Review

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# Influenza A Virus Non-structural 1 Protein: A Key Viral Weapon Against Host Pathways

Matloob Husain[1,*]

[1]Department of Microbiology and Immunology, University of Otago, 9054 Dunedin, New Zealand
*Correspondence: matloob.husain@otago.ac.nz (Matloob Husain)
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**Influenza A virus (IAV) is an obligatory intracellular microbial pathogen. It causes seasonal epidemics, occasional pandemics, and zoonotic outbreaks of an acute febrile respiratory disease called influenza, commonly known as flu, in humans. IAV is an enveloped virus and possesses a single-stranded, negative-sense RNA genome which has a linear but segmented configuration and is composed of eight gene segments. The non-structural 1 (NS1) protein is encoded by the eighth gene segment and, as the name suggests, is not packaged into IAV particles but found only in infected cells. NS1 is an IAV virulence factor, and the IAV mutants lacking NS1 exhibit attenuated phenotype. NS1 is composed of two major domains—the N-terminal RNA-binding domain and the C-terminal effector domain, which are joined by a small linker region, and the effector domain is flanked by a C-terminal tail. NS1 is truly a multi-functional protein and exploits or subverts multiple host pathways, e.g., mRNA splicing, nuclear export and translation, innate antiviral response, phosphatidylinositol-3-kinase signaling, to facilitate IAV multiplication and pathogenesis. This review summarizes the targeting of those host pathways and their components by NS1 and highlights the potential of NS1 as an antiviral drug target and the development of IAV NS1 mutants as a flu vaccine.**

**Keywords:** influenza A virus; non-structural 1; virulence factor; RNA binding; mRNA splicing; interferons; retinoic acid-inducible gene I

## Introduction

Influenza A virus (IAV) is the prototypic member of *Orthomyxoviridae* family of viruses that also includes influenza B virus, influenza C virus and influenza D virus [1]. The influenza A, B, and C viruses cause an acute febrile respiratory disease, commonly known as flu, in humans. However, IAV is the most significant because it causes all three events in humans, i.e., recurring seasonal epidemics, occasional pandemics, and zoonotic outbreaks. Furthermore, IAV has a broad host range and, in addition to humans, it also infects other mammals, e.g., bats, cats, cattle, dogs, horses, pigs, seals, as well as different species of birds [1,2]. Consequently, IAV has been a significant burden on global public and animal health [3–7].

IAV is an enveloped virus and contains a linear but segmented, single-stranded, negative-sense RNA genome, which comprises eight gene segments [8]. The gene segments are numbered 1–8 in the order of their decreasing nucleotide lengths, where segments 1 and 2 are jointly longest (each with 2341 nucleotides) and segment 8 is the shortest (with 890 nucleotides). IAV maximizes the coding potential of its eight gene segments which, together, encode up to 22 viral proteins through alternative initiation and splicing and ribosomal frameshifting of mRNA [9]. Specifically, segment 1 encodes two proteins: polymerase basic 2 (PB2) and PB2 Spliced-1 (PB2-S1); segment 2 encodes three proteins: polymerase basic 1 (PB1), PB1-N40 and PB1-F2; segment 3 encodes four proteins: polymerase acidic (PA), PA-X, PA-N155 and PA-N182; segment 4 encodes just one protein: haemagglutinin (HA); segment 5 encodes two proteins: nucleoprotein (NP) and elongated NP (eNP); segment 6 encodes two proteins: neuraminidase (NA) and NA43; segment 7 encodes three proteins: matrix protein M1, M2 and M42; and segment 8 encodes five proteins: non-structural 1 (NS1), NS2 (now known as nuclear export protein or NEP), NS3, negative-strand polypeptide (NSP) and truncated NS1 (tNS1) [9]. Of these, ten proteins, PB1, PB2, PA, HA, NP, NA, M1, M2, NS1, and NEP, form the core IAV proteome and are critical for the effective IAV multiplication [9]. The rest of the proteins form the accessory IAV proteome, but a function is known for only some of them. These proteins play accessory roles in the viral immune evasion, antagonism and host shut-off activity [9,10].

The PB1, PB2, PA, HA, NP, NA, M1, M2, and NEP are structural proteins and are incorporated in IAV particles, which are enveloped; however, NS1, as the name indicates, is a non-structural protein and only found in infected cells [11]. The HA, NA, and M2 are membrane proteins and are inserted in the envelope of the IAV particle [11]. Based on the antigenic properties and sequences of HA and NA, IAV is further divided into subtypes, e.g., H1N1, H3N2, H5N1. The M1 forms a skeleton underneath the envelope,

which encapsulates eight viral ribonucleoprotein (vRNP) complexes. Each vRNP is comprised of PB1, PB2, and PA, which together form viral RNA polymerase, and NP bound to one of the eight viral RNA gene segments [11].

The IAV life cycle in the host cell starts with HA, a receptor-binding protein, binding the sialic acid receptor on the host cell plasma membrane and facilitating the attachment of IAV particles to host cell surface. IAV particles then enter the host cell, primarily by endocytosis. Within the endosome, the M2 protein forms the ion channels in the viral envelope, facilitating protons influx into the IAV particle. This acidification leads to the fusion of the viral envelope with the endosomal membrane, followed by uncoating of the IAV particle and the release of vRNPs into the cytoplasm. The NP in vRNPs then interacts with the cytoplasmic nuclear receptor, importins through its nuclear-localization signals, and the vRNPs are imported into the nucleus. In the nucleus, viral RNA polymerase (PB1, PB2, and PA) replicates and transcribes each viral RNA segment into viral RNA copies and mRNA, respectively. The viral mRNAs are then exported from the nucleus to the cytoplasm and translated into viral proteins. Of these proteins, PB1, PB2, PA, NP, M1, NS1, and NEP are imported back into the nucleus, whereas HA, NA, and M2 are transported to the plasma membrane. In the nucleus, PB1, PB2, PA, NP, and viral RNAs are assembled into vRNPs and subsequently exported to the cytoplasm with the help of M1 and NEP. Subsequently, the vRNPs, M1, and NEP are trafficked to the plasma membrane, where IAV progeny are assembled and released by budding through the enzymatic action of NA [12].

