

## Review

## The multifunctional NS1 protein of influenza A viruses

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The non-structural (NS1) protein of influenza A viruses is a non-essential virulence factor that has multiple accessory functions during viral infection. In recent years, the major role ascribed to NS1 has been its inhibition of host immune responses, especially the limitation of both interferon (IFN) production and the antiviral effects of IFN-induced proteins, such as dsRNA-dependent protein kinase R (PKR) and 2'5'-oligoadenylate synthetase (OAS)/RNase L. However, it is clear that NS1 also acts directly to modulate other important aspects of the virus replication cycle, including viral RNA replication, viral protein synthesis, and general host-cell physiology. Here, we review the current literature on this remarkably multifunctional viral protein. In the first part of this article, we summarize the basic biochemistry of NS1, in particular its synthesis, structure, and intracellular localization. We then discuss the various roles NS1 has in regulating viral replication mechanisms, host innate/adaptive immune responses, and cellular signalling pathways. We focus on the NS1–RNA and NS1–protein interactions that are fundamental to these processes, and highlight apparent strain-specific ways in which different NS1 proteins may act. In this regard, the contributions of certain NS1 functions to the pathogenicity of human and animal influenza A viruses are also discussed. Finally, we outline practical applications that future studies on NS1 may lead to, including the rational design and manufacture of influenza vaccines, the development of novel antiviral drugs, and the use of oncolytic influenza A viruses as potential anti-cancer agents.

## Influenza A viruses

Influenza A viruses are enveloped viruses within the family *Orthomyxoviridae* and are further classified into subtypes depending upon their surface glycoproteins, haemagglutinin (HA) and neuraminidase (NA). They contain a single-stranded, negative sense, segmented RNA genome consisting of eight segments of viral RNA (vRNA), which encode 11 known proteins (Palese & Shaw, 2007). Influenza A viruses are important pathogens with worldwide prevalence. Their natural host is waterfowl; however, they also infect humans and other animals such as pigs, horses and various avian species (Webster *et al.*, 1992). It is this zoonotic characteristic that allows the generation of potentially pandemic strains. Three human pandemics occurred during the last century, with the 1918 'Spanish' influenza pandemic resulting in up to 40 million deaths (Simonsen *et al.*, 1997). Influenza viruses also cause seasonal epidemics, which are due to the acquisition of mutations in the viral surface glycoproteins. Disease severity caused by these strains is limited in the general population; however, they can be fatal in elderly patients

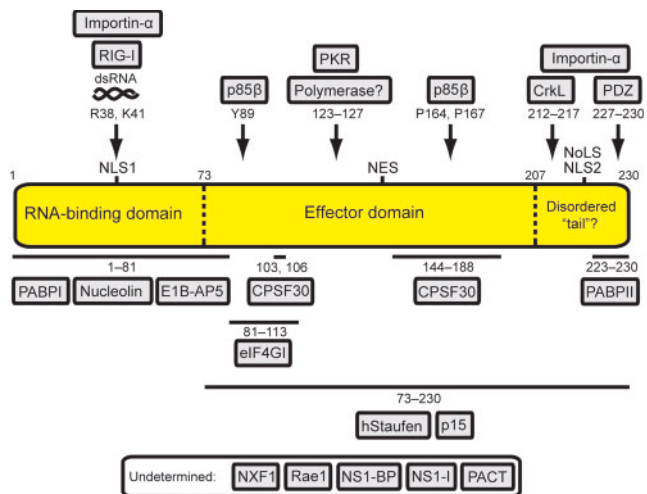
and those with underlying pulmonary or cardiac diseases (Barker & Mullooly, 1982).

To restrict virus proliferation, virus-infected cells usually mount a potent and diverse antiviral response (Randall & Goodbourn, 2008). Thus, to survive in nature, influenza A viruses have evolved multiple mechanisms to circumvent these defences. Some strategies are strain-specific, such as increased replication speed (Grimm *et al.*, 2007; Kurokawa *et al.*, 1999), or decreased sensitivity to host-cell antiviral effectors (Dittmann *et al.*, 2008). The viral NS1 protein is widely regarded as the common factor by which all influenza A viruses antagonize host immune responses (Egorov *et al.*, 1998; Garcia-Sastre *et al.*, 1998; Kochs *et al.*, 2007b). Indeed, mutant influenza A viruses unable to express NS1 only display high pathogenicity in mice lacking antiviral mediators such as STAT1 (Garcia-Sastre *et al.*, 1998), or the dsRNA-activated protein kinase, PKR (Bergmann *et al.*, 2000; Kochs *et al.*, 2007b). Thus, the available data strongly indicate that the major function of NS1 in current *in vivo* models is to antagonize IFN- $\alpha/\beta$ -mediated antiviral responses. However, NS1 is a multifunctional protein that performs a plethora of activities, which may additionally contribute towards efficient virus replication and virulence during infection. These include:

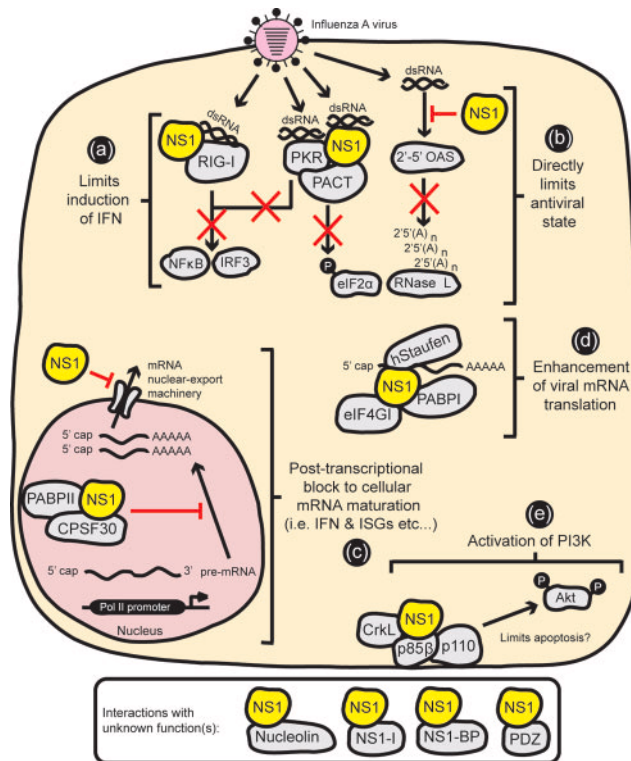
(i) temporal regulation of viral RNA synthesis; (ii) control of viral mRNA splicing; (iii) enhancement of viral mRNA translation; (iv) regulation of virus particle morphogenesis; (v) suppression of host immune/apoptotic responses; (vi) activation of phosphoinositide 3-kinase (PI3K); and (vii) involvement in strain-dependent pathogenesis. All of these functions of NS1 rely on its ability to participate in a multitude of protein–protein and protein–RNA interactions (summarized in Figs 1 and 2). Here, we review the various roles of NS1 during the replication cycle of influenza A viruses. We highlight the potential importance of each individual function and discuss how a single protein might have such a multifunctional nature.

### Synthesis and biochemistry of NS1

The NS1 protein of influenza A viruses is not a structural component of the virion, but is expressed at very high



**Fig. 1.** Schematic representation of the NS1 protein, together with its known interactors. NS1 (yellow) is 230–237 amino acids long depending upon the strain. The N-terminal 73 amino acids form a functional RNA-binding domain, whilst the effector domain predominantly mediates interactions with host-cell proteins. The final C-terminal ~20 amino acids may be natively unstructured. NS1 contains two nuclear localization sequences (NLS1 and NLS2), and a nuclear export sequence (NES). A nucleolar localization sequence (NoLS) has been reported for some strains, and is concomitant with NLS2. Residues involved in RNA-binding (Arg-38 [R38] and Lys-41 [K41]) are implicated in the inhibition of OAS/RNase L, Jun N-terminal kinase, and RIG-I. Additionally, NS1 contains binding sites for: poly(A)-binding protein I (PABPI), p85β, importin-α, nucleolin, NS1-BP, eIF4GI, hStaufen, NS1-I, PKR, PACT, CPSF30, poly(A)-binding protein II (PABPII), Crk/CrkL, PDZ domain-containing proteins, the viral polymerase, and components of the cellular mRNA nuclear export machinery (E1B-AP5, p15, NXF1, and Rae1). The interactions of NS1 with nucleolin, and NS1-I (17β-oestriol dehydrogenase precursor protein) (Wolff *et al.*, 1996) have so far been poorly characterized, but all other interactions are discussed in greater detail within the main text.



**Fig. 2.** Schematic diagram of the multiple functions of NS1 within infected cells. (a) Pre-transcriptional limitation of IFN-β induction. (b) Inhibition of the antiviral properties of PKR and OAS/RNase L. (c) Post-transcriptional block to processing and nuclear export of all cellular mRNAs. (d) Enhancement of viral mRNA translation. (e) Activation of PI3K. Interactions with unknown consequences and/or localizations are detailed in the lower box.

levels in infected cells (Krug & Etkind, 1973; Palese & Shaw, 2007). It is encoded on a collinear mRNA derived from vRNA segment eight, which upon splicing results in the synthesis of the nuclear export protein mRNA (NEP, previously termed NS2) (Inglis *et al.*, 1979; Lamb & Choppin, 1979). Both mRNA species share 56 nucleotides at the 5' end, resulting in NS1 and NEP sharing 10 N-terminal amino acids (Lamb & Lai, 1980). In infected cells, the steady-state amount of spliced NEP mRNA is only approximately 10% that of unspliced NS1 mRNA (Lamb *et al.*, 1980). As described below, regulation of splicing is controlled, in part, by the viral NS1 protein itself (Garaigorta & Ortin, 2007) and may represent a mechanism for autoregulation of protein levels within infected cells.

