-185), Madin Darby Canine Kidney (MDCK) (ATCC CCL-34) and Chicken Embryo Fibroblast (CEF) (ATCC CRL-1590) cells were grown in Dulbecco's modified Eagle's medium (DMEM), while Mink Lung cells (MiLu) were maintained in Eagle's minimum essential medium with non-essential amino acids. These media were supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U mL^{-1} penicillin, 100 mg mL^{-1} streptomycin and incubated at 37 °C with 5% CO_2 in a humidified (95%) atmosphere.

3.3 Construction of expression plasmids

The NS1 expression plasmids used in Papers I, II, III and IV were constructed from full-length cDNA of segment 8, using primers: NS1-*KpnI*-Fw and NS1-*XbaI*-Rev (see the individual paper for details). The individual domains (RBD and ED) of alleles A and B of NS1 protein of H6N8 were also cloned. All of these products were digested with *KpnI* and *XbaI* at their 5′ and 3′ ends, respectively, and cloned into a mammalian expression vector pcDNA3.1+ (Invitrogen) at their respective restriction sites (*KpnI* and *XbaI*).

For the construction of chimeric NS1 (H6N8 chiNS1 A/B), the RNA binding domains of H6N8 allele A (A-C-RNA) and ED of H6N8 allele B (B-C-ED) were amplified using primers as described in paper II. The resulting products were digested and ligated to each other at *EcoRI* sites. Similarly, H6N8 chiNS1 B/A was constructed by digestion and ligation of the RNA binding domain of H6N8 allele B (B-C-RNA) and ED of H6N8 allele A (A-C-ED). The subsequent products were cleaved at *KpnI* at the 5′ end and *XbaI* at the 3′ end, and were subsequently cloned into pcDNA3.1+ (Invitrogen) (Papers II, III and IV). To create a mutation at nucleotide position 308 in the NS1 construct of H6N8 alleles A (T308A) and B (A308T), the M-T308A and M-A308T mutagenesis primers were used, respectively (Paper II). A QuikChange Site-Directed Mutagenesis kit (Stratagene) was applied to create specific mutations.

The constructs used in Paper V were synthesized using NS1-*XbaI*-Fw and NS1-*KpnI*-Rev. The resultant products were cloned into pCMV-FLAG®-MAT-Tag®-1 (Sigma-Aldrich), another mammalian expression vector carrying a flag tag at the N-terminus.

3.4 Reporter plasmids

A set of reporter plasmids was used to screen for different signaling pathways. Each reporter plasmid carries a *cis*-acting enhancer element, responsive to the activation of a specific signaling pathway by a suitable stimulus, and tagged with a luciferase reporter gene. The reporter plasmids used in these studies are summarized in Table 2.

Table 2. Characteristics of the reporter plasmids used in this thesis.

Reporter plasmid	*Cis*-acting enhancer element	Transcription factor	Pathway	Source	Stimulus	Paper
pISRE-Luc	Interferon stimulated response element	ISRE	Proliferation/ Inflammation	Clontech	dsRNA	I, II, V
pNF-κB-Luc	Nuclear factor κB	NF-κB	Proliferation/ Inflammation /Apoptosis	Clontech	dsRNA/TNF-α	II
pAP1-Luc	Activator protein -1	c-jun/c-fos	JNK Proliferation/ Inflammation	Clontech	dsRNA	III
pTA-Luc[a]	N/A[b]	N/A	N/A	Clontech	N/A	I, II, III, IV
4xIRF3-Luc	Interferon regulatory factor 3 (PRD I/III)	IRF3	Proliferation/ Inflammation	Dr. S. Ludwig[d]	dsRNA	V
p125-Luc	IFN-β	ISRE	Proliferation/ Inflammation	Dr. Hongxia Li[e]	dsRNA	V
pGL4.74[c]	N/A	N/A	N/A	Dr. Magnus Johansson[f]	N/A	V

[a] contains a *renilla* luciferase gene and was used as an internal control
[b] not applicable
[c] contains a *renilla* luciferase gene and was used as a transfection control to standardize all reporter assays
[d] Institute of Molecular Virology, Centre for Molecular Biology of Inflammation (ZMBE), Germany
[e] WuHan Institute of Virology, Chinese Academy of Sciences, China
[f] School of Life Sciences, Södertörns University, Sweden

3.5 Transfections and reporter assays

All of the luciferase assays were performed in 24-well transfection plates (Nunc). The transfection complexes for reporter and expression plasmids were prepared with an equal amount of the corresponding plasmids using FuGENE6 (Roche) (Papers I, II, III and IV). In all of the luciferase assays reported in

Paper V, the transfections were performed using Lipofectamine 2000 (Invitrogen) in A549 and CEF cells, according to the manufacturer's instructions. In all of the studies, freshly passaged A549, MiLu and CEF cells were grown in 24-well plates at a density of ~$2.5x10^4$ cells per well, to attain 80% confluency by the next day for transfection. After 24 hours of transfection (determined from a prior optimization of the assay), the cells were stimulated with either poly I:C (dsRNA) (Invivogen) or TNF-α (Invitro) in duplicate wells for each sample. At the indicated time-points post stimulation, the cells were lysed with 300 µl of lysis buffer from the Luciferase Assay Kit (Stratagene) (Paper I), or 100 µl of One Glo Luciferase Substrate (Promega) (Paper II, III and IV) or 100 µl of 1x passive lysis buffer in the Dual-Luciferase Reporter Assay System (Promega) (Paper V). The luciferase activity was measured in luminometer plates (Nunc), and analysed by reading luminescence in a Wallac Victor$^{2™}$ 1420 Multilabel counter (Wallac Sverige AB). Each transfection experiment was repeated at least three times, and the average value of the luminescence readings was calculated and normalized with *renilla* luciferase activity.

In Paper II, A549 cells were transfected with the ISRE reporter plasmid for 24 hours, followed by infection with influenza viruses at a multiplicity of infection (MOI) of 5. *Firefly* luciferase activity was determined in the cell lysates using One Glo Luciferase Substrate, as described above.

3.6 *In situ* proximity ligation assay (*in situ* PLA)

A549 cells were seeded in 8-well chambers (VWR international) for 24 hours, followed by transfection with the indicated amount of NS1 constructs using Lipofectamine 2000 (Invitrogen). After the indicated time post transfection, cells were fixed and permeabilized. The cells were incubated with primary antibodies raised against the C-terminus of the NS1 protein (ProSci) (Papers II, III and IV) or anti-flag (Sigma-Aldrich) (Paper V). Afterwards, the cells were incubated with anti-rabbit PLUS and anti-rabbit MINUS (Papers II, III and IV) or anti-mouse PLUS and anti-mouse MINUS (Paper V) proximity-probes at 37° C for 2 hours. Thereafter, the probes were hybridized and the ends ligated, followed by amplification of the ligated probes using rolling cycle amplification. Finally, the cells were incubated in a detection solution containing a fluorescently labeled detection oligonucleotide. Slides were visualized under standard immunofluorescence microscope (Nikon) using suitable filters for Texas red (PLA) and DAPI (nuclear) staining.

To quantify the *in situ* expression levels of the NS1 proteins, BlobFinder software V3.0 (Uppsala University, Uppsala, Sweden) was used.

3.7 Western blotting

For the detection of NS1 protein expression, transfections of the indicated NS1 constructs were performed in 24-well plates using FuGENE6 (Roche) (Paper I) or Lipofectamine 2000 (Invitrogen) (Papers II, III, IV and V). At the indicated times, cells were washed and lysed using Bio-Plex cells lysis kit (Bio-Rad Laboratories) (Papers I, II, III and IV) or NP40 lysis buffer (Paper V). Concentration and quality of the lysate were measured using Nanodrop ND1000 (Nanodrop Tec.). A total of 50 µg of lysate was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) in Ready Gel J 7.5% (Bio-Rad Laboratories) and then transferred to a polyvinylidene difluoride (PVDF) membrane (GE Healthcare). The membranes were blocked (PBS, 2% (wt/vol) bovine serum albumin) at room temperature for 1 hour. Thereafter, the membranes were incubated with NS1 antibodies raised in goat against a peptide mapping near the C-terminus of influenza A NS1 (Santa Cruz Biotechnology) (Paper I) or NS1 antibodies raised in rabbit against the C terminus of NS1 protein (ProSci) (Papers II, III and IV), or antibodies raised in mouse against flag tag (Paper V) and β-actin from Sigma-Aldrich (Paper I) or from ProSci (Paper V), at 4 °C overnight. After washing with PBST (PBS, 0.2% Tween 20), membranes were incubated with horseradish peroxidase (HRP)-conjugated anti-rabbit or anti-mouse or anti-goat secondary antibodies for 2 hours at room temperature with continuous agitation. The blots were developed by an ECL advance kit from GE Healthcare, and visualized by ChemDoc XRS system from Bio-Rad with Quantity One® software.