Parallel to the IAV life cycle, a concerted host innate immune response comprising multiple host pathways and a plethora of host restriction factors is activated, which interferes with different stages of the IAV life cycle [13,14]. In turn, IAV employs the NS1 to antagonize many of those pathways and restriction factors to facilitate its multiplication and pathogenesis [13]. In addition, NS1 promotes several viral processes over the host cell processes and exploits other host pathways and factors to facilitate IAV multiplication. This review summarizes those NS1 functions during the IAV life cycle.

## NS1

NS1 was first identified in 1971 as a 25 kilodalton protein in the nuclear fraction of infected cells and initially called as NNP (nonstructural nucleus-associated protein) [15]. Later, it was discovered that NS1 is encoded by the smallest viral gene segment, segment 8 [16]. It was renamed as NS1 when it was found that segment 8 also encodes another smaller protein, then termed as NS2 but now called NEP, via mRNA splicing [17,18]. NS1 is translated from the unspliced mRNA of segment 8 through initiation by the first AUG codon. NS1 polypeptide is around 230 amino acids long but its length can vary between 217 to 237 amino acids in different IAV strains of mammalian and avian origins [19]. The nucleotide insertions or deletions in NS1, mainly at the C-terminus, during IAV evolution due to selection pressure are the primary reasons for its varied lengths [20,21]. On average, recently isolated human and avian IAV strains possess a 219- and 230-amino acid long NS1, respectively [21]. Based on NS1 length, IAV strains can be divided into two phylogenetic groups or subtypes: non-structural gene group/subtype A and B. The group/subtype A contains both mammalian- and avian-origin IAV strains but group/subtype B contains only avian-origin IAV strains [19,22,23].

### *Structure*

NS1 contains two conserved functional domains: an N-terminal domain composed of 1–73 amino acids and a C-terminal domain composed of 86–205 amino acids [24,25]. These domains are joined by a small linker region of 74–85 amino acids and the C-terminal domain is flanked by a C-terminal tail of varied length of 206 to 237 amino acids (Fig. 1) [25]. The N-terminal domain is called the 'RNA-binding domain', because it binds both single-stranded and double-stranded RNA (dsRNA) though the former in a sequence-dependent manner and the latter in a sequence-independent manner [26,27]. Nevertheless, NS1 exhibits higher affinity towards the partially double-stranded viral RNA, also called as panhandle RNA [26]. The C-terminal domain is called the 'effector domain' and binds to many host proteins during the IAV life cycle [25]. The varied-length C-terminal tail possesses a four-amino acid PDZ-binding motif (227–230), which binds to PDZ domain-containing host proteins (Fig. 1) [28,29]. NS1 exists as a homodimer and both the RNA-binding domain and the effector domain can independently form a dimer [30–32]. The dimerization of the RNA-binding domain forms a six-helix fold structure and is required for the binding of dsRNA [33–35].

### *Modifications*

NS1 is known to undergo multiple modifications, e.g., acetylation, ISGylation, methylation, phosphorylation, and SUMOylation. NS1 is acetylated at the N-terminus and at lysine 108 and methylated at arginine 193 [36,37]. The phosphorylation of NS1 occurs at multiple serine, threonine, tyrosine residues and at multiple sites, located in both RNA-binding and effector domains (Fig. 1). For example, NS1 is phosphorylated at serine 42 [38], 48 [38,39], 83 [40] and 205 [41], threonine 49 [42], 80 [43], 197 [39] and 215 [44], and tyrosine 73 [40]. Further, NS1 is ISGylated at multiple lysine residues located in the RNA-binding domain as well as the effector domain [45,46] and SUMOylated at the sites in the effector domain and C-terminal tail [47].

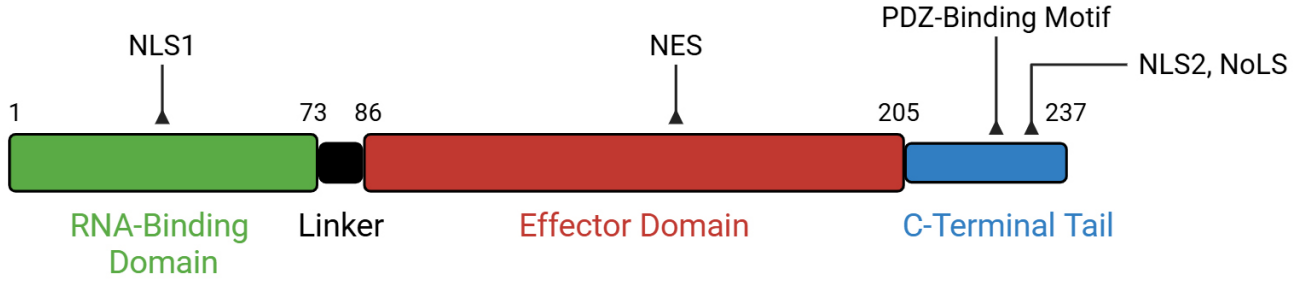


**Fig. 1. Non-structural 1 (NS1) protein domains and motifs.** Amino acids 1–73 comprise the RNA-binding domain, 74–85 linker region, 86–205 effector domain, and 206–237 varied-length C-terminal tail. NLS, nuclear localization signal; NoLS, nucleolar localization signal; NES, nuclear export signal (Illustration created with BioRender.com).