NS1 has a strain-specific length of 230–237 aa, and an approximate molecular mass of 26 kDa (Palese & Shaw, 2007). However, naturally occurring NS1 proteins with C-terminal truncations (~15–30 aa) are not uncommon (Suarez & Perdue, 1998). Furthermore, sequence analysis shows that, during the 1940s, the 230 aa long NS1 protein of circulating human H1N1 viruses gained a 7 aa

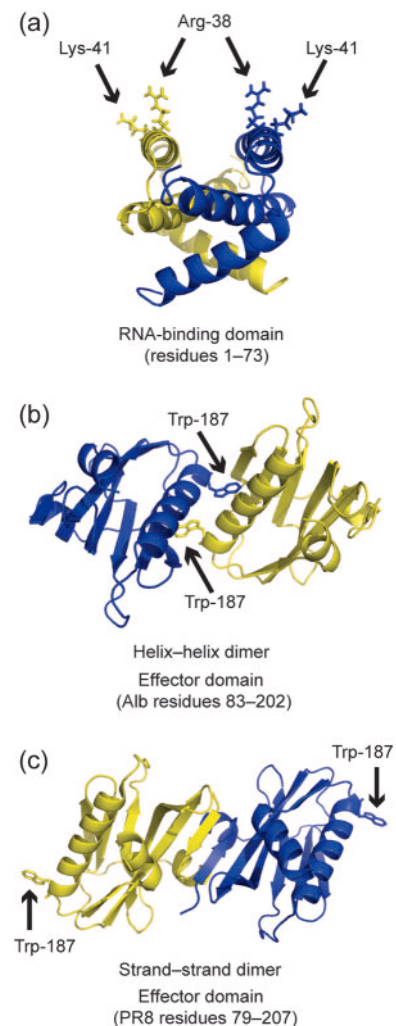
C-terminal extension via a single nucleotide mutation (Fig. 4). This extension was subsequently retained in human H1N1, H2N2 and H3N2 viruses until the 1980s, when both co-circulating H1N1 and H3N2 viruses lost the extension via reversion of the original mutation. The significance of the extension and why it was subsequently lost is not entirely clear, although it has recently been functionally implicated in the nuclear and nucleolar localization of NS1 (Melen *et al.*, 2007). Given the variability in NS1 length, the general importance of reported interactions between the NS1 C terminus and various cellular proteins, such as poly(A)-binding protein I (PABPI) and PDZ domain-containing proteins, remains unclear.

Phylogenetic analysis of NS1 amino acid sequences has also indicated that NS1 proteins can be divided into two major groups, originally termed alleles A and B (Treanor *et al.*, 1989; Ludwig *et al.*, 1991). A number of NS1 proteins from avian influenza viruses together with those of all human, swine and equine influenza viruses are described as allele A NS1 proteins, whereas those of allele B are exclusively from avian viruses. The level of homology within each allele is 93–100%; however, between alleles it can be as little as 62%. When a recombinant human virus containing an allele B NS1 protein was used to infect squirrel monkeys, there was a decrease in the ability of the virus to replicate in the respiratory tract compared with wild-type (wt) virus (Treanor *et al.*, 1989). This suggested that allele A NS1 proteins have a replicative advantage in mammalian hosts. Further analysis revealed that allele A NS1 proteins are under continual selection pressure to mutate, whereas those of allele B are not (Ludwig *et al.*, 1991). It is possible that allele B NS1 proteins represent the archaic version of this protein and that, after entering the human influenza virus population via reassortment events, NS1 has been under a strong selection pressure to mutate, giving rise to the allele A NS1 proteins. The large degree of evolutionary divergence between the two alleles may indicate that there are significant functional constraints on NS1 proteins between host species. The significance of NS1 alleles for the virulence and pathogenicity of certain influenza viruses is not clear; however, the majority of highly pathogenic avian influenza viruses isolated from humans have contained an allele A NS1 protein (Zohari *et al.*, 2008).

Post-translational modification of NS1 proteins may also be a strain-specific virus polymorphism. Indeed, phosphorylation of NS1 has been reported for only some influenza A viruses (Petri *et al.*, 1982), and at least two distinct sites of modification have been proposed based upon biochemical and structural work: Ser-195 and Thr-197 (Bornholdt & Prasad, 2006; Privalsky & Penhoet, 1981). However, phosphorylation of these two residues has yet to be experimentally confirmed. NS1 phosphorylation appears to occur rapidly after translation, within the cell nucleus (Privalsky & Penhoet, 1981). It is still unknown if any physiological role for NS1 phosphorylation exists.

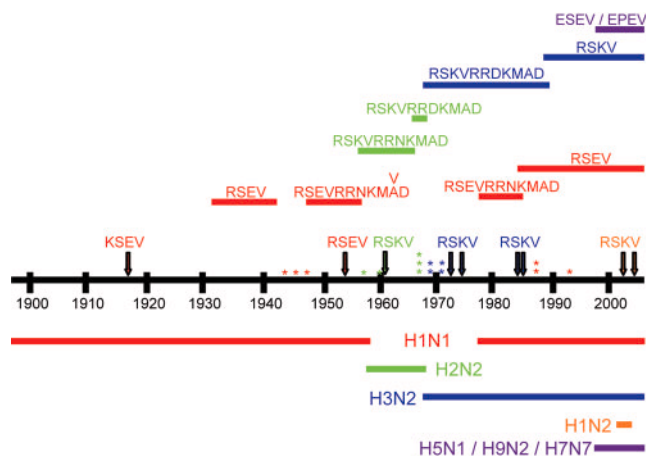
## Structure of the NS1 protein

NS1 is notionally divided into two distinct functional domains: an N-terminal RNA-binding domain (residues 1–73) (Fig. 3a), which *in vitro* binds with low affinity to several RNA species in a sequence independent manner (Chien *et al.*, 2004; Hatada & Fukuda, 1992; Qian *et al.*, 1995), and a C-terminal ‘effector’ domain (residues 74–



**Fig. 3.** Structure of the influenza A virus NS1 protein. (a) Cartoon ribbon representation of the dimeric RNA-binding domain (A/Udorn/72 [H3N2]; residues 1–73). Arg-38 and Lys-41, which are critical for RNA-binding, are highlighted. (b) and (c) Cartoon ribbon representations of the two proposed effector domain dimerization conformations. (b) A/Duck/Albany/76 [H12N5] residues 83–202 (helix–helix dimer). (c) A/Puerto Rico/8/34 [H1N1] residues 79–207 (strand–strand dimer). Trp-187, which has been shown experimentally to be essential for dimerization of the avian NS1 protein effector domain, is highlighted in both structures. For all structures (a–c), monomers are coloured blue and yellow. Images were prepared using MacPyMol (PDB files: 1NS1, 2GX9, 3D6R).





**Fig. 4.** The C-terminal amino acid sequences of human NS1 proteins. A schematic diagram displaying the C-terminal amino acid residues 227–230/237 of the NS1 protein from all human influenza viruses isolated since 1918. The circulating human influenza A virus subtypes are shown below the timeline. Colours represent different circulating subtypes: red, H1N1; green, H2N2; blue, H3N2; orange, H1N2; and purple, human H5N1/H9N2/H7N7 viruses derived from avian sources. C-terminal sequences from amino acid 227 are shown above the timeline with the first isolate from 1918 displaying the sequence KSEV. During the late 1940s the NS1 protein was extended by seven amino acids (RRNKMAD) and this extension was subsequently retained in all human influenza virus subtypes until it was lost from both the co-circulating H1N1 and H3N2 viruses in the late 1980s. During this time a number of influenza viruses were isolated containing naturally occurring C-terminal NS1 truncations, indicated by asterisks. The avian derived sequences of ESEV and EPEV fit the consensus sequence of a PDZ domain ligand (PL).

230) (Fig. 3b, c), which predominantly mediates interactions with host-cell proteins, but also functionally stabilizes the RNA-binding domain (Wang *et al.*, 2002). Full-length NS1 likely exists as a homodimer, with both the RNA-binding and effector domains contributing to multimerization (Nemeroff *et al.*, 1995).

The RNA-binding domain alone is a symmetrical homodimer with each monomer consisting of three  $\alpha$ -helices (Chien *et al.*, 1997; Liu *et al.*, 1997). Dimerization is essential for binding dsRNA (Wang *et al.*, 1999) and the stoichiometry of dimer:dsRNA is 1:1 (Chien *et al.*, 2004). Two identical helices from each NS1 monomer contribute towards dsRNA-binding by forming antiparallel 'tracks' on either side of a deep cleft (Liu *et al.*, 1997). The 'tracks' consist of conserved basic and hydrophilic residues that appear to form complementary contacts with the polyphosphate backbone of dsRNA (Yin *et al.*, 2007). Residues in NS1 that mediate this interaction, either directly or via improving complex stability, include Thr-5, Pro-31, Asp-34, Arg-35, Arg-38, Lys-41, Gly-45, Arg-46 and Thr-49 (Wang *et al.*, 1999; Yin *et al.*, 2007). It should be noted that

alanines are commonly substituted for both Arg-38 and Lys-41 in many experimental studies in order to abrogate the RNA-binding activity of NS1 (Fig. 3a).