3.8 Quantification of IFN-β by ELISA

To measure the secreted IFN-β in cell supernatants, a commercially available VeriKine sandwich ELISA kit (PBL Biomedical Laboratories) was applied according to the manufacturer's instructions. A standard curve was established, and the titer of IFN was calculated using MasterPlex ReaderFit software.

3.9 Quantitative real-time PCR (qPCR) for pre- and mature-IFN-β mRNA

To measure the level of pre- and mature-IFN-β mRNA inhibition by the NS1 proteins of different influenza A subtypes, A549 cells were seeded into six-well plates. Cells were transfected with the indicated NS1 constructs and stimulated with dsRNA. The cells were lysed using TRIzol Reagent (Invitrogen) at 0, 2, 4, 8, 16 and 24 hours post stimulation. RNA was extracted using the RNeasy Mini kit, and treated with DNase using on column DNase

Digestion Kit (Qiagen) to remove any DNA contamination. To measure the IFN-β pre-mRNA, the total RNA was reverse transcribed into cDNAs using 20-mer oligonucleotides which target the sequence upstream the poly(A) tail (Paper V). For mature-IFN-β mRNA quantification, the total RNA was reverse transcribed using Oligo(dT)20 (Invitrogen), which targets the poly(A) tail of mature IFN-β mRNA (Paper V). Both, pre- and mature-mRNAs were quantified using Brilliant II SYBR Green QRTPCR Master Mix Kit (Agilent Technologies) with oligonucleotides for IFN-β (Papers II and V), as per the manufacturer's instructions. To normalize the loading cDNA, β-actin was run in parallel at all time points using forward (5′-TGG GTC AGA AGG ACT CCT ATG-3′) and reverse (5′-AGA AGA GCT ATG AGC TGC CTG-3′) primers. The results were presented as fold increase in IFN-β mRNA using the $2^{-\Delta\Delta T}$ method.

3.10 VSV-GFP IFN bioassay

For quantitative measure of IFN-β inhibition by the NS1 proteins of H9N2 and H5N1, A549 cells were seeded in 8-well chamber slides (VWR international). Transfections were performed using Lipofectamine 2000 (Invitrogen). Eighteen hours post transfection incubation period, cells were stimulated and infected with vesicular stomatitis virus (VSV) expressing GFP (VSV-GFP) with an MOI of 2. The fluorescence intensities were measured at 18 hours post infection. Images were taken using an immunofluorescence microscope (Nikon) (Paper V).

3.11 IRF-3 nuclear translocation assay

The NS1 constructs for H9N2 and H5N1 were transfected in A549 and MDCK cells in 8-well chamber slides (VWR international) using Lipofectamine 2000 (Invitrogen). At 24 hours post-transfection incubation period, phosphorylation of IRF3 was stimulated with 10 μg of dsRNA. At 24 hours post stimulation, cells were fixed in 1% paraformaldehyde for 40 minutes. Then the cells were incubated with anti-pIRF3 (Ser396) rabbit monoclonal antibodies (Millipore), followed by incubation with Cy3-mouse anti-rabbit IgG as secondary antibodies (Jackson Immuno Research). The nuclear localization of activated IRF-3 was examined with a standard immunofluorescence microscope (Nikon) using suitable filters for Texas red (IRF3) and DAPI (nuclear) staining (Paper V).

3.12 Genetic characterization and phylogenetic analysis

The H9N2 influenza virus was isolated from backyard poultry flocks, and was biologically characterized using standard hemagglutination-inhibition (HI), neuraminidase inhibition assay and intravenous pathogenicity index (IVPI) at the Poultry Research Institute (PRI), Lahore, Pakistan. For genetic characterization, the eluted RNA from impregnated Qiacard FTA Indicator (Qiagen) was used to sequence all of the gene segments at SLU, Uppsala, Sweden. The phylogenetic analysis was performed on the basis of hemagglutinin, neuraminidase and non-structural genes in the Molecular Evolutionary Genetics Analysis (MEGA, version 5) software package (CEMI, Tempe, AZ, USA) using the neighbor-joining (NJ) method. To confirm the genetic pattern demonstrated by the NJ method, the trees were also constructed using Bayesian Inference with the program MrBayes version 3.1.2 (Paper V).

3.13 Statistical analysis

Data were analyzed for statistical significance applying Student *t*-test. P values of ≤ 0.05 were deemed to be statistically significant.

4 Results and discussion

4.1 Strategies of allele A and B NS1 proteins to counteract IFN-β production and their contributions in viral pathogenicity (Papers I and II)

In the early 1980s, a H10N4 subtype of influenza A viruses (IAV) caused severe infection in mink on the South-East coast of Sweden. Considering that the outbreak was caused by an avian virus (Englund *et al.*, 1986; Klingeborn *et al.*, 1985), an *in vivo* study was conducted in which mink were intranasally infected with mink (mink/84) and avian (mallard/85, fowl/85, chicken/49) isolates (Berg *et al.*, 1990). Interestingly, all isolates caused disease and were transmitted by contact except chicken/49. Moreover, there was a difference in the abilities of mink/84 and chicken/49 to induce interferon (IFN) response (Englund, 2000; Englund & Hard af Segerstad, 1998).

In a recent study conducted to establish the reason behind this apparent difference in pathogenicity and virulence, it was revealed that the isolates (mink/84, chicken/49) have different NS gene segments, where mink/84 was found to carry allele A and chicken/49 carries allele B NS gene segments (Zohari *et al.*, 2010). Until then, this division of influenza A viruses (allele A and B) had been based purely on the putative amino acid sequence of the NS gene (Treanor *et al.*, 1989). However, it had been established that allele B exclusively contained strains of avian origin, while allele A comprised strains of both avian and mammalian (human, equine and swine) origin (Ludwig *et al.*, 1991), with one exception. Despite being a mammalian virus, A/equine/Jilin/1/1989/H3N8 was found to belong to allele B (Guo *et al.*, 1992). Recently, another virus originating from swine (A/Swine/Saskatchewan/18789/2002/H1N1) was also placed in the allele B category of influenza A viruses (Zohari *et al.*, 2008a). However, it should be

noted that all of the eight segments of these two influenza A isolates show high identity to that of avian influenza A viruses, an indication of their origin from an avian source.

It was plausible to hypothesize that the difference in the pathogenicity between mink/84 and chicken/49 might be NS1 allele dependent, primarily, due to the fact that allele A NS1 protein differs from allele B NS1 protein by over 30% of their amino acids (Figure 15). In line with this, it was reported that highly pathogenic avian influenza viruses, with the potential for human direct transmission, mainly contain the allele A NS1 protein (Zohari *et al.*, 2008b). Applying a model system in which A549 cells were co-transfected with NS1 protein expression plasmids, and a pISRE-Luc reporter plasmid under the stimulation of poly I:C (dsRNA), it was possible to demonstrate that mink/84 NS1 causes 85.3% inhibition of ISRE promoter activity. In contrast, chicken/49 NS1 caused a considerably weaker inhibition of ISRE promoter activity with only 20.8%. A similar pattern was observed in a homologous cell culture system, mink lung cells (MiLu). To exclude the possibility that the difference was due to IFN-β mRNA transcription or IFN-β signaling, RT-PCR and ELISA were applied, respectively, which confirmed the results demonstrated in the luciferase assays (Paper I).

Thus, it was confirmed that mink/84 and chicken/49 inhibit IFN-β production in a manner dependent on allele type, and the concept of the essential role of allele A and B NS1s in the pathogenicity of influenza A viruses was established. Nonetheless, it was obvious that the NS1 protein can act as an essential determinant of influenza virus pathogenesis and virulence. However, there were several concerns to be addressed in more detail: (i) since the mink/84 (H10N4) and chicken/49 (H10N7) carry different neuraminidase (NA) genes, the apparent difference in pathogenicity might be species-specific; (ii) the contribution of alleles (A and B) in the inhibition of type I IFN promoter activities might be influenza A virus subtype-specific; (iii) the NS1 protein is a multifunctional protein, which interferes in type I IFN production at multiple steps in ways more than one, and the molecular mechanism behind this allele-dependent type I IFN inhibition would be of special interest; (iv) this inhibition of type I IFN promoter activity might be cell-type specific; (v) the structural motifs in the NS1 proteins (allele A and allele B) responsible for this difference are still unknown; (vi) apart from IFN inhibition, what is the implication of allele A and B NS1 protein in the pathogenicity of influenza A viruses; and finally (vii) what is the biological importance of such discrepancies in allele A and allele B dependent viral replication?