### *Intracellular Localization*

NS1 is a highly expressed protein and was initially identified as a nuclear protein in IAV-infected cells [15,18]. Therefore, NS1 is predominantly localized to the nucleus and possesses two nuclear localization signals (NLS): one (NLS1) in the RNA-binding domain (residues 35–41) and the other (NLS2) in the C-terminal tail (residues 219–237) (Fig. 1) [48]. In addition, NS1 of IAV H5N1 strains possesses another NLS at residues 19–21 [49]. Also, NS1 is localized to the nucleolus, and the NLS2 in the C-terminal tail concurrently possesses the nucleolar localization signal (NoLS) (Fig. 1) [18,50]. However, the NLS2 and NoLS in the effector domain are lost in some human IAV strains isolated after 1989 and all avian IAV strains due to C-terminal truncations [51]. Further, NS1 possesses a nuclear export signal (NES) in the effector domain (residues 138–147) (Fig. 1), and a subpopulation of NS1 is localized to the cytoplasm and shuttles between the cytoplasm and nucleus [52–54].

## Virulence Function

NS1 is not essential for the multiplication of IAV but is required for its pathogenesis and virulence [55,56]. The IAV mutants lacking whole NS1 gene or containing mutations or deletions in NS1 RNA-binding domain, effector domain, or linker region exhibit restricted growth and attenuated phenotype in interferon-competent cells and animals, respectively. However, these IAV mutants have normal growth and are virulent in interferon-deficient cells and animals, respectively [53,55,57,58]. This indicates that NS1 is the main viral protein which helps IAV to evade host innate antiviral response. Furthermore, the NS1 of highly pathogenic IAVs, like 1918 pandemic strain and avian H5N1 subtype is more efficient in evading this response [59–61].

In addition, the naturally occurring or adaptive mutations, deletions, and truncations in NS1 gene can decrease or increase the growth and virulence of circulating and emerging IAV strains in different host species. The C-terminal tail and the linker region of NS1 from different IAV strains have been most prone to naturally occurring or adaptive mutations and truncations. For example, the NS1 of IAV strains isolated from 1950 to 1989 acquired 7 amino acids at the C-terminus, which extended its length to 237 amino acids and helped acquire NLS2 [21]. However, this extension was lost in the IAV strains isolated after 1989 and the majority of them possessed 230-amino acid long NS1. Then, the 2009 pandemic IAV H1N1 strain lost a further 11 amino acids from the NS1 C-terminus and contained a 219-amino acid long NS1 [62]. This truncation increased its virulence in mice as recombinant IAV with 219-amino acid long NS1 was more lethal than the recombinant IAV with 230-amino acid long NS1 [63]. However, this truncation did not change the viral growth kinetics as both recombinant viruses grew to similar titres in mammalian cells [62,63]. Similarly, the amino acid composition of PDZ-binding motif at NS1 C-terminus may determine the virulence of IAV strains in different species. The consensus sequence of PDZ-binding motif in the NS1 of the majority of human IAV strains is Arg-Ser-X-Val (RSXV) but in the majority of avian IAV strains, it is Glu-Ser-X-Val (ESXV) [28]. The recombinant IAV strains containing ESXV sequence in NS1 exhibited more lethality in mice than the ones containing RSXV sequence [64,65]. However, the recombinant IAV strains containing ESXV sequence in NS1 exhibited attenuated growth in human, mouse, and duck cells [66]. Further, most IAV H5N1 strains isolated in 2000 or later acquired a five-amino acid (80–84) deletion in the NS1 linker region that was associated with their increased growth and virulence in chickens but not in mice [67–69].

Some naturally acquired point mutations in NS1 RNA-binding and effector domains also contribute to the virulence and adaptation of some IAV strains in a different host species [70]. Conversely, some such mutations also lead to the attenuation or act as a barrier to interspecies adaptation of some IAV strains. For example, the avian H5N1 strain, which emerged in humans in 1997/98, acquired Phe103Leu and Met106Ile mutations in NS1 to adapt to the mammalian host [71,72]. Similarly, Pro42Ser mutation in the NS1 of a H5N1 chicken isolate contributed to its adaptation in mice. Furthermore, Pro212Ser and Val178Ile

mutations in the NS1 of H7N9 subtype, which emerged in humans in 2013, contributed to its adaptation in mammals [73]. Conversely, Glu55Lys, Glu66Lys and Phe133Cys mutations in the NS1 of a H5N1 chicken isolate attenuated it in human cells [74]. Similarly, Ile64Thr, Asp189Asn and Val194Ile mutations in the NS1 of seasonal H3N2 virus attenuated it in mice [75]. Further, Ala149Val mutation in the NS1 of a H5N1 goose isolate attenuated it in chickens [76]. Furthermore, a deletion of amino acids 191–195 in the NS1 of H5N1 pig isolates attenuated them in chickens and mice.

Finally, protein modifications like acetylation, phosphorylation, ISGylation and SUMOylation of NS1 also influence its function and, consequently, the IAV pathogenesis [36,45]. The phosphorylation of NS1 plays a differential role in its virulence function. For example, phosphorylation of NS1 at tyrosine 73, serine 83 and 205, and threonine 215 enhances its virulence function [38,40,41,44], whereas the phosphorylation of NS1 at threonine 49 and 80 suppresses its virulence function [42,43].

Therefore, NS1 is the main virulence factor of IAV and determines its pathogenesis in different host species. To exert its virulence function, NS1 employs multiple mechanisms which can be categorized into three main parts: (1) promotion of IAV life cycle, (2) antagonism of host innate antiviral response, and (3) manipulation of other host pathways and factors.

## Promotion of IAV Life Cycle

NS1 promotes IAV life cycle by promoting the viral RNA synthesis [77,78] and viral mRNA nuclear export and translation and inhibiting the host gene expression by interfering with host mRNA splicing, nuclear export and translation. In addition, NS1 shuts off the host gene expression by binding to host cell DNA through its effector domain and blocking the access of transcription machinery [79,80].