Crystallographic studies revealed that the C-terminal effector domain of both a human and avian NS1 protein (residues 74–230) can independently homodimerize, with each monomer consisting of seven  $\beta$ -strands and three  $\alpha$ -helices (Bornholdt & Prasad, 2006; Hale *et al.*, 2008a). Within each monomer, the  $\beta$ -strands form a twisted, crescent-like, anti-parallel  $\beta$ -sheet around a long, central  $\alpha$ -helix. There is currently no structure available for the C-terminal ~25 amino acids of NS1, a region which is involved in many strain-specific functions (Fig. 1). It is possible that this stretch of NS1 is intrinsically disordered, and thus may only adopt an ordered structure upon binding the appropriate ligand. Such intrinsic disorder is noteworthy given the C-terminal variability in the lengths of many NS1 proteins.

The precise dimeric assembly of the NS1 effector domain has yet to be fully established, as two dimer conformations have recently been proposed: strand–strand (Bornholdt & Prasad, 2006), and helix–helix (Hale *et al.*, 2008a) (Fig. 3b, c). Amino acids involved at both dimer interfaces appear reasonably well-conserved; however, biochemical evidence indicates that Trp-187 (a residue located at the helix–helix interface) is essential for dimerization of an avian NS1 effector domain in solution (Hale *et al.*, 2008a). This suggests that the helix–helix dimer, at least for the avian NS1 protein used, is likely to be biologically relevant (Fig. 3b, c). It should be noted that the published human effector domain structure is from an allele A NS1 protein (Bornholdt & Prasad, 2006), whilst that published for an avian influenza virus is from an allele B NS1 protein (Hale *et al.*, 2008a). Thus, it may be that the structural differences observed in these two studies are NS1 allele-specific. Interestingly, very recent data suggest that a third dimeric state of the NS1 effector domain also probably exists (PDB ID: 2RHK). As a full-length NS1 protein structure has yet to be determined, the actual conformation of the complete NS1 dimer may differ significantly from that already published for the two individual domains. Alternatively, it may be that NS1 has various dimeric states that occur in either a strain- or ligand-specific manner, a mode of action that would clearly contribute to the multifunctional nature of NS1.

### Intracellular localization of NS1

A number of studies have reported various intra-cellular localization patterns for NS1. In infected cells, the distribution of NS1 may be dependent on several factors, including: (i) virus strain; (ii) expression level of NS1; (iii) cell fixation procedure; (iv) cell type used; (v) cell polarity; and (vi) time post-infection (Li *et al.*, 1998; Newby *et al.*, 2007). Nevertheless, in virus-infected cells NS1 predominantly localizes to the nucleus, but a significant proportion can also be found in the cytoplasm (Greenspan *et al.*, 1988; Newby *et al.*, 2007), particularly at later times post-

infection (Garaigorta *et al.*, 2005; Melen *et al.*, 2007). Within the nucleus, NS1 has been shown to localize to ND10 structures (Sato *et al.*, 2003).

Depending on the viral strain, NS1 contains one or two nuclear localization sequences (NLS) (Fig. 1) (Greenspan *et al.*, 1988), which mediate the active nuclear import of NS1 via binding to cellular importin- $\alpha$  (Melen *et al.*, 2007). As such, translocation of NS1 into the nucleus is extremely rapid (Privalsky & Penhoet, 1981). NLS1 is highly conserved, monopartite, and involves three residues also involved in binding dsRNA (Arg-35, Arg-38 and Lys-41). In contrast, the bipartite NLS2 comprises specific amino acids (Lys-219, Arg-220, Arg-231 and Arg-232) found at the C-termini of some NS1 proteins (Melen *et al.*, 2007). As NLS2 is absent from the NS1 proteins of a large number of virus strains, it is difficult to ascribe a function to this sequence with regard to viral replication. Concurrent with NLS2 is a functional nucleolar localization signal (NoLS), which includes additional basic residues (Arg-224 and Arg-229) (Melen *et al.*, 2007) (Fig. 1). Interestingly, NS1 has recently been shown to interact with nucleolin (Murayama *et al.*, 2007), a major multifunctional nucleolar protein (Fig. 1). Despite this, the nucleolar function of NS1 is unknown; however, a mutant influenza A virus expressing a truncated NS1 protein unable to localize to nucleoli was not attenuated for replication in tissue culture (Melen *et al.*, 2007).

Cytoplasmic localization of a subpopulation of NS1 is potentially regulated by three mechanisms. It is possible that newly synthesized NS1 is initially sequestered in the cytoplasm by a cellular or viral binding partner that acts by masking the NLS. Alternatively, it has been reported that a latent nuclear export signal (NES) in NS1 causes its nucleocytoplasmic transport (Li *et al.*, 1998). The NES lies within residues 138–147, requires leucines at positions 144 and 146, and is normally ‘masked’ by residues 148–161 which lie adjacent to it (Li *et al.*, 1998). Thus, during infection the NES probably requires ‘unmasking’ in the nucleus for cytoplasmic localization of NS1 to occur. Additionally, it is possible that competition between the NLS and NES exists, such that the NES only becomes dominant after the NLS itself has also been masked by a nuclear NS1-binding partner. The molecular events that govern these three putative mechanisms have yet to be established, but it is likely that specific cellular factors play key roles in determining the intracellular localization of NS1. For example, regulation by phosphorylation of NS1 is a possibility, given that mutation of Ser-195, a potential phosphorylation site in NS1 (Bornholdt & Prasad, 2006), appears to contribute to the nuclear retention of NS1 (Garaigorta *et al.*, 2005). Varied intracellular distribution of NS1 during infection may be essential for its ability to perform different functions.

### Role of NS1 in regulating splicing of segment eight mRNAs

As discussed above, the influenza A virus vRNA segment eight encodes two proteins: NS1, via the full-length

collinear mRNA transcript, and NEP, via a spliced mRNA. Expression *in trans* of NS1 has been shown to inhibit the splicing of vRNA segment eight encoded pre-mRNA both *in vitro* and *in vivo* (Fortes *et al.*, 1994; Lu *et al.*, 1994). Although the segment eight pre-mRNA is able to form spliceosomes, the subsequent catalytic steps appear to be inhibited by NS1 in a process requiring specific basic residues within the RNA-binding domain (Lu *et al.*, 1994). This has recently been confirmed by experiments in which NS1 was synthesized from functional vRNPs, resulting in the inhibition of segment eight mRNA splicing (Garaigorta & Ortin, 2007). Such inhibition required the N-terminal region of NS1, but appeared independent of RNA-binding. It was also found that NS1 specifically downregulated nuclear export of its own mRNA by a process requiring NS1 RNA-binding activity (Alonso-Caplen & Krug, 1991; Alonso-Caplen *et al.*, 1992; Garaigorta & Ortin, 2007). The biological reasons for this are currently unknown.

The mechanism by which NS1 inhibits segment eight mRNA splicing has yet to be fully established. However, it is possible that a novel cellular ~70 kDa NS1-binding protein, termed NS1-BP, may be involved. NS1-BP was initially identified as an interaction partner for NS1 in yeast two-hybrid screens (Wolff *et al.*, 1998). Given that NS1-BP predominantly co-localizes with the spliceosome assembly factor SC35, it was suggested that this protein is normally involved in cellular mRNA splicing. During influenza A virus infection, the cytoplasmic fraction of NS1-BP redistributes to the nucleus, and apparently co-localizes with NS1 (Wolff *et al.*, 1998). Similar immunofluorescence experiments have demonstrated that NS1 expression causes redistribution of cellular splicing factors in nuclei of infected cells (Fortes *et al.*, 1995). These reports, together with findings that NS1 can bind and disrupt complexes between specific small nuclear RNAs (snRNAs) (essential components of spliceosomes), highlight likely biological interactions between NS1 and the cellular mRNA splicing machinery (Lu *et al.*, 1994; Qiu *et al.*, 1995; Wang & Krug, 1998).

### Effects of NS1 expression on virus-specific RNA and protein synthesis

#### Temporal regulation of viral RNA synthesis

Temperature-sensitive mutations in vRNA segment eight have been shown to reduce levels of all vRNA segments in infected cells, without affecting the total amounts of mRNA or cRNA (vRNA template) (Wolstenholme *et al.*, 1980). This observation was confirmed using viruses expressing NS1 proteins with C-terminal truncations; however, such a phenotype appeared to be strain-specific (Falcon *et al.*, 2004). These reports gave the first indications that NS1 plays a role in controlling viral RNA replication during infection. Substitution of residues 123 and 124 in NS1 was shown to prevent the NS1-mediated binding and inhibition of the dsRNA-activated antiviral protein kinase, PKR (Min *et al.*, 2007). Thus, this mutant

induced PKR activation and exhibited reduced viral protein synthesis at late times post-infection. However, the mutant virus was not attenuated in tissue-culture due to enhanced vRNA synthesis at early times post-infection, which resulted in increased viral mRNA transcription and early viral protein synthesis. Given that these effects were apparently independent of PKR, it was speculated that the same residues in wt NS1 normally act to temporally regulate vRNA synthesis during infection. At the mechanistic level, NS1 has previously been reported to interact with the viral polymerase complex (Marion *et al.*, 1997b) and has a high affinity for dsRNA in the form of vRNA-like panhandle structures (Hatada & Fukuda, 1992; Hatada *et al.*, 1997). Indeed, evidence is accumulating for a number of functional interactions between NS1 and replicating RNPs during infection (Twu *et al.*, 2007).

### Selective translation of viral mRNAs

It has been reported that, during influenza A virus infection, there is selective translation of viral mRNAs over cellular mRNAs, a process possibly mediated by sequences in the 5'UTR of viral mRNAs (Garfinkel & Katze, 1993). A number of proteins appear to bind the 5'UTR of viral mRNAs, including NS1 (Park & Katze, 1995), and many studies have attempted to determine the effect of NS1 expression on viral protein synthesis. It was reported that NS1 increases translation initiation of viral mRNAs within transfected cells, but does not affect the translation of non-viral mRNAs (de la Luna *et al.*, 1995). It was shown that the 5'UTR sequences of viral mRNAs were responsible for this selective translation. Similarly, Enami *et al.* (1994) demonstrated that NS1 does not affect viral mRNA transcription, but rather enhances translation in a viral 5'UTR-dependent manner. However, unlike de la Luna *et al.*, these authors were unable to observe an effect of NS1 on translation of mRNAs containing the 5'UTR from vRNA segment eight (Enami *et al.*, 1994). Thus, it is possible that NS1-enhanced viral mRNA translation is vRNA segment-specific.