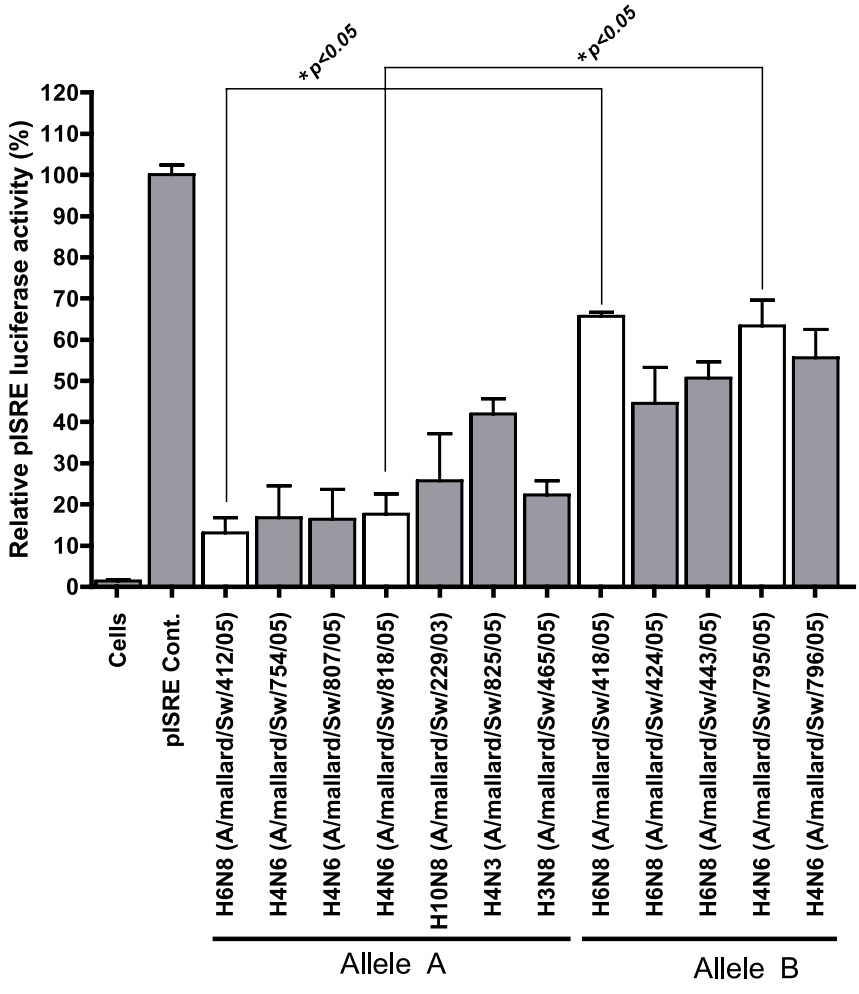

Figure 14. Screening of several subtypes of influenza A viruses for the ability of their NS1 protein to block dsRNA-induced pISRE activity. Human A549 cells were co-transfected for 24 hours with NS1 expression plasmids and pISRE-Luc reporter plasmids. ISRE promoter was stimulated with poly I:C for the next 24 hours. Luciferase activity was measured 24 hours post stimulation. The data represents the average of three independent experiments performed in duplicate. Allele A NS1s can clearly be differentiated from allele B NS1 proteins based on their level of ISRE promoter inhibition. * indicates significant differences between the compared isolates.

In order to address some of these concerns, an extensive study was designed especially to understand the molecular mechanism behind the difference in IFN-β inhibition (Paper II). In an initial screening, NS1 proteins from various avian influenza A subtypes (H6N8, H4N6, H10N8, H4N3, H3N8, H1N1) representing both allele A and allele B, were evaluated for their ability to inhibit dsRNA-dependent ISRE promoter activation. Based on the level of

inhibition, allele A influenza A viruses can clearly be differentiated from allele B, where the level of ISRE promoter inhibition was stronger (up to 87%) in allele A than in allele B (up to 45%) (Figure 14). For deeper analysis, two subtypes (H6N8 and H4N6), each comprised of isolates having both alleles A and B NS1 proteins were chosen. These viruses were identical regarding other viral proteins (with few amino acid differences at uncharacterized positions) except for the NS gene segment.

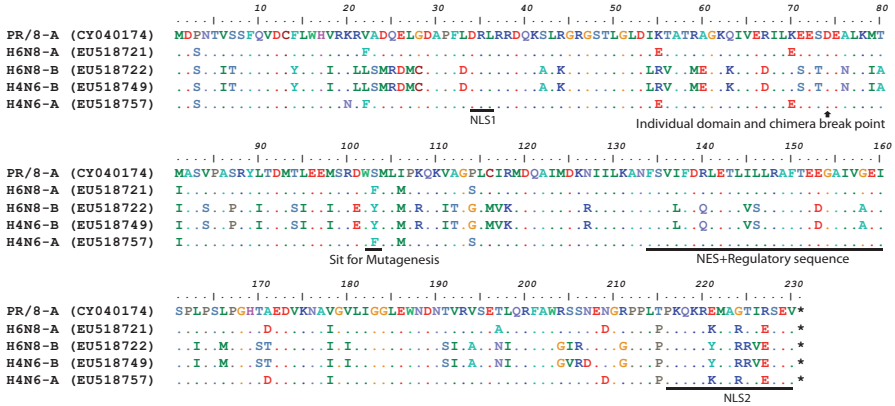

Figure 15. Alignment of the amino acid sequences for alleles A and B of H6N8 and H4N6 along with PR/8 used in this study (Papers I, II, III and IV). The dots represent identical residues to that of PR/8. NLS1 (aa 34–38), NES+regulatory sequence (aa 134–161), NLS2 (aa 216–230) and site of mutagenesis (aa 103) are underlined. The arrow indicates the NS1 chimeras's and individual domain's break points. The amino acids having similar properties were assigned the same color.

As outlined in Figure 7 (introduction section), the ISRE promoter is downstream of IFN transcription, IFN production and subsequent signaling. It was of interest to investigate the abilities of allele A and B NS1 proteins to interfer with IFN production at the specific level. It has recently been demonstrated that the expression of NS1 induces up-regulation of the SOCS1 and SOCS3, which are potent JAK/STAT inhibitors (Jia *et al.*, 2010). Since alleles A and B differ in the suppression of the ISRE promoter, it is plausible to hypothesize that this difference is due to the interactions and inhibition of the JAK/STAT pathway, downstream to IFN receptors, which might reflect the additional effects of the immune evasion potential of different NS1s. However, quantification of the IFN-β protein in NS1 transfected and dsRNA stimulated cells demonstrated that the difference between alleles A and B was due to a difference in the mechanism(s) involved in IFN-β production in the presence of NS1, and not due to signaling from the IFN receptors (Figure 16 A), which was further confirmed by the results of luciferase assay at the corresponding time

points (Figure 16 B). Further, upstream to IFN-β production is the transcription of IFN-β mRNA, primarily initiated by the cumulative action of three transcription factors (IRF3, NF-κB and AP-1) (Maniatis *et al.*, 1998). The results of qRT-PCR showed that the difference in IFN-β protein expression was due to alteration in the IFN-β mRNA transcription, thus indicating that the underlying difference resides in differential NS1 suppression of IFN induction (pre-transcription IFN suppression). However, the biological implications and the mechanisms underlying the differences between allele A and B NS1 proteins in counteracting the IFN-α/β response warrant further investigation.

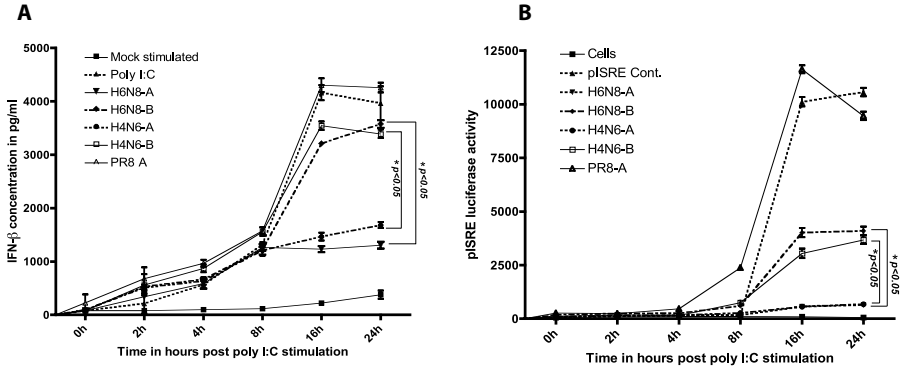

Figure 16. Inhibition of dsRNA-induced IFN-β protein expression and ISRE promoter inhibition by allele A and B NS1 proteins of H6N8 and H4N6. **(A)** A549 cells were either mock-treated or transfected with corresponding NS1 expression plasmids. Cell supernatants were collected at 0, 2, 4, 8, 16 and 24 h post dsRNA stimulation, and were subjected to an ELISA. **(B)** A549 cells were either mock-treated or transfected with ISRE signaling plasmid or co-transfected with ISRE signaling plasmid and NS1 expression plasmids for 24 h, and luciferase activity was measured at 0, 2, 4, 8, 16 and 24 h post dsRNA stimulation.