### *Host mRNA Splicing, Nuclear Export and Translation*

NS1 inhibits the splicing of host pre-mRNA [81,82] by binding, sequestering, or interfering with the function of several components of spliceosome (Fig. 2) [83,84]. Specifically, NS1 binds, through its RNA-binding domain, to small nuclear RNAs in small nuclear ribonucleoprotein particles (snRNPs) [82,85,86] and UAP56 [87]. Further, it binds to cleavage and polyadenylation specificity factor 30 (CPSF30) [88,89] and poly(A)-binding protein II (PABII) [90] through its effector domain. Also, NS1 alters the localization of spliceosome components in the nucleus [91]. In addition, NS1 binds to the introns of pre-mRNAs through its RNA-binding domain and inhibits their removal [92]. However, the NS1 of initial H5N1, 2009 pandemic H1N1 and H7N9 isolates were defective in binding to CPSF30 due to naturally occurring mutations and, consequently, inhibiting the host gene expression [93]. Similarly, the C-terminally truncated NS1 of the 2009 pandemic H1N1 strain is defective in binding to PABII and blocking the host gene expression [63].

NS1 also inhibits the nuclear export of host mRNA because nuclear export of mRNA is coupled with splicing [81]. For this, NS1 binds to poly(A) sequence of mRNA through its RNA-binding domain and sequesters the mRNA in the nucleus (Fig. 2) [24,94]. In addition, NS1 binds several components of nuclear mRNA export machinery, particularly NXF1-NXT1 receptor in an RNA-independent manner and blocks its interaction with nucleoporins, thereby inhibiting the export of host mRNA into the cytoplasm (Fig. 2) [95,96].

Finally, NS1 inhibits the translation of host mRNA by outcompeting the host mRNA and preferentially initiating the translation of viral mRNA [97,98].

### *Viral mRNA Splicing, Nuclear Export and Translation*

Unlike host mRNA, NS1 does not inhibit the splicing of viral mRNA [82]. Instead, it regulates the splicing of viral M mRNA to maintain the ratio of viral M1 and M2 mRNAs [99]. Also, NS1 promotes the nuclear export of unspliced M1 mRNA [100].

Furthermore, NS1 promotes viral protein synthesis by promoting the translation of viral mRNAs over the host mRNA [101]. For this, NS1 interacts with eukaryotic translation initiation factor 4GI (eIF4GI), poly(A)-binding protein 1 (PABP1) and human Staufen (hStaufen), and then recruits the translation initiation complex to 5′ untranslated region (5′ UTR) of viral mRNA [97,102,103].

## Antagonism of Host Innate Antiviral Response

For a host, the first line of defence against IAV infection is the innate antiviral response, which inhibits IAV cycle at various stages. Innate antiviral response can be divided into three main parts: (1) sensing of virus particles by host cells, (2) expression of interferons and other cytokines, and (3) expression of interferon-stimulated genes [14]. NS1 antagonizes all three parts of host innate antiviral response against IAV.

### *Sensing*

Host cells employ pattern recognition receptors (PRRs) to sense infecting virus particles and infection through pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), respectively. To sense IAV particles and infection, human cells employ membrane-bound Toll-like receptors (TLRs) and cytosol-bound retinoic acid-inducible gene-like receptors (RLRs), nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs) and Z-DNA-binding protein 1 (ZBP1) [14].