Studies using temperature-sensitive influenza A viruses with mutations in NS1 demonstrated a reduction in viral protein synthesis (Hatada *et al.*, 1990). It has also been reported that viral protein synthesis in Madin-Darby canine kidney (MDCK) or Madin-Darby bovine kidney cells (MDBK) cells infected with mutant viruses encoding C-terminally truncated NS1 proteins is significantly reduced compared with that in wt virus-infected cells (Egorov *et al.*, 1998; Enami & Enami, 2000). However, these observations may be cell-type specific, as viral protein levels do not differ much between truncated-NS1 and wt virus-infected Vero cells (Egorov *et al.*, 1998; Salvatore *et al.*, 2002). Thus, the normal inhibitory effect of NS1 on host antiviral responses, which are severely impaired in Vero cells, may indirectly contribute towards efficient viral mRNA translation. For example, in IFN-competent cells, IFN induced by viruses encoding truncated NS1 proteins

could stimulate activation of antiviral proteins that lead to a reduction in viral protein synthesis.

Marion *et al.* (1997a) reported that the N-terminal 113 residues of NS1 were required for direct stimulation of viral mRNA translation in transfected COS-1 cells. Although binding of NS1 to the 5'UTR of viral mRNAs may correlate with NS1-mediated enhancement of viral protein synthesis, it is likely that interactions between NS1 and cellular proteins are also required for this effect. During infection, viral mRNAs were shown to be efficiently translated even in the presence of low levels of the cellular eIF4F cap-binding complex (Feigenblum & Schneider, 1993). It was subsequently reported that residues 81–113 of NS1 can interact with eIF4GI, the large subunit of eIF4F (Aragon *et al.*, 2000). Given that mutant NS1 proteins unable to bind eIF4GI are also defective in enhancing viral mRNA translation (Aragon *et al.*, 2000; Marion *et al.*, 1997a), it may be that NS1 normally recruits eIF4GI, and thus eIF4F, to the 5'UTR of viral mRNAs, thereby preferentially increasing viral translation. Furthermore, the N-terminal 81 aa of NS1 have been shown to interact with PABPI, a known interactor of eIF4GI, independently of RNA (Burgui *et al.*, 2003) and mapping studies suggested that a heterotrimeric NS1–PABPI–eIF4GI complex might be possible (Aragon *et al.*, 2000; Burgui *et al.*, 2003). In addition, NS1 can interact with and cause the redistribution of hStaufen, a dsRNA- and tubulin-binding protein related to PKR (Falcon *et al.*, 1999). As hStaufen normally contributes towards microtubular transport of cellular mRNAs to sites of enhanced translation, such as polysomes, it may be that interaction with NS1 promotes efficient viral mRNA translation. In support of this, a proportion of both NS1 and hStaufen have previously been found to co-fractionate with cytoplasmic polysomes in influenza A virus-infected cells (Falcon *et al.*, 1999; Krug & Etkind, 1973). Thus, to increase viral protein synthesis, NS1 appears to interact with viral 5'UTRs, hStaufen, eIF4GI and PABPI to recruit viral mRNAs (at the expense of cellular mRNAs) to multi-protein translation-initiation complexes (Figs 1 and 2). It is still not clear if the observed binding of NS1 to poly(A) sequences (Qiu & Krug, 1994) has any role to play in viral mRNA translation.

### NS1 and the host innate immune response

The host innate interferon (IFN) response is a potent antiviral mechanism that can limit virus replication and spread. Type I IFNs, such as IFN- $\alpha$  or IFN- $\beta$ , are soluble cytokines that are synthesized and secreted by cells in response to virus infection, and act in both an autocrine and paracrine manner to upregulate the expression of >300 IFN-stimulated antiviral genes (Randall & Goodbourn, 2008). Although a major function of NS1 is to antagonize host innate immune responses, as detailed below, the mechanisms and targets for NS1 are varied and strain-specific (Hayman *et al.*, 2006; Kochs *et al.*, 2007a; Twu *et al.*, 2007).



### NS1 is essential for antagonizing IFN- $\alpha/\beta$ -dependent responses

The generation of influenza A viruses unable to express NS1 (delNS1), or that express truncated forms of NS1, revealed the crucial role for this protein in counteracting the host IFN response (Egorov *et al.*, 1998; Garcia-Sastre *et al.*, 1998; Kochs *et al.*, 2007b). DelNS1 viruses induce large amounts of IFN in infected cells, and are consequently attenuated in IFN- $\alpha/\beta$ -competent systems. Not surprisingly, delNS1 viruses replicate more efficiently in IFN- $\alpha/\beta$ -deficient tissues such as Vero cells; however, virus titres are approximately 10–100-fold lower than for wt (Garcia-Sastre *et al.*, 1998; Kochs *et al.*, 2007b). This may be due to effects of cytokines other than IFN- $\alpha/\beta$  and cytokine-independent or IRF3-dependent responses. The lack of other 'IFN-independent' functions of NS1 also probably contributes to this attenuated phenotype.

### NS1 limits IFN- $\beta$ production

A number of studies have attempted to demonstrate how NS1 acts to limit the production of IFN- $\beta$ . Although such reports have often seemed contradictory, it is now apparent that the IFN-antagonistic properties of different NS1 proteins are strain-specific (Geiss *et al.*, 2002; Hayman *et al.*, 2006; Kochs *et al.*, 2007a). Current evidence indicates that NS1 proteins may have acquired the ability to limit IFN- $\beta$  induction by both pre-transcriptional (cytoplasmic) and/or post-transcriptional (nuclear) processes. Thus, it has been proposed that the existence and evolution of two such synergistic anti-IFN mechanisms could increase the capacity of some influenza A viruses to adapt to new hosts (Kochs *et al.*, 2007a). In this regard, it is also possible that certain virus strains may have lost one or other of these mechanisms, either naturally or during laboratory passage. For example, the NS1 protein of A/Puerto Rico/8/34 (PR8) clearly limits pre-transcriptional events associated with IFN- $\beta$  induction, but unlike many other NS1 proteins is apparently unable to block post-transcriptional processing of IFN- $\beta$  pre-mRNAs (Hayman *et al.*, 2006; Kochs *et al.*, 2007a). The two strategies by which NS1 proteins appear to intercede with the IFN-induction pathway are outlined below.

**(i) Pre-transcriptional limitation of IFN- $\beta$  induction by NS1.** Studies using PR8/NS1 demonstrated that this protein prevents dsRNA- and virus-mediated activation of the IRF-3, NF $\kappa$ B and c-Jun/ATF-2 transcription factors, which are otherwise essential for IFN- $\beta$  induction (Ludwig *et al.*, 2002; Talon *et al.*, 2000a; Wang *et al.*, 2000). Such inhibition was shown to occur pre-transcriptionally, and to require two residues in NS1 that strongly contribute to RNA-binding: Arg-38 and Lys-41 (Talon *et al.*, 2000a) (Figs 1 and 3a). It was originally postulated that PR8/NS1 may act by sequestering aberrant viral dsRNA away from host-encoded sensors (Talon *et al.*, 2000a). However, dsRNA has yet to be detected in influenza A virus-infected cells (Weber *et al.*, 2006), and it is now evident

that unique components of the influenza virus ssRNA genome can be directly recognized by the cytoplasmic pathogen sensor, RIG-I (Pichlmair *et al.*, 2006). As such, recent work now indicates that PR8/NS1 may mediate its pre-transcriptional block on IFN- $\beta$  induction by forming a complex with RIG-I (Guo *et al.*, 2007; Mibayashi *et al.*, 2007; Opitz *et al.*, 2007; Pichlmair *et al.*, 2006). Consistent with initial observations (Talon *et al.*, 2000a), co-precipitation of RIG-I with PR8/NS1 is largely dependent upon Arg-38 and Lys-41 in PR8/NS1 (Pichlmair *et al.*, 2006), suggesting that these two residues are involved in a potential protein–protein interaction, or that RNA acts as an intermediary component (Fig. 2). Indeed, direct binding of PR8/NS1 to RIG-I has yet to be demonstrated (Mibayashi *et al.*, 2007), and the presence of 5'-triphosphorylated ssRNA clearly enhances stability of PR8/NS1–RIG-I complexes (Pichlmair *et al.*, 2006). Intriguingly, PR8/NS1 has also been reported to block the function of both a constitutively active RIG-I construct lacking its RNA-binding helicase domain, and IPS-1, a downstream effector of RIG-I (Mibayashi *et al.*, 2007). These data indicate that PR8/NS1-mediated inhibition of the RIG-I/IPS-1 signalling pathway probably occurs by a complex molecular mechanism that has yet to be fully established.