Despite the fact that most of previously characterized residues are identical in both alleles, genetic characterization of allele A and B NS1 proteins of H6N8 and H4N6 showed the presence of two substitutions at two critical regions (Zohari *et al.*, 2008b). One substitution was observed in the NS1 protein in which it interacts with the CPSF30. This interaction with the CPSF30 inhibits 3´-end processing of cellular pre-mRNA and thus leads to its nuclear retention (Li *et al.*, 2001; Chen *et al.*, 1999; Nemeroff *et al.*, 1998). Two distinct domains mediate this function: one around residue 186 (Li *et al.*, 2001) and the other around residues 103 and 106 (Kochs *et al.*, 2007). It was further shown that mutations at the NS1-CPSF30 interaction sites dramatically changed the effect of the NS1 controlling host gene expression. The NS1 protein of allele A possessed the amino acids Glu186, Phe103 and Met106, whereas the NS1 protein of allele B possessed Glu186, Tyr103 and Met106. Therefore, to test

the contribution of the amino acid at position 103 in post-transcriptional pre-mRNA processing, this position was exchanged both in allele A (Phe103 to Tyr103) and in allele B (Tyr103 to Phe103). The luciferase activity evaluated for the ISRE promoter in A549 and MiLu cells transfected with mutant clones did not differ significantly from the wildtype, consistent with a previous report (Twu *et al.*, 2006), though weak variation was observed in A549 cells (Paper II).

Another substitution was observed at position 221, a site that has been demonstrated to be crucial for NLS and NoLS (Han *et al.*, 2010). At this position, allele A of both H6N8 and H4N6 strains has a lysine (K) residue, while allele B has tyrosine (Y), apart from other differences in both NES and NLS (Figure 15). Moreover, it has also been suggested that the function of the NS1 protein is very much influenced by the nuclear localization pattern of the NS1 protein (Volmer *et al.*, 2010). The observed difference at earlier time points post-transfection might interfere with the processing and export of newly synthesized mRNA. A similar pattern has been observed for A/Udorn/72 and A/Victoria/3/75, to which allele A shows high sequence similarity (Hayman *et al.*, 2006; Qian *et al.*, 1994). It was further demonstrated that accumulation of A/Victoria/3/75 NS1 in the nucleus of COS-1 cells results in the nuclear retention of mRNA (Fortes *et al.*, 1994; Portela *et al.*, 1985). The localization pattern was found to be different (cytoplasmic) during infection of influenza A viruses than transfection of NS1 constructs (nuclear) (Wang *et al.*, 2010), which warrants further investigation to ascertain the biological relevance of this nuclear location pattern in viral replication and pathogenicity.

In order to map the structural basis of the IFN-β inhibition, each domain of the NS1 proteins of both allele A and B H6N8 subtype was constructed. Results based on the dsRNA-induced ISRE promoter activities in both A549 and MiLu cells indicated that the ability to block ISRE promoter is exclusively mapped into the ED, whereas the RBD alone is unable to block ISRE promoter activity. This is in accordance with earlier reports where it was demonstrated that dimerization of NS1 protein is a pre-requisite for the functionality of RNA binding domain (Wang *et al.*, 1999). It was further shown that the ability of multimerization/dimerization was mapped to the effector domain of the NS1 protein from amino acid 134-161 (Wang *et al.*, 2002; Nemeroff *et al.*, 1998), and that the amino acid at position 149 plays a crucial role for this dimerization and subsequent pathogenicity by antagonizing interferon induction (Li *et al.*, 2006b). Therefore, it is obvious that the RNA binding domain alone remained non-functional, and hence did not affect the dsRNA induced ISRE promoter activities.

These results were further confirmed by the analysis of the chimeric NS1 proteins carrying either RBD of allele A and ED of allele B (H6N8 chiNS1 A/B) or vice versa (H6N8 chiNS1 B/A). In either of these chimeric variants, the NS1 protein re-gained the ability to inhibit type I IFN induced promoter activity. These results further validate the idea that the two domains are probably functionally interactive in a co-operative manner resulting in their overall IFN antagonism properties. It is also likely that the difference between the two NS1 proteins influences the three-dimensional structure and dimerization of the NS1 protein, which affects the function of NS1 in the suppression of IFN-β promoter activation. It is noteworthy that both allele A and B NS1 proteins possess an eight continuous amino acids motif (^{21}RFADQELG^{28} and ^{21}LLSMRDMC^{28}). This motif most likely reflects differences in the respective protein function, and would be interesting to study in terms of the biological significance in the context of viral infections.

While these experiments were in progress, an increase was observed in research on the contribution of the allele A and B NS gene segment in viral pathogenicity, virulence and replication. A study by Ma *et al.* (2010) showed that A/FPV/Rostock/34 (H7N1), when carrying allele B of A/Goose/ Guangdong/96 (H5N1), had increased infectivity in mice and replicated more efficiently in human and mouse cell lines than wild-type H7N1. In contrast, an earlier study demonstrated that an avian virus (A/FPV/Rostock/34, H7N1), containing an allele A NS1, replicates efficiently in mammalian cells, while reassortants, containing an allele B NS gene, were shown to replicate poorly in the upper and lower respiratory tract of squirrel monkeys (Treanor *et al.*, 1989). Consistent with the previous reports that the presence of allele B NS gene segment facilitates the replication of influenza A viruses, recently van Wielink *et al.* (2011) showed that MDCK cells expressing the allele B NS1 protein increased the delNS1 influenza virus replication to significantly higher titers, and thus established the basis for commercial vaccine production with the added advantage of DIVA strategies (van Wielink *et al.*, 2011). Studies have also highlighted that the NS gene segment of avian influenza A viruses plays essential roles in host-adaptation, cell-tropism and virulence of influenza A viruses (Ma *et al.*, 2010; Wang *et al.*, 2010).

All these facts lead to a general concept that the NS1 protein of allele A is genotypically and phenotypically different from allele B NS1 proteins of influenza A viruses. This raised interest to investigate previously characterized functions of NS1 protein in the context of allele A and B contributions.

4.2 Regulation of NF-κB and AP-1 transcription factors by the allele A and B NS1 proteins of influenza A viruses (Papers III and IV)

In the first part of the study, we established a concept that allele A and B NS1 proteins differentially regulate IFN-β production, as demonstrated by ISRE promoter activation, IFN-β mRNA transcription and IFN-β protein expression, despite the efficient expression of both allele A and B NS1 proteins. The maximal production of IFN-β is regulated by different transcription factors, namely interferon regulatory factor 3/7 (IRF3/7), nuclear factor κB (NF-κB) and activating protein 1 (AP-1) (Maniatis *et al.*, 1998). The binding of IRF3/7 to positive regulatory domains I and III (PRD I and III), NF-κB on PRD II and AP-1 on PRD IV initiates the transcription of IFN-β mRNA. The endogenous dsRNA, a by-product of viral replication, or synthetic dsRNA (poly I:C) activates all of these transcription factors through a complicated set of pathways (Iwamura *et al.*, 2001).

It is likely that the allele A NS1 proteins of influenza A viruses interfere in these transcription pathways more strongly than allele B NS1 proteins, and hence result in stronger inhibition of IFN-β mRNA transcription. To address this question, reporter plasmids that carry the luciferase gene under the control of a synthetic NF-κB binding site (pNF-κB-Luc) or AP-1 binding site (pAP-1-Luc) were used. The effect of allele A and B NS1 proteins of H6N8 and H4N6 (the same isolates used in the previous study, in order to maintain consistency) expression was evaluated on dsRNA-induced NF-κB and AP-1 promoter activation.

The results showed that there was a high activation of NF-κB promoter activation in mock-NS1 transfected A549 cells. However, with the addition of NS1 from either of the subtypes or alleles clearly diminished the NF-κB promoter activation. Although the level of inhibition of NF-κB promoter was evident for both alleles (A and B), surprisingly it was significantly higher in allele A (~80%) compared to allele B (H6N8-B=30% and H4N6-B=40%) ($p < 0.05$) (Figure 17). In order to exclude the possibility that this role of allele A and B NS1 proteins might be species-specific, this pathway was further examined in mink lung cells (MiLu). A similar pattern was seen, where allele A NS1 proteins of both influenza A subtypes were consistently better (84% and 80% in H6N8 and H4N6) in blocking the induction of NF-κB promoter, compared to allele B NS1 proteins (46% in H6N8 and 37% in H4N6) (Figure 17).