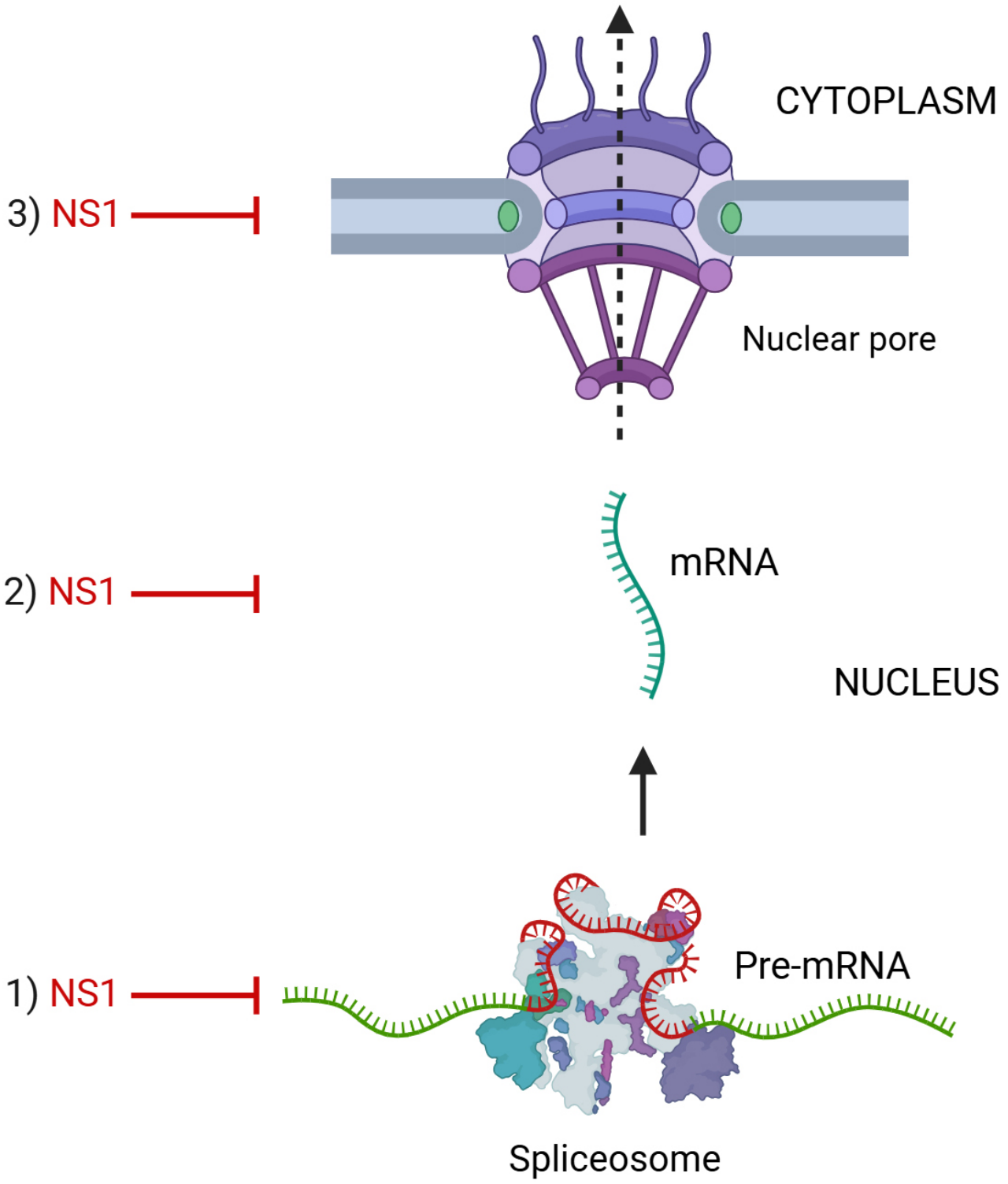


**Fig. 2. NS1 inhibits host mRNA splicing, nuclear export and translation.** NS1 (1) inhibits host pre-mRNA splicing by binding several components of spliceosome, (2) binds and sequesters mature host mRNA in the nucleus, and (3) inhibits host mRNA nuclear export to the cytoplasm by binding several components of nuclear mRNA export machinery (Illustration created with BioRender.com).

NS1 antagonizes TLR3, which senses IAV infection in endosomes, indirectly by binding to Hippo signaling pathway molecules, Yes-associated protein (YAP) and transcriptional coactivator with PDZ-binding motif (TAZ) (YAP/TAZ) and promoting their nuclear localization. In the nucleus, YAP/TAZ binds to the promoter of TLR3 and downregulates its expression [104]. Further, NS1 antagonizes retinoic acid-inducible gene I (RIG-I)-mediated sens-

ing of IAV infection in multiple ways [105–107]. RIG-I is one of the main RLRs that senses IAV infection by recognizing partially double-stranded RNA, also known as panhandle RNA, in IAV-infected cells [14]. First, NS1 downregulates the expression of RIG-I in infected cells by (1) recruiting the transcriptional repressor, CCAAT/enhancer binding protein beta (C/EBP$\beta$) to RIG-I promoter [108], (2) interfering with RIG-I pre-mRNA processing [92], and (3) destabilizing the RIG-I mRNA via monocyte chemotactic protein-induced protein 1 (MCPIP1) RNase activity [109]. Second, NS1 directly binds RIG-I via its RNA-binding domain and sequesters it away from IAV panhandle RNA [110–112]. Third, NS1 binds to viral dsRNA in IAV-infected cells and sequesters it from the recognition by RIG-I [113]. Finally, NS1 destabilizes RIG-I by binding to RIG-I-stabilizing protein, NLRC5 [114]. However, the phosphorylation of NS1 at threonine 49 and 80 inhibits its binding to RIG-I [42,43]. Furthermore, naturally occurring mutation (Arg21Gln) and deletion (amino acids 191–195) in the NS1 of IAV 1918 H1N1 and swine-origin H5N1 isolate, respectively, disrupt its binding to RIG-I [115]. Finally, NS1 directly interacts with NLRP3, the best characterized NLR, to antagonize the NLRP3 inflammasome-mediated sensing of IAV infection [116–119].

### *Expression of Interferons and Other Cytokines*

The sensing of IAV infection by different PRRs results in the activation of several downstream signaling pathways. All of these pathways culminate in the expression of interferons (IFNs) and other cytokines [14]. NS1 inhibits the expression of several of those IFNs and cytokines, as well as neutralises their anti-IAV effects [61].

TLRs signal via either MyD88-dependent pathway or TRIF-dependent pathway. These pathways culminate in the activation of ubiquitin ligases, tumor necrosis factor (TNF) receptor-associated factor (TRAF) 6 and 3, respectively [14]. The activated TRAFs then ubiquitinate and activate the kinases such as transforming growth factor-$\beta$-activated kinase 1 (TAK1) and inhibitor of $\kappa$B kinases (IKKs) which, in turn, activate the transcription factors, interferon regulatory factor (IRF) 3 and 7 and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-$\kappa$B). The IRFs 3 and 7 and NF-$\kappa$B then translocate to the nucleus and engage with their respective promoters on the host genome and stimulate the expression of IFNs and other cytokines [14]. NS1, through its effector domain, interacts with IKK and inhibits its kinase function and, consequently, inhibits the activation and nuclear translocation of IRF3 and NF-$\kappa$B [120,121]. Furthermore, NS1 downregulates the expression of sphingosine 1-phosphate lyase (SPL) which promotes the kinase activity of IKK [122]. In addition, NS1 directly interferes with the binding of NF-$\kappa$B to the promoter of type III IFN genes [123].