**(ii) Post-transcriptional limitation of IFN- $\beta$  induction by NS1.** It is not clear if co-precipitation of RIG-I is a feature exhibited by all influenza A virus NS1 proteins. Indeed, comparative studies between the NS1 proteins of PR8 and A/Texas/36/91 (Tx/NS1) revealed that Tx/NS1 interacts relatively poorly with RIG-I, and is partially limited in its ability to prevent IRF-3 dimerization/activation (Kochs *et al.*, 2007a). Despite this, Tx/NS1 completely blocks IFN- $\beta$  mRNA synthesis during infection (Kochs *et al.*, 2007a). The ability of NS1 to prevent the nuclear post-transcriptional processing of RNA polymerase II transcripts appears to be a common additional strategy that many influenza A virus strains use to limit IFN- $\beta$  production (Fortes *et al.*, 1994; Hayman *et al.*, 2006, 2007; Kochs *et al.*, 2007a; Lu *et al.*, 1994; Nemeroff *et al.*, 1998; Noah *et al.*, 2003; Qiu & Krug, 1994; Shimizu *et al.*, 1999; Twu *et al.*, 2007).

General inhibition of nucleo-cytoplasmic transport of all poly(A)-containing mRNAs was one of the first functions ascribed to NS1 (Fortes *et al.*, 1994; Qiu & Krug, 1994). At the time, it was speculated that global nuclear retention of cellular mRNAs by NS1 might provide a pool of cap-donors for the viral polymerase complex, thus increasing priming of viral mRNA transcription. However, it is now apparent that blocking cellular mRNA processing and transport may be an effective means to limit a number of host-cell processes, including the innate antiviral response. Given that NS1 does not prevent nuclear export of RNAs lacking poly(A) sequences, it was suggested that direct binding of NS1 to the 3' poly(A) tail of mRNAs was the mechanism by which this inhibition occurred (Qiu & Krug, 1994). However, viral mRNAs are not prevented from

leaving the nucleus of infected cells, despite them having a poly(A) tail. Therefore, interactions between NS1 and proteins directly involved in mRNA maturation and nucleo-cytoplasmic transport may play the greater and more specific role in cellular mRNA export inhibition.

Influenza virus A/Udorn/72 (Ud) has been extensively used to model the nuclear inhibition of cellular pre-mRNA processing by NS1. The C-terminal effector domain of Ud/NS1 binds directly to two zinc-finger regions in the 30 kDa subunit of cleavage and polyadenylation specificity factor (CPSF30) (Nemeroff *et al.*, 1998; Noah *et al.*, 2003; Twu *et al.*, 2006) and interacts with poly(A)-binding protein II (PABPII) (Chen *et al.*, 1999). Binding to PABPII requires residues 223–237 of Ud/NS1 (Li *et al.*, 2001), whilst binding to CPSF30 appears to require Phe-103, Met-106, Leu-144 and residues 184–188 of Ud/NS1 (Kochs *et al.*, 2007a; Li *et al.*, 2001; Noah *et al.*, 2003; Twu *et al.*, 2006, 2007) (Figs 1 and 2). Glu-96 may also be functionally significant (Shimizu *et al.*, 1999). PR8/NS1, which is unable to block the processing of RNA polymerase II transcripts, is unable to interact with CPSF30 due to amino acid substitutions at residues 103 and 106 (Kochs *et al.*, 2007a). The Ud/NS1–CPSF30 complex is thought to prevent CPSF30 from binding cellular pre-mRNAs, thereby inhibiting normal cleavage and polyadenylation of the 3'-end of host-cell mRNAs (Nemeroff *et al.*, 1998). As polyadenylation of influenza A virus mRNAs is independent of cellular 3'-end processing factors (Palese & Shaw, 2007), viral mRNAs are not affected by CPSF30 inhibition. Furthermore, the interaction of Ud/NS1 with PABPII may specifically block the nuclear export of fully processed mRNAs that partially escape 3'-end formation inhibition (Chen *et al.*, 1999). A recent study has also proposed a third method by which NS1 proteins may cause nuclear retention of host-cell mRNAs: NS1 appears to form an inhibitory complex with components of the cellular mRNA nuclear export machinery, specifically NXF1, p15, Rae1, E1B-AP5 and Nup98 (Satterly *et al.*, 2007) (Fig. 1). Viral mRNAs must overcome this global block on nucleo-cytoplasmic transport; however, it is still unclear how this occurs.

Although the multiple strategies by which NS1 inhibits host-cell mRNA processing seem somewhat mutually redundant, there may be unidentified benefits to the virus of efficiently targeting this cellular process. For example, some NS1 proteins have been reported to limit host-cell gene expression in response to IFN- $\alpha$  and tumour necrosis factor (TNF)- $\alpha$  (Geiss *et al.*, 2002; Hayman *et al.*, 2006, 2007; Kochs *et al.*, 2007a; Seo *et al.*, 2002), thus rendering the virus less sensitive to the antiviral effects of these cytokines. Ud viruses expressing NS1 proteins unable to bind CPSF30 have been shown to induce large amounts of both IFN- $\beta$  and cytokine-independent antiviral mRNAs (Noah *et al.*, 2003). Consequently, these mutant viruses are attenuated in both IFN- $\alpha/\beta$ -competent and IFN- $\alpha/\beta$ -deficient cells (Noah *et al.*, 2003; Twu *et al.*, 2006). Therefore the functional consequences of inhibiting host-

cell mRNA processing extend far beyond sole antagonism of IFN- $\beta$  production.

### NS1 limits the activity of PKR and OAS

NS1 can directly block the function of two cytoplasmic antiviral proteins: 2'-5'-oligoadenylate synthetase (OAS) (Min & Krug, 2006), and the dsRNA-dependent serine/threonine protein kinase R (PKR) (Min *et al.*, 2007) (Fig. 2). Both OAS/RNase L and PKR are key regulators of viral transcription/translation processes, but play additional roles in other innate defences such as IFN- $\beta$  induction and the host apoptotic response (Garcia *et al.*, 2006; Silverman, 2007).

OAS is activated by dsRNA, a putative by-product of viral replication, and polymerizes ATP into 2'-5' oligoadenylate chains. These chains cause dimerization and activation of the latent RNase, RNase L, which inhibits virus replication by degradation of RNA (Silverman, 2007). Data indicate that a predominant function of the NS1 RNA-binding domain is to out-compete OAS for interaction with dsRNA, thereby inhibiting this host antiviral strategy (Min & Krug, 2006). Given the role of RNase L in augmenting the production of IFN- $\beta$  (Silverman, 2007), it is possible that NS1-mediated OAS inactivation also contributes to suppression of IFN- $\beta$  synthesis (Donelan *et al.*, 2003; Talon *et al.*, 2000a).

dsRNA also binds and activates PKR, thereby releasing PKR auto-inhibition. A major substrate for activated PKR is the eukaryotic translation initiation factor 2 $\alpha$  (eIF2 $\alpha$ ), the phosphorylation of which leads to a reduction in both cellular and viral protein synthesis (Garcia *et al.*, 2006). *In vitro* experiments initially indicated that NS1 may also compete with PKR for binding dsRNA (Hatada *et al.*, 1999; Lu *et al.*, 1995). However, an RNA-binding defective NS1 protein efficiently limited PKR activation in response to dsRNA or PACT, a protein activator of PKR (Li *et al.*, 2006a). Furthermore, NS1 has been shown to interact with PKR in a dsRNA-independent manner, which required NS1 residues 123–127 (Li *et al.*, 2006a; Min *et al.*, 2007; Tan & Katze, 1998). Based on domain mapping studies, it has been proposed that NS1 binds to a linker region in PKR, and thereby prevents a conformational change that is normally required for release of PKR auto-inhibition (Li *et al.*, 2006a). Such a mechanism would allow NS1 to circumvent both dsRNA- and PACT-mediated inhibition of translation by PKR. However, it remains to be determined if an observed NS1–PACT interaction has any functional consequences (Li *et al.*, 2006a).

### NS1 and the host RNAi pathway

RNA interference (RNAi) is an RNA-guided cellular mechanism for downregulating expression of specific genes. Involvement of RNAi in the innate antiviral responses of mammalian cells is still controversial, but NS1 has already been proposed to antagonize such a



putative host-cell defence (Li *et al.*, 2004). Overexpression of NS1 inhibited the induction of RNAi in heterologous *Drosophila* and plant cell systems (Bucher *et al.*, 2004; Delgadillo *et al.*, 2004; Li *et al.*, 2004). However, a similar inhibitory effect has yet to be observed in mammalian cells (Kok & Jin, 2006). Thus, despite recent data suggesting that components of the mammalian RNAi pathway participate in innate anti-influenza virus responses (Matskevich & Moelling, 2007), a functional role for NS1 in RNAi-antagonism during virus infection awaits clarification.

### NS1 and the host adaptive immune response

Virus infections *in vivo* are detected by sentinel dendritic cells (DCs). Upon stimulation, DCs mature, release proinflammatory cytokines/chemokines and migrate to lymph nodes, where they present pathogen-specific antigens to cytotoxic and helper T-cells. This initiates an adaptive immune response specific for the invading pathogen: cytotoxic T-cells directly kill virus-infected cells, whilst helper T-cells augment this killing capacity by producing cytokines such as IFN- $\gamma$  and TNF- $\beta$ . In a mouse model, the NS1 protein of a human H5N1 influenza virus reduced systemic and pulmonary pro-inflammatory cytokines and prevented TNF- $\alpha$ -mediated bone marrow lymphocyte depletion (Hyland *et al.*, 2006). Furthermore, in human-derived primary DCs, PR8/NS1 was shown to limit induction of several genes involved in DC maturation and migration (Fernandez-Sesma *et al.*, 2006). Consequently, infected DCs were unable to mature, and failed to stimulate the secretion of IFN- $\gamma$  from helper T-cells. The limitation of gene-expression in DCs is specific only for certain genes, and mechanistically appears unrelated to suppression of IFN- $\beta$  production by PR8/NS1 (Fernandez-Sesma *et al.*, 2006). Given recent studies demonstrating that protection against influenza virus infection requires reactivation of memory T-cells by antigens presented on bone marrow-derived DCs (Castiglioni *et al.*, 2008), the prevention of DC maturation by NS1 may limit virus-clearance by the host. Thus, it will be essential to verify such profound immunosuppressive effects of NS1 in the context of virus infection using a relevant *in vivo* model.