The activated NF-κB pathway has been identified as essential for efficient viral replication (Nimmerjahn *et al.*, 2004) by increasing the vRNA synthesis (Kumar *et al.*, 2008). On the other hand, the NS1 protein of avian influenza A

viruses has been shown to inhibit activation of NF-κB (Wang *et al.*, 2000). This act is probably essential for balancing the optimal induction of NF-κB to prevent the hyper-activation of this transcription factor. Keeping in mind that the presence of the allele B NS1 protein facilitates viral replication (van Wielink *et al.*, 2011; Ma *et al.*, 2010), it is tempting to postulate that in the context of an influenza A virus infection weaker inhibition of NF-κB by allele B NS1 protein appears to have a supportive function for viral replication that is dominant over its antiviral activity.

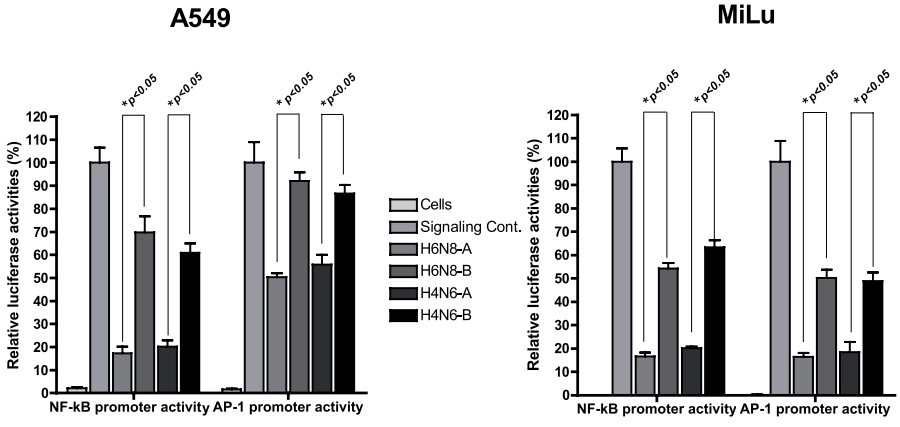

Figure 17. Differential inhibition of the NF-κB and AP-1 promoters by allele A and B NS1 proteins of avian influenza A viruses in A549 and MiLu. Both cell lines were co-transfected with each of the NS1 expression plasmids (H6N8-A, H6N8-B, H4N6-A or H4N6-B), along with either pNF-κB-Luc or pAP-1-Luc reporter plasmid or empty plasmid. After an incubation period of 24 hours, the cells were stimulated with dsRNA (10 μg/ml). After another 24 hours, cells were lysed and luciferase activity was determined. The mock treated control (Signaling Cont.) was set to 100%, and the level of inhibition was reported in percentage. The data presented here correspond to three experiments with transfections performed in duplicate. * Indicates a significant difference as determined by the Student's *t*-test, with p-values of <0.05.

To investigate the role of the NS1 protein of avian influenza A viruses against the induction of the AP-1 promoter, the expression vectors for allele A and B NS1 proteins of both subtypes of influenza A viruses were co-transfected with pAP-1-Luc reporter plasmids into A549 cells, and subjected to dsRNA stimulation before lysis for measurement of luciferase activity. The results showed that the activation of the AP-1 promoter was significantly inhibited by the expression of allele A NS1 proteins from both subtypes (~52%) ($p<0.05$) (Figure 17). The allele B NS1 proteins of corresponding subtypes were unable to inhibit this exogenously administered dsRNA-induced AP-1 promoter activity, although a low-level (statistically non-significant) reduction was obvious (H6N8-B=8% and H4N6-B=14%).

At this point, the results from the reporter assay demonstrated that the allele A NS1 protein inhibits the AP-1 and NF-κB transcription factors by interfering in the signal transduction pathways that lead to activation of AP-1 and NF-κB promoter activity, respectively. In contrast, the allele B NS1 proteins from the corresponding influenza A subtypes were weaker in this inhibition and hence only weakly interfered in the signaling pathways. The same trend was observed when the experiment was repeated in MiLu cells. These results demonstrated that differential regulation of IFN-β production by alleles A and B was mediated through all three transcription factors, and further confirmed that NF-κB and AP-1 are important regulators of IFN-β. However, the individual and cumulative effects of these IFN-β regulators, in term of influenza infection, remain to be investigated.

Next, the structural basis of the inhibition of the NF-κB and AP-1 promoters was investigated. The results showed that the RNA binding domain from neither allele A nor B supported NF-κB and AP-1 promoter inhibition, being non-significant when compared to positive control. On the contrary, the effector domain from both alleles A and B led to a significant suppression of NF-κB and AP-1 promoter activation ($p<0.05$). In MiLu cells, a similar pattern was experienced. To further confirm this characteristic of RBD and ED, two previously described chimeric NS1s were used, containing either the RNA binding domain of allele A and the effector domain of allele B (H6N8 chiNS1 A/B) or vice versa (H6N8 chiNS1 B/A). Interestingly, NF-κB and AP-1 promoter inhibition was reversed in both chimeric NS1s, and was comparable to their wildtype (wt). This ability of chimeric NS1 was reproducible in MiLu cells, and was non-significant when compared to corresponding wt NS1 protein. Taken together, these data demonstrated that the two domains are probably functionally interactive in a co-operative manner to result in their overall NF-κB and AP-1 promoter inhibition.

The conclusion that the ability to block dsRNA-induced NF-κB and AP-1 signaling is mapped into the effector domain, and that the RNA binding domain alone is unable to block NF-κB and AP-1 promoter activity in both A549 and MiLu cells, confirms our previous study (Paper II). It has already been demonstrated that the effector domain of the NS1 protein is not only involved in regulation of cellular activities by protein-protein interactions but also essential for the dimerization and stability of RNA domain (Hale *et al.*, 2008c; Kochs *et al.*, 2007; Donelan *et al.*, 2003; Wang *et al.*, 2002). Consistent with these results, it is likely that the lack of effector domain in the NS1 protein influences the three-dimensional structure and dimerization of the RNA domain, which affects the function in the suppression of NF-κB and AP-1 promoter activation. Moreover, it should be noted that within the RNA binding

domain, a unique motif of eight continuous amino acids might have biological relevance, which warrants further investigation by mutagenesis studies.

4.3 Contribution of HPAIV H5N1-like NS1 protein to the pathogenicity of a recombinant H9N2 influenza virus (Paper V)

A great wealth of earlier reports and our comprehensive studies have demonstrated that the NS1 protein regulates a plethora of cellular activities. Among them, the NS1 protein plays an indispensible role in antagonizing the host IFN system. Therefore, it is plausible to speculate that low pathogenic avian influenza viruses (LPAIV) may use this protein as a shield to be able to escape the host immune response. In this regard, it has been demonstrated that reassortant H9N2 viruses (LPAIV) acquire genotypic characteristics of highly pathogenic H5N1 influenza A viruses (Iqbal *et al.*, 2009). In an attempt to screen backyard poultry for pathogens, a H9N2 virus (A/Chicken/Pakistan/BYP/2010) was isolated and analyzed genetically and biologically. The phylogenetic analysis, based on the hemagglutinin (HA) and neuraminidase (NA) genes showed that this isolate belongs to the G1 group of H9N2 influenza A viruses, whereas based on non-structural (NS) gene segment it clusters into a clade 2.2 with highly pathogenic H5N1 of Z-genotype. Based on this interesting outcome, and results demonstrated previously (Geiss *et al.*, 2002; Basler *et al.*, 2001), it is tempting to hypothesize that reassortant viruses of subtype H9N2 have acquired enhanced pathogenicity, replication and transmission abilities due to recruitment of a new NS1. Among others, as described before, inhibition of IFN-β is one of the best-characterized strategies of NS1 protein primarily for increased virulence and host range determination (Hale *et al.*, 2008c; Li *et al.*, 2006b). Therefore, we attempted to screen NS1 proteins of H9N2 (H9N2/NS1) and HPAIV H5N1 (H5N1/NS1) in parallel for their abilities to regulate different signaling pathways, and investigated the molecular mechanisms in IFN-β production in human and avian cells.

The combined activity of three transcription factors (IRF3, NF-κB and AP-1) is essential to initiate the transcription of IFN-β (Maniatis *et al.*, 1998). To evade such a response, the NS1 protein has evolved to inhibit all of these central activators of type I IFN (Hale *et al.*, 2008c). However, little is known about how the NS1 protein of recombinant H9N2 performs such maneuvers. Initially, the role of the NS1 protein of H9N2/NS1 and H5N1/NS1 was evaluated to resolve this question using 4xIRF3, pAP-1-Luc and pNF-κB-Luc reporter plasmids, which are under the control of IRF3, AP-1 and NF-κB promoters, respectively (Table 2). The results demonstrated that both NS1

proteins of H9N2 and H5N1 are equally potent in blocking dsRNA-induced IRF3 (Figure 18 A, D), AP-1 promoters (Figure 18 B, E) and TNF-***α*** induced NF-***κ***B promoter (Figure 18 C, F). Therefore, I conclude that the understudy NS1 protein of H9N2 viruses has strong abilities to down-regulate IFN-***β*** production from all major possible ways when compared to HPAIV H5N1 subtype. Further, this inhibition was found to be species independent when compared in human A549 and CEF cells.