RIG-I exists in an inactive phosphorylated and acetylated form in the uninfected cells. RIG-I is activated by dephosphorylation and deacetylation and subsequently ubiquitinated by ubiquitin ligases, Riplet and tripartite motif containing 25 (TRIM25). Next, RIG-I forms a complex with mitochondrial antiviral-signaling protein (MAVS) which, in turn, forms a complex with TRAF3. Then, TRAF3 facilitates the activation and nuclear translocation of IRFs 3 and 7 and NF-$\kappa$B, which lead to the expression of IFNs and other cytokines [14]. NS1 antagonizes this signaling by interacting and interfering with Riplet and TRIM25 activities (Fig. 3) [124–126]. However, the NS1-TRIM25 interaction occurs in a host species-specific and IAV strain-specific manner [115,127]. Furthermore, the phosphorylation of NS1 at threonine 49 impairs this interaction [42]. NS1, through its effector domain, also interacts with TRAF3 and inhibits its activation (Fig. 3) [128,129]. Consequently, NS1 inhibits the RIG-I-mediated activation of NF-$\kappa$B (Fig. 3) [130]. In addition, NS1 competes with RIG-I for binding to host proteins, protein activator of protein kinase R (PACT) and 14-3-3epsilon, which promote RIG-I signaling [131,132]. Further, NS1 induces and reduces the expression of host proteins, A20 and OTUB1, respectively, which inhibit and promote RIG-I signaling, respectively, in IAV-infected cells [133,134].

Finally, NS1 interferes with NLRP3 inflammasome assembly [117,135] and inhibits the activation of proinflammatory response [119].

### *Expression of Interferon-Stimulated Genes*

Interferon-stimulated genes (ISGs) are expressed through Janus kinase–signal transducer and activator of the transcription (JAK-STAT) pathway. This pathway is activated when IFNs and other cytokines, expressed through downstream signaling pathways mentioned above, are secreted and bind to their cognate receptors on host cell surface. Subsequently, the JAK kinases and transcription factors STATs are recruited to cytokine-receptor complex and JAKs phosphorylate STATs. The phosphorylated STATs then dissociate themselves from the complex and form a transcription factor complex, which translocates to the nucleus. In the nucleus, this transcription factor complex stimulates the expression of hundreds of ISGs, which create an ‘antiviral state’ in IAV-infected cells [14]. NS1 inhibits JAK-STAT pathway by inducing the expression of suppressor of cytokine signaling (SOCS) 1 and 3 proteins, which interfere with the formation of cytokine-receptor-JAK complex and phosphorylation of STATs [136]. Furthermore, NS1 antagonizes the antiviral function of several individual ISGs. For example, NS1 interferes with the function of human guanylate-binding protein 1 (hGBP1) [137], 2′,5′-oligoadenylate synthetase (OAS), which degrades viral RNA via RNase L [138], and protein kinase R (PKR) [139,140] and zinc finger antiviral protein short (ZAPS) [141], both of which inhibit viral protein synthesis.

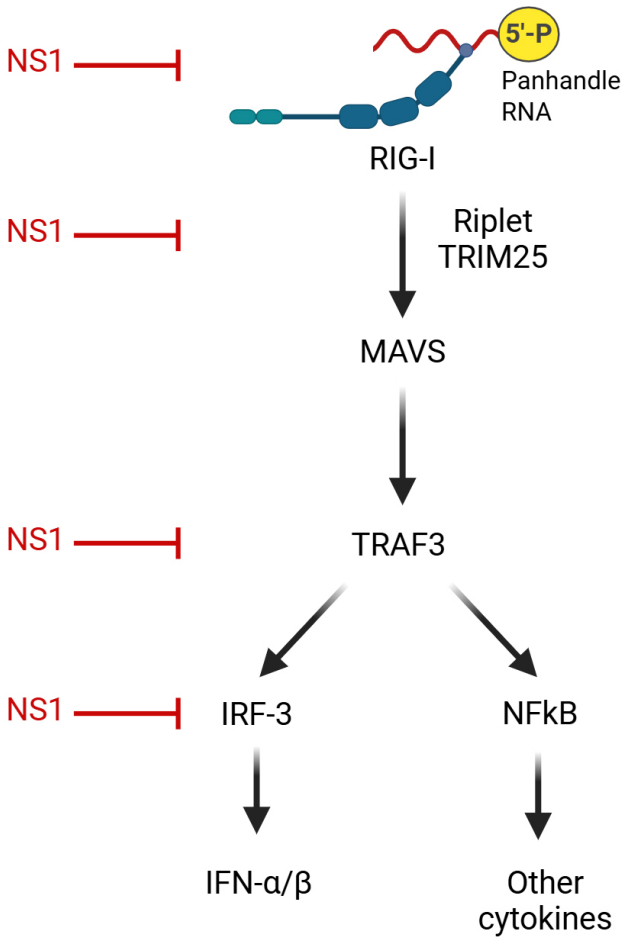


**Fig. 3. NS1 interferes with influenza A virus (IAV)-induced retinoic acid-inducible gene I (RIG-I) signaling at multiple stages.** 5'-P, 5'-phosphate; TRIM25, tripartite motif containing 25; MAVS, mitochondrial antiviral-signaling protein; TRAF3, tumor necrosis factor (TNF) receptor-associated factor 3; IRF-3, interferon regulatory factor 3; NF-$\kappa$B, nuclear factor kappa-light-chain-enhancer of activated B cells; IFN, interferon. (Illustration created with BioRender.com).

## Manipulation of Other Host Pathways and Factors

In addition to the above, NS1 targets programmed cell death, metabolic pathways, stress response, and other antiviral factors to promote IAV multiplication.

### *Programmed Cell Death*

There are three known programmed cell death pathways: apoptosis, necroptosis, and pyroptosis. Apoptosis is a regulated form of cell death and is characterized by DNA fragmentation and cell shrinkage. Necroptosis is a proinflammatory form of cell death and is characterized by organelle swelling and membrane rupture. Whereas pyroptosis is a highly proinflammatory form of cell death and is characterized by the formation of pores in the plasma membrane by gasdermin proteins. Lately, all three pathways have been integrated into one term, PANoptosis. IAV infection induces PANoptosis in infected cells, which aids in viral clearance but, paradoxically, also contributes to tissue damage and flu severity in the host [142].

NS1 contributes to this paradoxical role of PANoptosis and has been described to promote apoptosis though other reports describe NS1 inhibiting the apoptosis [29]. Similarly, NS1 induces necroptosis [143] but inhibits pyroptosis [117].