### NS1 and the host apoptotic response

The biological function of apoptosis during influenza A virus infection is unclear, although it is often considered to be a cellular antiviral mechanism that limits virus replication. As such, influenza viruses have developed various means by which to delay this apparent host defence strategy (Ehrhardt *et al.*, 2007; Kurokawa *et al.*, 1999; Zhirnov & Klenk, 2007; Zhirnov *et al.*, 2002). However, cellular pro-apoptotic factors also promote the efficient propagation of influenza viruses, and certain viral proteins, such as NA and PB1-F2, have pro-apoptotic functions (Palese & Shaw, 2007). Thus, the overall temporal regulation of both pro- and anti-apoptotic mechanisms

may be critical for the virus. Limiting apoptosis early during infection could promote events such as genome replication, whilst enhancing apoptosis later may lead to increased release of progeny virions. Apoptosis after viral replication may also increase the phagocytic clearance of infected cells, which might otherwise stimulate cell-mediated cytotoxic responses.

The role of NS1 in apoptosis has not been fully established, as NS1 is reported to have both pro- and anti-apoptotic functions (Ehrhardt *et al.*, 2007; Lam *et al.*, 2008; Schultz-Cherry *et al.*, 2001; Shin *et al.*, 2007b; Stasakova *et al.*, 2005; Zhirnov *et al.*, 2002). Such conflicting data may be a consequence of the specific experimental protocol, cell-type or virus strain used. Alternatively, an intriguing hypothesis is that NS1 contributes temporally to both 'early' suppression of apoptosis and 'late' induction of cell death.

During virus infection, NS1 clearly displays anti-apoptotic functions which are linked to its ability to limit the production and downstream effects of IFN (Zhirnov *et al.*, 2002). Thus, in IFN-competent MDCK cells, PR8 delNS1 virus induced higher levels of apoptosis than wt PR8 (Zhirnov *et al.*, 2002). However, in Vero cells, which lack IFN- $\alpha/\beta$  genes, both viruses induced similar levels of apoptosis, but at a much slower rate than that observed in MDCK cells (Zhirnov *et al.*, 2002). It is not known if Vero cells are defective in pathways and genes other than IFN- $\alpha/\beta$ , therefore one can only speculate that IFN- $\alpha/\beta$ -antagonism by NS1 is the most important factor in limiting apoptosis. As catalytically active PKR is reported to play a role in apoptosis during influenza virus infection (Takizawa *et al.*, 1996), the direct binding and inhibition of PKR by NS1 could also lead to cell-death suppression. The same may be true for NS1-mediated inhibition of pro-apoptotic OAS/RNase L (Min & Krug, 2006), or the JNK/AP-1 stress pathway (Ludwig *et al.*, 2002). As described below, activation of the host-cell PI3K pathway has recently been described as an additional direct method by which NS1 may limit induction of apoptosis (Ehrhardt *et al.*, 2007; Shin *et al.*, 2007a; Zhirnov & Klenk, 2007).

### NS1 and the PI3K signalling pathway

PI3K is a heterodimeric lipid kinase consisting of an 85 kDa regulatory subunit (p85) and a 110 kDa catalytic subunit (p110). When active, this kinase generates the intracellular second messenger PIP<sub>3</sub>, which causes the specific membrane-recruitment of a diverse range of signalling proteins (Hawkins *et al.*, 2006). The serine/threonine protein kinase Akt (protein kinase B; PKB) is perhaps one of the best-studied PIP<sub>3</sub>-binding PI3K effectors. Akt is ubiquitously expressed in nearly all cell-types and has over 100 protein substrates. Consequently, PI3K/Akt plays an important role in numerous host-cell processes, including anti-apoptosis, cell growth, proliferation and cytokine production/signalling.

For influenza A viruses, PI3K activation, as determined by Akt phosphorylation, was shown to occur in the first 8 h of infection (Zhirnov & Klenk, 2007) and is caused by expression of the viral NS1 protein (Ehrhardt *et al.*, 2007; Hale *et al.*, 2006; Shin *et al.*, 2007a) (Fig. 2). The C-terminal effector domain of NS1 binds specifically and directly to the p85 $\beta$  regulatory isoform of PI3K (Hale *et al.*, 2006, 2008b), although weak co-precipitation of NS1 with p85 $\alpha$  has been noted from infected cells (Ehrhardt *et al.*, 2007). To date, residues of NS1 implicated in p85 $\beta$ -binding include Tyr-89/Met-93 (Hale *et al.*, 2006), Pro-164/Pro-167 (Shin *et al.*, 2007b) and Leu-141/Glu-142 (Li *et al.*, 2008), which are all adjacent to one another in the NS1 monomer (Bornholdt & Prasad, 2006; Hale *et al.*, 2008a) (Figs 1 and 5). The molecular mechanism of NS1-mediated PI3K activation has yet to be fully determined, although a current model suggests that NS1 binds the inter-SH2 domain of p85 $\beta$ , thereby blocking normal inhibitory contacts between p85 $\beta$  and p110 (Hale *et al.*, 2008b; Li *et al.*, 2008). However, interactions between NS1 and other domains of p85 $\alpha/\beta$  may also contribute towards PI3K stimulation (Shin *et al.*, 2007a, b).

Recent data suggest that the NS1 proteins of avian, but not human, influenza viruses hyperactivate PI3K by binding the N-terminal SH3 domains of Crk and/or CrkL, two highly related human signalling proteins (Heikkinen *et al.*, 2008) (Fig. 2). The interaction is mediated by a consensus class II SH3 domain-binding motif in NS1 (Finkelstein *et al.*, 2007; Heikkinen *et al.*, 2008), which encompasses NS1 residues 212–217. Binding of avian NS1 proteins to Crk/CrkL increases the phosphorylation of Akt, and requires proline at residue 215 (Heikkinen *et al.*, 2008) (Fig. 1). Threonine is present at this residue in all human NS1 proteins except that of the 1918 virus (Finkelstein *et al.*, 2007). Substitution of threonine for proline at position 215 in the 1918 NS1 protein abolished NS1–Crk/CrkL binding, and double mutation of Pro-212 and Pro-215 was shown to prevent NS1 from inducing large amounts of Akt phosphorylation (Heikkinen *et al.*, 2008). The role of avian NS1 proteins in coordinating a functional interaction between PI3K and Crk/CrkL is currently unclear, and it may be that Crk/CrkL-binding has additional consequences for virus replication which are PI3K-independent.

Chemical inhibition of PI3K using LY294002 or wortmannin results in the impaired propagation of influenza A viruses in tissue culture (Ehrhardt *et al.*, 2006; Hale *et al.*, 2006), and induces apoptosis in virus-infected cells (Ehrhardt *et al.*, 2007; Zhirnov & Klenk, 2007). However, studies using these compounds are difficult to interpret given that they (i) globally inhibit nearly all classes of PI3K (Hawkins *et al.*, 2006), (ii) limit viral entry into cells (Ehrhardt *et al.*, 2006), and (iii) display remarkable binding and inhibitory promiscuity towards other kinases (Gharbi *et al.*, 2007). A recombinant PR8 virus engineered to express NS1 containing alanine substitutions at Pro-164/Pro-167 was reported to be attenuated for growth in MDCK cells (Shin *et al.*, 2007b). The mutant NS1 protein

did not bind or activate PI3K, and mutant virus-infected cells appeared to enter apoptosis much earlier than wt virus-infected cells. This indicated a direct role for NS1-activated PI3K in limiting host-cell apoptosis, and confirmed the indirect findings of previous studies using LY294002 and wortmannin (Ehrhardt *et al.*, 2007; Zhirnov & Klenk, 2007). However, such results have yet to be fully resolved with the observation that a PR8 delNS1 virus induced similar levels of apoptosis to wt virus in IFN- $\alpha/\beta$ -deficient Vero cells (Zhirnov *et al.*, 2002).

Recombinant Ud and WSN viruses expressing NS1 proteins containing a single tyrosine to phenylalanine substitution at residue 89 (Y89F) revealed an apparent strain-specific requirement for PI3K activation in MDCK cells (Hale *et al.*, 2006). The mutation completely abrogated binding of NS1 to p85 $\beta$ , and the mutant viruses were unable to induce the phosphorylation of Akt. The mutant WSN virus demonstrated growth kinetics similar to those of wt virus, whereas the Ud mutant replicated to titres approximately tenfold lower than wt. Thus, it would appear that the WSN virus does not require the PI3K-activating functions of its NS1 protein. The reasons for this are unclear, but it will be necessary to determine if this is a strain- or host-cell-specific phenomenon.

Given the diverse array of PI3K-regulated physiological processes, it is possible that other apoptosis-independent, cell type-specific consequences of NS1-activated PI3K exist. Indeed, PI3K activation has been implicated in many cellular functions that are also regulated by NS1, such as cytokine-downregulation in DCs, enhancement of mRNA translation and suppression of innate signalling pathways (Fukao & Koyasu, 2003; Plataniias, 2005). Further work is therefore required to validate the plethora of potential roles that NS1-activated PI3K may have during infection.