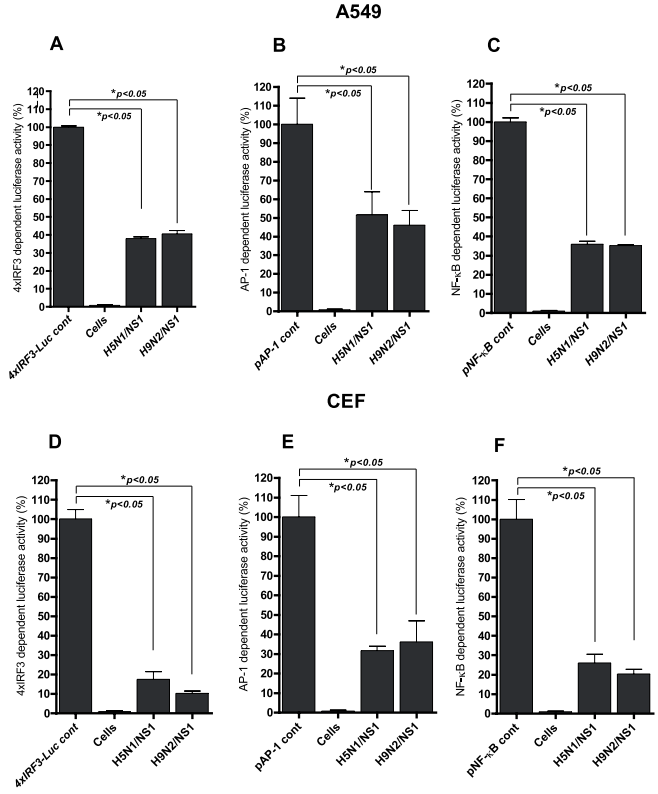

Figure 18. Inhibition of dsRNA- or TNF-***α*** induced pathways by expression of the NS1 protein of influenza A viruses. A549 and CEF cells were co-transfected with the expression plasmids for NS1 protein derived from H9N2 (H9N2/NS1) and H5N1 (H5N1/NS1) with 10 ng of pGL4.74 together with either 4xIRF3-Luc **(A,D)**, pAP-1-Luc **(B,E)** pNF-***κ***B-Luc **(C,F)** reporter plasmids, or normalized with empty flag vector (cont.) or left untreated (cells). After a 24 hours post-transfection incubation period, the A549 cells were either stimulated with 10 μg of dsRNA (poly I:C) per ml (4xIRF3 and pAP-1) or 25 ng/ml of TNF-***α*** (NF-***κ***B) or left untreated (cells). After an additional 24 hours of incubation period, the cell extracts were prepared and the luciferase activity was measured using the Dual-Luciferase Assay System (Promega). The values in mock-transfected cells were normalized and were set to 100% (4xIRF3 cont., pAP-1 cont. and NF-***κ***B cont.). Error bars indicate standard deviations. The data shown here are representative for three experiments with transfections performed in duplicate. * indicates a significant difference as determined by the Student's *t*-test, with p-values of < 0.05.

A substitution was observed at a functionally important site (aa position 221) in the C-terminus of the NS1 protein of H9N2 and H5N1 (Volmer *et al.*, 2010). The sequence analysis of both NS1 proteins showed that the NS1 protein of H9N2 carries lysine (K) at position 221, whereas the NS1 protein of H5N1 carries glutamic acid (E) at this position. Despite these substitutions, there was no difference in the function, expression and cellular localization pattern, in accordance with previous reports (Wang *et al.*, 2010). However, it has been observed that the localization pattern of NS1 protein is different (cytoplasmic) during influenza A virus infection compared to transfection of NS1 constructs (nuclear). The nuclear localization pattern has been suggested to correlate with the pathogenicity of influenza A viruses (Volmer *et al.*, 2010), and therefore it would be of interest to investigate the contribution of this substitution in the context of viral infection and replication.

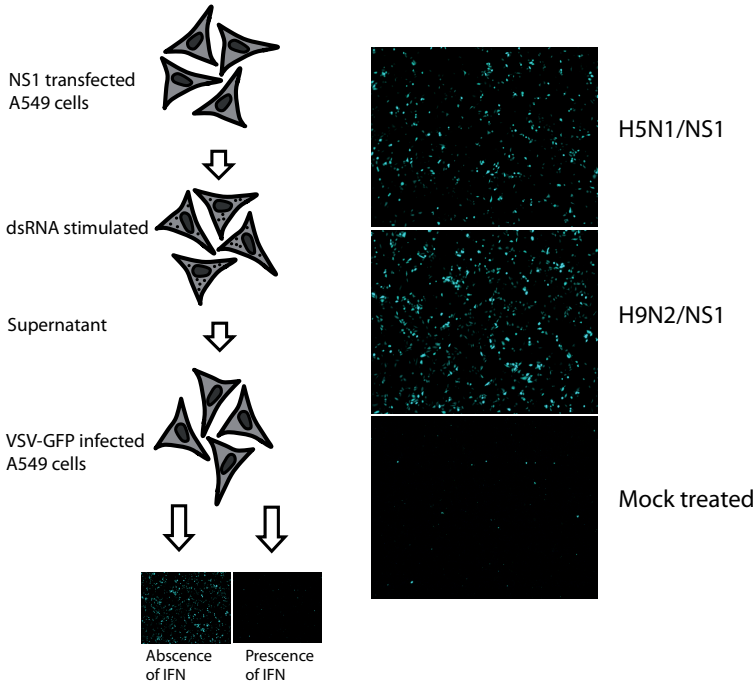

Figure 19. A VSV-GFP based IFN bioassay to detect the level of inhibition of IFN-*β* gene expression in the presence of H9N2/NS1 and H5N1/NS1 proteins. **(Left panel)** The stimulation of IFN-*β* by dsRNA is inhibited in the presence of NS1 proteins and thus leads to efficient replication of the VSV-GFP virus. **(Right panel)** A549 cells were transfected with 300 μg of expression plasmids encoding H9N2/NS1 and H5N1/NS1 proteins. After 18 hours post-transfection incubation period, the cells were either stimulated with 5 μg of dsRNA per ml or left untreated (mock treated). After an additional 24 hours, the cells were infected with VSV-GFP (MOI of 2). Fluorescence was measured after 18 hours of infection.

The NS1 protein is a multifunctional protein that inhibits IFN-β production at multiple steps (Figure 13, introduction section) (Hale *et al.*, 2008c; Kochs *et al.*, 2007). Therefore, the whole IFN-β pathway was evaluated, and it was observed that both proteins were equally potent in inhibiting IFN-β transcription, IFN-β synthesis (Figure 19) and IFN-β signaling, terminated at ISRE transcription factor. All of these characteristics are well defined for the NS1 protein HPAIV H5N1, suggesting a strong functional homology between the NS1 protein of H9N2 and H5N1. Furthermore, NS1 protein has been identified to inhibit nuclear localization of IRF3, as another strategy to abrogate IFN mRNA transcription (Talon *et al.*, 2000). Consistent with previous reports, it was found that both NS1 variants inhibited the nuclear localization of IRF3 transcription factor.

The NS1 protein of HPAIV H5N1 possessed a five amino acid deletion in the middle of the C-terminus, and it is believed that this deletion started appearing after the year of 2000 (Dundon & Capua, 2009). The last report of H5N1 NS1 protein without the five amino acid deletion appeared from Kazakhstan in 2006 (GenBank accession number ACJ53834) (Dundon & Capua, 2009). Although the biological significance of this deletion is ill defined, it was reported that H5N1 influenza A viruses with the deletion in the NS1 protein were more pathogenic than H5N1 without the deletion, in both chickens and mice (Long *et al.*, 2008). Also, it is now clear that the NS1 protein is an essential virulence factor for avian influenza A viruses in other hosts, and may serve as a fundamental marker for the pathogenicity (Ma *et al.*, 2010; Wang *et al.*, 2010; Li *et al.*, 2006b). Therefore, it is logical to think that H9N2 influenza A viruses adapt the NS gene segment from H5N1 to take advantage of this virulence factor. Moreover, it is obvious from the results demonstrated in this study that NS1 proteins of H9N2 and H5N1 have strong genetic and functional similarities. These characters may facilitate the increased virulence and pathogenicity in H9N2 virus infections. Therefore, these viruses should not be overlooked when investigating their epizootic and zoonotic potential.