### *Metabolic Pathways*

Phosphatidylinositol-3-kinase (PI3K) signaling is crucial for cellular metabolism and regulates cellular homeostasis under various pathological conditions and diseases. PI3Ks are a highly conserved group of lipid kinases which are activated by various stimuli. PI3Ks contain a catalytic subunit (p110 $\alpha$, $\beta$, $\gamma$, or $\delta$) and a regulatory adaptor subunit (p85$\alpha$, p85$\beta$, or p55$\gamma$). In response to a stimulus, PI3Ks phosphorylate the membrane phosphatidylinositol-4,5-bisphosphate (PIP2) into phosphatidylinositol (3,4,5) trisphosphate (PIP3), to which the effectors like Akt kinases are docked and induce downstream signaling cascade controlling cellular growth, division and migration [144,145].

NS1 binds p85$\beta$ subunit of PI3K through its effector domain and activates the downstream signaling cascade [144–146]. The full significance of the activation of PI3K signaling during IAV infection is still not clear though it has been shown to promote IAV multiplication [144,147]. Further, it is proposed to protect the infected cells from early apoptotic death [148]. However, some IAV H1N1 and H5N1 strains containing naturally occurring mutations in NS1 gene or bat-origin IAV strains are deficient in activating the PI3K signaling [149,150].

### *Stress Response*

In response to stress, like virus infection, cells form stress granules and processing (P)-bodies in the cytoplasm, which store, silence, or degrade translationally stalled mRNAs. Stress granules and P-bodies also contribute to host anti-IAV defence, because multiple host anti-IAV proteins, e.g., DEAD-box helicase 3 (DDX3), DEAD-box helicase 3 X-linked (DDX3X), Moloney leukemia virus 10 (MOV10), nuclear domain 10 (ND10), and RNA-associated protein 55 (RAP55), promote the formation of these sites, localize to these sites, and sequester viral RNA and proteins in these sites [151–154]. NS1 interacts with these proteins and inhibits the formation of stress granules and P-bodies [151–155]. Furthermore, NS1 modulates the activation of c-Jun N-terminal kinase (JNK), which regulates the expression of stress response genes. In addition, NS1 alleviates the endoplasmic reticulum stress in infected cells due to excessive production of IAV glycoproteins [156]. Finally, NS1 arrests host cell cycle at G0/G1 phase to potentially provide favourable environment for accumulation of viral proteins [157].

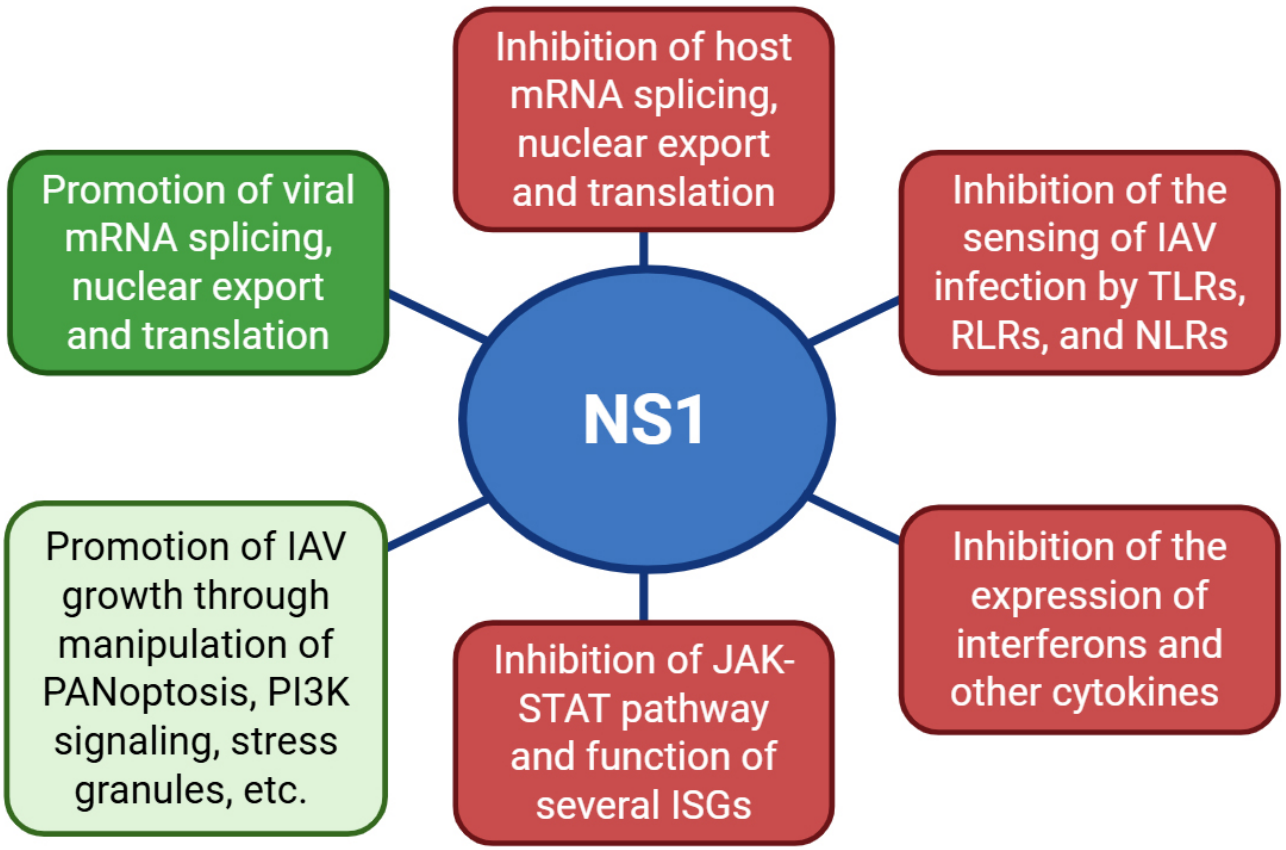


**Fig. 4. NS1 promotes (green boxes) or inhibits (red boxes) multiple viral and host pathways and components to promote IAV multiplication.** PI3K, phosphatidylinositol-3-kinase; TLRs, Toll-like receptors; RLRs, retinoic acid-inducible gene-like receptors; NLRs, nucleotide-binding oligomerization domain (NOD)-like receptors; JAK-STAT, Janus kinase–signal transducer and activator of the transcription; ISGs, interferon-stimulated genes. (Illustration created with BioRender.com).