### Contribution of NS1 to the pathogenicity and virulence of influenza A viruses

NS1 as a molecular determinant of virulence has been extensively studied in recent years. When the NS gene segment of the mouse-lethal WSN strain was exchanged for that of the 'Spanish influenza' 1918 pandemic virus, it was discovered that the recombinant virus replicated well in tissue-culture, but was attenuated in mice (Basler *et al.*, 2001). It was speculated that attenuation of the recombinant virus may be due to the human origin of the 1918 NS1 protein, which is adapted to function well in human cells, but is unable to work optimally in murine cells. Indeed, in a human lung cell-line (A549), a recombinant WSN virus containing the NS1 gene of the 1918 virus was more efficient at blocking expression of IFN-regulated genes than wt WSN (Geiss *et al.*, 2002). This underlined the strain-specific importance of NS1 in regulating host-cell responses triggered by infection. However, such apparent species adaptation has yet to be correlated with a role in virulence using relevant animal models.

### Cytokine deregulation and virulence

As stated previously, viruses unable to express an NS1 protein only replicate in cells or mice that have a compromised IFN response (Garcia-Sastre *et al.*, 1998; Kochs *et al.*, 2007b). Transcriptional profiling in infected cells indicated that lack of NS1 significantly increased virus-induced expression of NF- $\kappa$ B- and IRF3-regulated mRNAs (Geiss *et al.*, 2002). Furthermore, it was shown that H7N7 avian influenza viruses that encode NS1 proteins with large C-terminal deletions, or which lack NS1 altogether, were attenuated in mice and were strong inducers of IFN in mammalian and avian cells. Consequently, these viruses are highly pathogenic in mice lacking the IFN-inducible antiviral factor, Mx1 (Kochs *et al.*, 2007b). Similar results were found whilst studying swine influenza viruses that also encode C-terminally truncated NS1 proteins (Solorzano *et al.*, 2005). These mutants displayed attenuation in pigs, which correlated with an increase in IFN- $\alpha/\beta$  production in pig cells. Such results also appear true of turkey influenza viruses, where a C-terminal truncation of NS1 resulted in less severe lesions in infected chickens compared with wt virus infection (Cauthen *et al.*, 2007). Interestingly, although the virus expressing the mutant NS1 protein induced more IFN, both mutant and wt viruses were shown to be equally sensitive to the effects of IFN pre-treatment.

When the NS1 protein of a low pathogenic swine H5N1 virus containing a natural deletion of residues 191–195 was engineered into the background of a highly pathogenic virus, the recombinant virus was attenuated in chickens and unable to antagonize the host IFN response (Zhu *et al.*, 2008). Conversely, the virulence of the low pathogenic virus was increased when the five amino acids were engineered back into its NS1 protein. Deletion of residues 191–195, which are not in any known dimerization or protein-binding motif, were shown to reduce the stability of NS1 and to prevent its interaction with CPSF30, a property that likely accounts for the increase in IFN induction. Similar results regarding the apparent instability of C-terminally truncated NS1 proteins have been reported by others (Quinlivan *et al.*, 2005; Solorzano *et al.*, 2005).

Studies on the pathogenicity of goose influenza viruses in chickens demonstrated that an increase in IFN induction, and thus virus attenuation, could also be conferred by substitution of valine for alanine at residue 149 (A149V) in NS1 (Li *et al.*, 2006b). However, in their natural goose host, viruses with either valine or alanine at this position appear to replicate efficiently without inducing signs of disease. Presumably this allows both viruses to circulate undetected in the goose population. Jiao *et al.* (2008) later reported that the A149V mutation had no effect on the virulence of influenza viruses in mammals, but that substitution of serine for proline at residue 42 (P42S) of NS1 resulted in increased virus virulence. Interestingly, it had been reported earlier that substitution of glycine for serine at residue 42 (S42G) of WSN/NS1 increased the replication and virulence of an attenuated WSN virus lacking RNA-binding ability (Donelan *et al.*, 2003). Mechanistically, it is

clear that residue 42 can play an important role in the ability of NS1 to antagonize host IFN responses, including NF- $\kappa$ B and IRF-3 pathways, apparently in an RNA-binding independent manner (Donelan *et al.*, 2003; Jiao *et al.*, 2008). Thus, mutations in the NS1 RNA-binding domain also modulate influenza virus virulence.

The first reported human H5N1 highly pathogenic avian influenza (HPAI) outbreak in 1997 led to infection of 18 individuals with six fatalities (Subbarao *et al.*, 1998). The viruses responsible for the outbreak were potent inducers of pro-inflammatory cytokines, especially TNF- $\alpha$  (Cheung *et al.*, 2002), and the viral infection was characterized by hypercytokinaemia and reactive haemophagocytic syndrome (To *et al.*, 2001). In the background of a human influenza virus, the NS1 protein of an H5N1 virus was able to reduce levels of pro-inflammatory cytokine induction, and it was speculated that the outcome of disease may depend on the balance between pro-inflammatory cytokine production and the ability of NS1 to overcome it (Hyland *et al.*, 2006). It was shown that replication of a lethal H5N1 virus (HK/97) was resistant to the antiviral effects of IFN and TNF- $\alpha$  (Seo *et al.*, 2002) and that this resistance required glutamic acid at amino acid position 92 of NS1. Introducing the NS1 protein of HK/97 into a human influenza virus allowed the recombinant virus to replicate in the presence of cytokines, whereas the wt human virus, or a recombinant virus with aspartic acid at residue 92, did not replicate at all. The recombinant virus also resulted in increased pathogenicity in pigs. This mechanism of increased virulence is therefore distinct from that described above, in which deletions and truncations in NS1 led to higher IFN induction. The results of Seo *et al.* (2002) also suggest that the hypercytokinaemia associated with the HK/97 virus may have been caused by the host mounting a huge cytokine response against a virus that is completely resistant to it. In contrast to the results of Hyland *et al.* (2006), it was shown in a mouse model that infection with a recombinant PR8 virus encoding the NS1 protein of the HK/97 virus led to elevated levels of inflammatory cytokines/chemokines and a decrease in the anti-inflammatory cytokine IL-10 (Lipatov *et al.*, 2005). This cytokine imbalance required Glu-92 in NS1, and was consistent with the detailed post-mortem results of individuals that died during the 1997 H5N1 HPAI outbreak. However, it must be noted that, although the HPAI viruses isolated in 1997 contained the Glu-92 residue in NS1, H5N1 viruses containing this residue are no longer isolated naturally and Glu-92 has yet to be found in the NS1 proteins of other influenza A virus subtypes. In addition, it was recently reported that a deletion of amino acids 80–84 in NS1 enhances the virulence of H5N1 viruses (Long *et al.*, 2008). However, as this enhanced virulence was always associated with the Glu-92 mutation and could not be conferred to a virus containing Asp-92 in NS1, the effect of this deletion alone is unclear. In a previous study it was noted that certain viruses with deletion of residues 191–195 in NS1 also had a deletion of residues 80–84 (Zhu *et al.*, 2008). Given that one of these viruses appears non-



pathogenic, the importance of the 80–84 deletion as a virulence determinant remains to be determined.

### Cell signalling and virulence

Apart from overcoming the host IFN response and being able to replicate efficiently in the presence of cytokines, another mechanism by which NS1 may affect virulence is by binding to and interfering with cellular signalling proteins. From large-scale sequencing analysis it was observed that the C-termini of avian influenza virus NS1 proteins have the consensus sequence of a PDZ domain ligand (PL) (Obenauer *et al.*, 2006). PDZ domains are protein–protein recognition modules within a multitude of proteins that organize diverse cell-signalling assemblies. They specifically recognize and bind to short C-terminal peptide motifs of 4–5 amino acids, the PL. The PL of avian NS1 proteins consists of residues 227–230, with the sequence ESEV or EPEV. The NS1 C-terminal sequences of all human influenza A viruses isolated since 1918 are shown in Fig. 4. The avian PL sequence was not observed in the NS1 proteins of non-avian viruses, and for a large number of human NS1 proteins any potential PL was masked by a seven amino acid C-terminal extension. Obenauer *et al.* (2006) showed that avian NS1 proteins and that of the 1918 virus are able to bind to up to 30 human PDZ domain-containing proteins, whereas human NS1 proteins cannot. The effects of avian NS1 PL sequences on the virulence of a human influenza virus were recently reported (Jackson *et al.*, 2008). The introduction of avian or 1918 PL sequences into the NS1 protein of WSN increased the virulence of this virus in mice. Infection with viruses containing the avian-like PL in NS1 was characterized by a severe loss of body weight, decreased survival, decreased MLD<sub>50</sub>, severe alveolitis and increased viral spread in the infected lung. This work supported the hypothesis of Obenauer *et al.* that avian NS1 proteins, when present in human cells, may interact with PDZ domain-containing proteins to disrupt certain cellular pathways and cause increased virulence. The specific targets and mechanisms by which the avian NS1 PL motif achieves this effect remain to be identified.

As stated previously, a recent report suggests that avian NS1 proteins and that of the 1918 virus may bind to cellular Crk/CrkL proteins to hyperactivate PI3K signalling (Heikkinen *et al.*, 2008). It is not yet known whether this interaction affects the virulence or replication of avian influenza viruses in mammalian cells. However, given that the Crk/CrkL-binding motif of NS1 is almost exclusively found in viruses of avian origin, it may be important to determine its contribution to virus pathogenicity and/or inter-species transmission of HPAI viruses.

### Applied studies on NS1

#### Antiviral compounds targeting functions of the NS1 protein

Antiviral drugs will be an important initial defence against rapidly emerging novel strains of influenza A virus. Given

the numerous roles of NS1 during virus replication, one potential target for anti-influenza drug design may be to disrupt conserved interactions of NS1 with cellular and viral factors. In this regard, peptide-mediated inhibition of the NS1–CPSF30 interaction has recently been described as a ‘proof-of-principle’ approach to limit virus replication in tissue-culture (Twu *et al.*, 2006). Unfortunately, such a virus-specific strategy allows for virus mutation and the development of drug resistance. Nevertheless, this approach is attractive as compounds targeting virus–host interactions may be effective against many different virus strains. Further analysis of the recently determined structures of NS1 may provide targets for the rational design of antiviral compounds (Bornholdt & Prasad, 2006; Hale *et al.*, 2008a; Liu *et al.*, 1997; Yin *et al.*, 2007). Given that the NS1 proteins of certain influenza A viruses appear to have distinct host-cell protein-binding properties (Kochs *et al.*, 2007a), it may be necessary to evaluate the specific structures of several different NS1 proteins.