5 Conclusions

Based on these findings, it was possible to demonstrate that the NS1 protein, in a manner dependent on alleles, applies multiple mechanisms for manipulating the IFN signaling pathway, as measured by ISRE, NF-κB and AP-1 promoter activation, IFN-β mRNA transcription and IFN-β protein expression.

Specifically, the following conclusions can be drawn out from these studies:

- Despite the fact that the NS1 protein is a well-characterized virulence factor, the IFN antagonistic activity is attributed only to allele A NS1 proteins, whereas allele B is inert in this matter.
- This study provided insights showing that allele A and B NS1 proteins can differentially regulate the host NF-κB signaling pathway, which may offer some new perspectives on understanding the host regulation of viral RNA synthesis, especially when allele B is shown to facilitate viral replication.
- Based on the failure to inhibit AP-1 activation by allele B, it is presumed that the NS1 protein is likely to exert a similar effect in preventing the activation of NF-κB, which subsequently results in overall inhibition of IFN-β.
- Structural insights showed that inhibitory activities for ISRE, NF-κB and AP-1 promoters, and subsequently IFN-β inhibition, could be mapped to the C-terminal effector domain.
- Further evidence was provided that well matched ED and RBD are crucial for dsRNA-induced ISRE promoter activities, whereas ED alone, independent of RBD nature, is sufficient for interaction and prevention of the NF-κB and AP-1 promoter activities.
- It has become apparent that, as a result of co-circulation of different subtypes of influenza A viruses in the same host, LPAIV have

acquired gene products of HPAIV. This capability allows the viruses to replicate in a hostile environment by efficiently antagonizing the induction of antiviral mechanism mediated by type I IFN systems.

6 Future prospects

Although our knowledge about how the NS1 protein of influenza A viruses interferes in the IFN signaling pathways has increased significantly in recent years, there are several elements of this complicated phenomenon that still need to be resolved. It is undoubtedly essential to understand the precise molecular mechanisms underlying the versatile nature of NS1 proteins, and to determine the biological implications of allele A and B NS1 proteins in viral pathogenicity, virulence and host determination, especially in the worst-case scenario when most of the previously characterized residues are identical between both alleles of the NS1 protein.

It remains to address the factors that determine the selection of allele A or allele B NS gene segments in the same subtype of influenza A viruses, in the same bird population and in the same geographical region. Above all, such selection has been observed during the same period of time. It is likely that the evolutionary process for the NS gene is as complex interplay as that of HA and NA evolution. However, comparison of both of these influenza elements may provide genetic linkage between the selections of either gene. As has been predicted for HA and NA genes, a steady frequency of reassortment over a certain period of time may elevate fitness of specific HA-NA combinations; and it is likely that such a phenomenon exists for NS gene segments, and may explain the evolutionary dynamics in their natural wild bird hosts. However, it can be speculated that AIV exists in a large pool of transient and functionally equivalent gene segments, and that providing a complex pattern of host immunity results in successive changes in each allele, primarily to intricate fitness for survival which would be harmonious with the virus's divergence under sympatry. Such an hypothesis has been employed for several HA subtypes of influenza A viruses, such as H14 that exists only in Russia, and H16 that has been isolated only from gulls. This restriction is linked to existing immunity against a specific subtype. Therefore, research into the factors

responsible for allele selection will help to better understand the evolution and ecology of influenza A viruses, and may help to generate a novel model of cell-tropism and host adaptation.

The stable adaptation of AIV to several novel hosts (horses, swine and humans), within which transmission is frequent, has largely been studied. This adaptation is mainly associated with acquisition of mutations that make an individual virus distinct from the rest of the viral population. Provided that adaptation of AIV to novel hosts restricts its return back to avian hosts, mainly due to the genetic constellations in the new host (Dugan *et al.*, 2008; Swayne, 2007), it is likely that adaptability of allele A to allele B may be responsible for the low overall prevalence of allele B containing influenza A viruses, and therefore limit (with two exceptions) their presence in avian species.

Given that the production of type I IFNs is directly associated with the replication and attenuation of influenza A viruses (Geiss *et al.*, 2002; Basler *et al.*, 2001), weak ability of the allele B NS1 protein to abrogate IFN production can be exploited for the host's benefit. A selection of strains carrying the allele B NS gene segment may result in optimal inhibition of IFN production, which allows the replication of viruses to the extent of efficient immunogenicity but not sufficient to cause disease. Alternatively, such a suggestion can be employed using suitably truncated and mutated NS1 protein, which would allow the viruses to be attenuated and lead to establishment of a live attenuated vaccine.

It is difficult to study all of the roles of the NS1 protein entirely in cell-culture systems (*in vitro*), and such studies might not be a true image of the biological nature of this multifunctional and complicated protein. Therefore, it is essential to use suitable and relevant animal models (*in vivo*), to better understand the role of allele A and B NS1 proteins in influenza A viruses' pathogenicity, host-adaptation, cell-tropism and virulence in the context of live virus infection.

There is a wealth of reports describing not only the multi-functionality of the NS1 proteins in abrogating IFN responses, but also numerous other functions in the regulation of host-cell mechanisms. However, the majority of these reports describe only single functions at the same time. Therefore, it is of great importance to rule out whether NS1 can interact with a single cellular partner or regulate multiple partners at the same time. Such studies are essential to highlight the priorities of the NS1 protein during the course of influenza A virus pathogenesis, so that possibilities to target specific functions of NS1 protein will be evaluated. Moreover, using established models characterized for the functionality of NS1 protein, it is possible to incorporate allele A and B NS1 proteins, to cumulatively understand the implication of

allele division in the pathogenesis and replication of influenza A viruses, apart from their obvious role in regulation of IFN-β production. This might further be required to unanimously resolve the complex interplay between the multifunctional NS1 protein and the hostile cellular environment.

It has been reported that inhibition of the NF-κB pathway blocks the replication of influenza A viruses in otherwise susceptible cells (Nimmerjahn *et al.*, 2004). This indicates that an intact NF-κB pathway is a pre-requisite for efficient viral infection. To further address the underlying molecular mechanism, it was revealed that NF-κB regulates viral RNA synthesis and hence facilitates its replication (Kumar *et al.*, 2008). The NS1 protein, on the other hand, blocks the activation of the NF-κB pathway, presumably to prevent an over-expression of IFN-β (Wang *et al.*, 2000). Given that allele B does not completely block NF-κB and an intact NF-κB is required for influenza viral replication, it is hypothetically possible that increased replication of influenza A viruses in the presence of the allele B NS segment might be due to active NF-κB. In favor of this, it is noted that influenza A viruses with a NS segment of allele B type have a tendency to replicate efficiently compared to their naïve or recombinant forms (carrying allele A NS1 or delNS1) (van Wielink *et al.*, 2011; Wang *et al.*, 2010). Therefore, a molecular understanding of this phenomenon may lead to a better understanding of host regulation of viral RNA synthesis, and may establish the basis for therapeutic approaches to combat influenza infection.

In conclusion, despite significant advancements, several fundamental questions about the dynamics of NS1 protein functionality, in particular the contribution of the alleles dimension, remain to be addressed. Moreover, the properties of alleles A and B AIV in viral infectivity, pathogenicity, host adaptabilities and viral pathogenesis are poorly understood, and therefore warrant further investigations.

7 Populärvetenskaplig sammanfattning

Interferoner är små äggviteämnen (proteiner), vilka är naturligt förekommande i kroppen som utgör en första försvarslinje mot virus eller vissa bakterier, och produceras även i närvaro av immunstimulerande faktorer. I människokroppen produceras tre familjer av interferoner. De interferoner som är av intresse för oss är typ I interferoner; interferon-alfa från vita blodkroppar, interferon-beta från fibroblaster samt typ II interferoner så kallade interferon-gamma från en särskild vit blodkropp som kallas lymfocyt. Typ I interferoner har också en rad effekter på celler i immunsystemet. Det är känt sedan tidigare att interferoner kan förstärka immunsvaret mot proteiner, men det är osäkert vilken roll interferoner spelar för uppkomsten av adaptivt immunsvar under virusinfektion.

Alla angripande virus är mer eller mindre känsliga för interferoner. Interferonerna bildas mycket tidigt i en infektion, ofta inom loppet av några timmar efter virusets första framträdande och kan påverka virusets förmåga att föröka sig inuti cellen. Därför är det viktig för ett angripande virus att ha resurser att kunna ”ta hand om” värddjurets interferonsvar. Influensavirus är inget undantag. Man har på senare tid identifierat ett virusprotein, icke-strukturellt protein (non-structural protein) 1 (NS1) som en av de faktorer som är viktiga för att påverka värdens immunsvar.