*Other Antiviral Factors*

NS1 competitively binds to DDX21 RNA helicase, which inhibits IAV RNA synthesis by binding to PB1 [41,158]. Further, NS1 downregulates the expression of DDB1- and CUL4-associated factor 7 (DCAF7), which interferes with IAV polymerase activity [159]. Also, NS1 degrades the anti-IAV E3 ligase, beta-transducin repeat-containing protein ($\beta$-TrCP) [160].

## Targeting NS1 to Prevent and Treat Flu

As described above, the IAV NS1 mutants are attenuated, and some naturally-occurring mutations in NS1 help various IAV strains in interspecies transmission and adaptation and exhibit different pathogenicity in different host species [115,127,161–165]. Consequently, NS1 mutants have been developed as flu vaccine candidates [166–168] and NS1 has been explored as an antiviral drug target [169] to prevent and treat the flu, respectively.

*Vaccine*

The concept of using IAV NS1 mutants as a flu vaccine came about when it was learned that such mutants were attenuated in animals [170,171], but induced a protective response in those animals and protected them from wild-type IAV challenge [170–174]. Since then, different preparations of NS1-deficient IAV have been developed to use as live-attenuated flu vaccines [166–168,175–178]. IAV strains with deleted NS1 induce cross-protective neutralizing response in mice, monkeys, and humans [166,179–181]. In addition, these viruses also induce cross-protective T cell immunity in mice [166]. Similarly, IAV strains with truncated NS1 induced a protective humoral and cellular immune response and protected the mice, monkeys, pigs, and chickens against wild-type IAV challenge [171–176,178,182–184]. Furthermore, IAV with mutated NS1 or NS1 which is designed to be degraded in infected cells, also induces similar adaptive immune response and protection against wild-type IAV challenge [167]. Using this approach, a trivalent NS1-deleted vaccine underwent Phase I/II human trials, and a bivalent NS1-truncated vaccine was approved as Ingelvac Provenza™ for use in pigs [179,185]. However, NS1-based vaccines face some challenges to their wider use in humans. The NS1-deficient IAV strains are difficult to grow in commonly used vaccine production platforms using IFN-competent embryonated chicken

eggs and Madin-Darby canine kidney (MDCK) cells and can only grow in IFN-deficient Vero cells [186]. Therefore, new and updated platforms need to be developed for the production of NS1-based IAV vaccines. Furthermore, there is a risk of a live attenuated NS1-based vaccine strain reassorting with a circulating IAV strain, which may naturally infect the vaccinated person, and generating a new IAV variant [180].

### *Antiviral Drug Target*

Early on, four antiviral compounds targeting NS1 were identified using an NS1-expressing yeast strain [187]. One of those compounds, named NSC125044, led to the synthesis of additional analogous compounds A9 (JJ3297) and A22, which potently inhibited the IAV replication [188,189]. A9 and A22 interact with the NS1 effector domain and interfere with its binding to CPSF30 [190]. NSC125044 derivative compound, 3-hydroxy-N-[(Z)-1-(5,6,7,8-tetrahydronaphthalen-2-yl)ethylideneamino]naphthalene-2 carboxamide (HENC) and traditional Chinese medicine compounds also interfere with NS1-CPSF30 binding [191,192]. In addition, epigallocatechin gallate (EGCG) and its derivative compounds interacting with the NS1 RNA-binding domain and interfering with its binding to dsRNA have also been identified [193–195]. Furthermore, several other compounds, like pyrazolopyridines, quinoline carboxylic acid, and baicalin, have been identified to target other functions of NS1 and reverse the NS1-mediated inhibition of host innate antiviral response, mRNA nuclear export and PI3K signaling [196–199]. NS1 could be an ideal antiviral drug target to inhibit and treat IAV infection though a broad-spectrum antiviral drug targeting NS1 is yet to be developed. One of the reasons for this is that the structures of full-length NS1 in its native form as well as in complex with its many binding partners, are yet to be solved [25,200]. Furthermore, as described above, many functions of NS1 are IAV strain-specific and associated with the sequence variations, e.g., in linker region and at the C-terminus, and the functional differences of different NS1 variants are yet to be elucidated. Nevertheless, IAV can always mutate the NS1 to become resistant to any broad-spectrum anti-NS1 drug that is developed.

## Conclusion

In summary, NS1 is a phenomenal multi-domain and multi-functional viral protein which targets multiple host pathways and components (Fig. 4) to promote IAV multiplication and pathogenesis. Consequently, NS1, with careful and focused design, can be exploited as the target of flu vaccines and antiviral drugs.

## Availability of Data and Materials

Not applicable.

## Author Contributions

MH is the sole contributor to this manuscript. The author confirms sole responsibility for the conception and design of the study; the acquisition, analysis, and interpretation of data; the preparation of the manuscript; and being accountable for all aspects of the work.

## Ethics Approval and Consent to Participate

Not applicable.

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## Conflict of Interest

The author declares no conflict of interest. MH is serving as one of the Editorial Board members of this journal. MH declares that he had no involvement in the peer review of this article and has no access to information regarding its peer review. Further, Figs. 1,2,3,4 were created using BioRender.com. The author has no financial or personal relationship with BioRender.com, and the use of this tool does not imply any endorsement.

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