Inhibition of NS1-activated signalling cascades, such as PI3K, could also be a useful way of restricting influenza A virus replication. Similarly, compounds that augment the activities of NS1-deregulated antiviral pathways may titrate out the functions of NS1. Such host-directed strategies may be less susceptible to virus mutation and drug resistance; however, there could be unknown toxic side-effects for uninfected tissues. Despite this, given that PI3K inhibitors and IFN (or IFN-agonists) are already under investigation as potential long-term therapies for several chronic disorders and/or viral infections, it may be possible to rapidly develop similar compounds for use as short-term anti-influenza drugs. This may be particularly relevant for prophylaxis and treatment in the event of an emerging influenza A virus outbreak.

#### NS1 and vaccine design

The use of recombinant influenza viruses with truncated or mutated NS1 proteins as promising live-attenuated virus vaccines has been demonstrated previously (Baskin *et al.*, 2007; Falcon *et al.*, 2005; Ferko *et al.*, 2004; Quinlivan *et al.*, 2005; Richt *et al.*, 2006; Solorzano *et al.*, 2005; Talon *et al.*, 2000b; Vincent *et al.*, 2007). Such viruses are partially debilitated in their ability to counteract the host IFN response, but are able to replicate to high titres in suitable IFN-deficient systems, such as Vero cells (Talon *et al.*, 2000b). These vaccine candidates have multiple benefits: (i) they can be administered intranasally, as unlike viruses lacking the complete NS1 ORF they are replication competent in the host; and (ii) NS1-truncated viruses retain immunogenicity, and thus elicit antibody, cell-mediated, and mucosal protective immune responses (Fenko *et al.*, 2004; Talon *et al.*, 2000b).

Another strategy to produce live-attenuated influenza vaccines would be to mutate specific residues of NS1 involved in its various functions, thus generating recombinant viruses with full-length ‘designer’ NS1 molecules.

Although such viruses may be unstable and prone to genotypic/phenotypic reversion, it could be advantageous to have a vaccine strain possessing a full-length NS segment in which NS1 functions are 'finely tuned'. However, a benefit of NS1-truncated viruses is that they are likely to display increased genetic stability (Falcon *et al.*, 2005; Ferko *et al.*, 2004; Quinlivan *et al.*, 2005; Richt *et al.*, 2006; Talon *et al.*, 2000b). Given that NS1-truncated viruses are generally attenuated, except in cells lacking the ability to produce IFN, vaccine production on an industrial scale could be problematic and require a suitable complementary IFN-deficient host-cell technology. Temperature-sensitive NS1 mutants may overcome this issue (Falcon *et al.*, 2005). Alternatively, it may be more commercially viable to produce vaccine candidate strains in cell lines such as Vero cells, or those engineered to have properties akin to the multiple functions of NS1 (such as active PI3K, and inactive IRF-3/PKR/RNase L). This could involve generating cell-lines that stably express NS1, or which express proteins with analogous functions (Young *et al.*, 2003). A better understanding of the multiple functions of NS1 will clearly aid in the design of such technologies.

### Recombinant viruses with mutated NS1 genes as oncolytic therapies

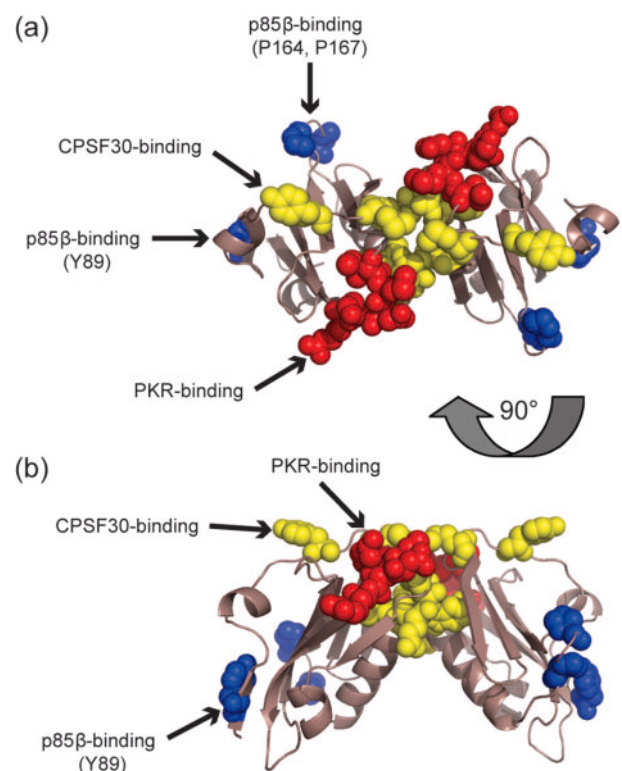
Conditionally replicating oncolytic viruses may be useful clinical tools for eradicating tumour cells with specific gene defects. Such engineered viruses are unable to replicate in normal cells due to mutation of a viral host-modulating function. However, in cells that are already altered in such a function, the mutant virus should readily replicate and consequently kill the tissue. Many tumours have genomic alterations that prevent their ability to produce or respond to IFN- $\alpha/\beta$  (Russell & Peng, 2007). In addition, components of the PI3K signalling pathway are very frequently activated in human cancers. Thus, recombinant influenza A viruses expressing defective NS1 proteins unable to counter host innate immunity and/or activate PI3K may be able to infect and lyse cells with particular tumorigenic properties. Although many potential oncolytic viruses have already been described (Russell & Peng, 2007), it is possible that engineered oncolytic influenza A viruses can be particularly useful in targeting a subset of cancerous cells with specific genetic defects.

Previously, a delNS1 virus was shown to replicate efficiently and cause cell death in cells expressing high levels of oncogenic Ras, whereas replication was restricted in non-malignant cells (Bergmann *et al.*, 2001). Active Ras is commonly found in many human cancers, and is functionally linked to the inhibition of PKR and to the activation of PI3K. Thus, it is not surprising that cells expressing constitutively active Ras are complementary to the replication of delNS1 influenza A viruses (which will be unable to block PKR or activate PI3K). Similarly, it has been demonstrated that delNS1 viruses are only active as conditionally cytolytic viruses in IFN-resistant cell-lines,

which are unable to mount a full antiviral response (Muster *et al.*, 2004). In addition, induction of cell-mediated immune responses by influenza A viruses expressing mutated NS1 proteins has been reported to boost activation of the host's own tumour-lytic cytotoxic T-lymphocytes, which may augment any therapy by killing uninfected tumour cells (Efferson *et al.*, 2006). Despite the demonstrated potential benefits of using mutant influenza A viruses as oncolytic agents, further work is still required to develop such viruses into viable therapies.

### Concluding remarks

The extreme multifunctional nature of NS1 is in many ways surprising given its relatively low molecular mass (~26 kDa). As NS1 does not seem to possess any intrinsic enzymic activity, it may be that NS1 simply acts as an array of small protein-binding epitopes to allow interaction with many different partners. This means that, to perform most of its inhibitory activities, NS1 would probably have to be in molar excess of its interactors in order to sequester and/or inhibit them efficiently (e.g.



**Fig. 5.** Binding sites for cellular proteins on the NS1 effector domain. Cartoon ribbon representations of the dimeric effector domain of A/Duck/Albany/76 [H12N5]. Residues implicated in binding CPSF30 (103, 106, 184–188; yellow), PKR (123–127; red) and p85 $\beta$  (89, 164, 167; blue) are highlighted as spheres. Views are arbitrarily designated top (a), and side (b). Images were prepared using MacPyMol (PDB file: 3D6R).

dsRNA, CPSF30, PKR). In contrast, not all available cellular PI3K will have to be engaged by NS1 for the virus to activate this signalling cascade. Furthermore, the simultaneous binding of such a small protein to multiple cellular factors may not be possible as the binding sites on NS1 could overlap each other. This is highlighted by the structural analysis of NS1 shown in Fig. 5, which demonstrates that the CPSF30- and PKR-binding sites of NS1 are in very close proximity. It is also not known whether NS1 can interact with multiple cellular partners at one time, or whether the binding of NS1 to one partner, e.g. dsRNA, is a pre-requisite (or anti-requisite) for its binding to another. This poses the question as to whether there is functional or temporal hierarchy in the activities performed by NS1. For example, it is unclear in terms of the virus replication cycle if it is more important for NS1 to first activate PI3K (or inhibit RIG-I activity) before globally inhibiting the processing and nuclear export of all cellular mRNAs. It may be that intra-cellular concentration and/or localization of NS1 contributes towards any possible hierarchy of binding, or NS1 may intrinsically have different affinities towards its binding partners. Thus, despite our substantial knowledge of this amazing and fascinating protein, much still remains to be learnt of its roles in the virus replication cycle. Future insights may require a move away from studies in traditionally used cell-lines, such as MDCK cells, in favour of more relevant host-cell types and perhaps even primary cell cultures. However, it will be difficult to evaluate all the roles of NS1 solely in tissue-culture cells, and a better understanding of influenza A virus pathogenesis in suitably relevant animal models is needed. Unfortunately, such studies are complicated by that fact that some functions of NS1 are strain- or cell-type specific, and will no doubt influence virus pathogenicity and host range.

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