I våra studier har vi fokuserat på förmågan hos NS1 att nedreglera värdcellens immunsvar. Vår huvudhypotes är att NS1 har utvecklats för att “ta hand om” den värd? som influensavirus lever i.

När vi tidigare har tittat på den genetiska variation som finns hos olika influensavirus, visar det sig att variationen är ganska begränsad. Vi fann dock virus som har en genvariant av NS1, som kallas allel A, och virus som har en annan genvariant kallat allel B. Av dessa hittas allel A hos influensavirus som infekterar olika däggdjur (människa, gris och häst), medan allel B i princip uteslutande hittas hos influensavirus som infekterar fåglar. Majoriteten av de

virus vi studerade hade NS1 av varianten allel A (87 %) och enbart 13 % hade allel B. Denna uppdelning är helt baserat på genetisk karaktärisering och deras funktionella konsekvenser är till stor del okända. I den här studien har vi därför försökt att bringa insikter om strukturella och funktionella skillnader som kan finnas mellan dessa två genvarianter av NS1.

För att kunna studera på vilka sätt de två varianterna av NS1 påverkar värdens immunsvar har vi satt upp olika cellkultursystem. Vi använder oss av plasmider (gen-kassetter) som uttrycker de olika NS1-proteinerna. Dessa plasmider introduceras i cellerna tillsammans med olika reporterplasmider, för att kunna mäta nedreglering av immunassocierade gener. På så sätt kan vi jämföra de olika NS1-genernas förmåga att nedreglera t ex interferon, som är en mycket viktig virusbekämpande molekyl, i celler från olika värddjur.

Våra resultat visar att NS1 av allel A har en starkare förmåga att hämma produktionen av interferon-beta i humana celler, jämfört med NS1 av typen allel B från motsvarande influensasubtyper. Vi kunde bekräfta detta genom att studera hur dessa proteiner kan hämma aktiveringen av flera cellulära faktorer som är av stor betydelse för immunsvaret (ISRE, IRF3, NF-κB, AP-1, samt interferon-beta på mRNA- och proteinnivå).

Det har tidigare observerats att virus med olika varianter av NS1 visar en varierad grad av sjukdomsframkallande förmåga hos minkar (*Mustela vison*). Vi undersökte material från tidigare infektionsstudier på mink där influensavirus som bar på NS1 av allel A verkade vara mer sjukdomsframkallande än virus med NS1 av allel B. En fråga som vi ställde oss var om det fanns strukturella skillnader mellan de två NS1-varianterna. Våra studier tyder på att interaktion mellan NS1 och olika cellulära komponenter förmedlas av det som kallas effektordomänen på proteinets C-terminal.

Sammantaget visar våra studier på den mångsidiga natur och återbekräftar den oumbärliga roll som NS1-proteinet spelar för influensavirus.

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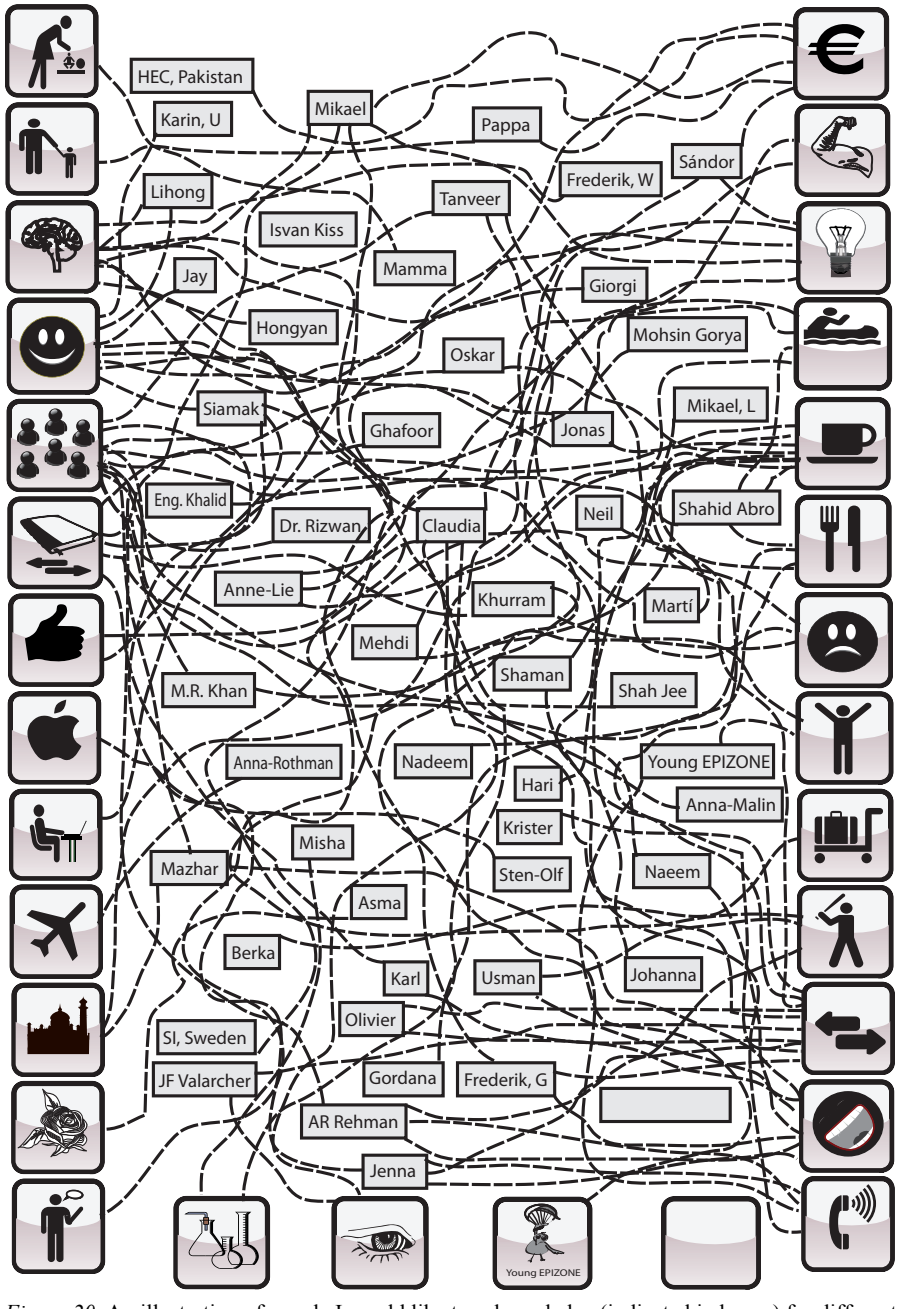

Figure 20. An illustration of people I would like to acknowledge (indicated in boxes) for different activities (indicated by icons). Missing people are welcome to fill in their name into the empty box and a suitable activity into the empty icon. If really interested, try to convert a dotted line to a solid line, starting from your name icon. By doing so, you will realize the complexity of signaling, a main object of this thesis.

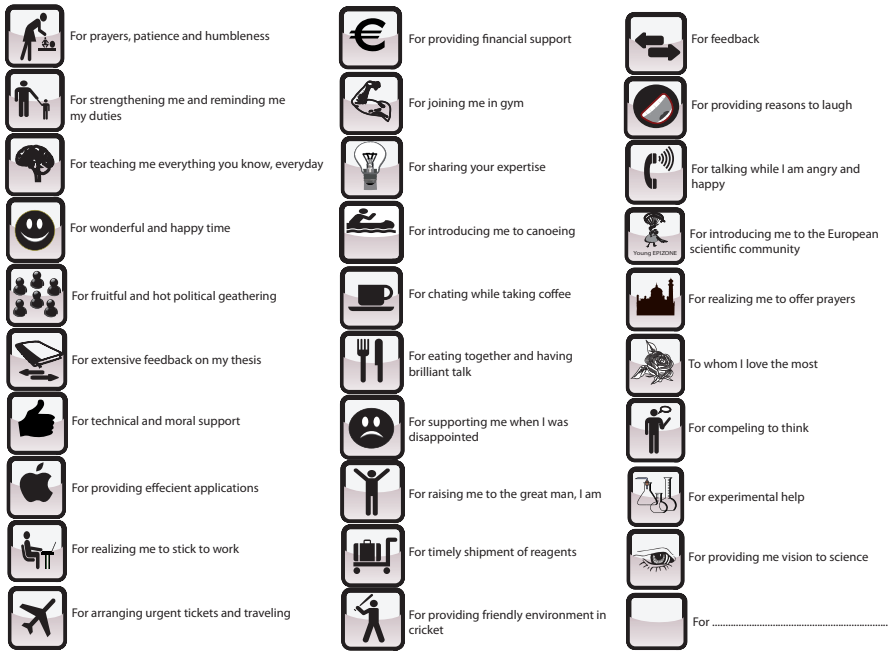

Figure 21. An explanation of the activities.