REGULATORY MECHANISMS FOR HOST INNATE IMMUNITY TO INFLUENZA

A VIRUS

AN ABSTRACT

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WENZHUO HAO

Approved:

Shitao Li, PhD Director

Lucy C. Freytag, PhD

Kevin Zuezdaryk, PhD

Kislay Parvatiyar, PhD

Zhen Lin, MD, PhD
ABSTRACT

Influenza A virus (IAV) is a highly contagious respiratory pathogen and threatens global public health with considerable economic impact. Upon invading host cells, Influenza viral RNA is sensed by the host pattern recognition receptor (PRR)-retinoic acid-inducible gene I (RIG-I), which instigates the IRFs and NF-kB signaling pathways inducing the expression of type I interferon (IFN) and inflammatory genes. The RIG-I pathway can be suppressed by viral proteins that obstruct signaling cascades and host factors for a successful infection, which is tightly regulated by the interactions between IAV and the host. A comprehensive understanding of antiviral host factors and host-IAV interactions will be invaluable in revealing IAV pathogenesis and identifying potential therapies. In this study, we first focused on the host protein FK506-binding protein 5 (FKBP5), an interacting protein of the IKK complex. FKBP5 preferentially binds IKKα. Knockout of FKBP5 increases susceptibility to IAV infection and impairs RIG-I-mediated type I IFN activation, suggesting that FKBP5 restricts IAV infection by activating RIG-I-mediated NF-kB signaling. Second, we found that the transcription and splicing factor SNW domain-containing protein 1 (SNW1) interacts with HOIP (HOIL1-interacting protein, also known as RNF31). HOIP is the E3 ubiquitin ligase for linear ubiquitination that plays a key role in regulating the RIG-I signaling pathway and effectively prevents IAV infection. Our study revealed a new mechanism that HOIP forms a complex with SNW1, elongation factor p-TEFb, and RNA polymerase II subunit B to facilitate p65 and IRF3 transcriptional
activity, thereby promoting innate immune defense to IAV. Furthermore, the IAV non-structural protein 1 (NS1) interacts with SNW1. Interestingly, NS1 perturbs the SNW1-HOIP complex to impair mRNA expression of IFN and ISGs. Our data suggest that the SNW1-HOIP complex facilitates p65- or IRF3-mediated gene transcription, whereas NS1 inhibits their transcriptional activity. Our study has uncovered three new host factors, namely FKBP5, SNW1, and HOIP, that restrict IAV infection. Furthermore, we have discovered various mechanisms of how these factors regulate the RIG-I pathway and antiviral gene expression. In addition, our study sheds light on a new way that IAV evades detection by disrupting the interaction between SNW1 and HOIP, which is mediated by NS1. The knowledge obtained from our study can be harnessed to boost innate immune responses and impede influenza infection.
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A DISSERTATION

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CHAPTER 1. OVERVIEW OF THE INNATE IMMUNE RESPONSES TO INFLUENZA A VIRUS.

1.1. Introduction of Influenza A virus

1.1.1. Influenza A virus biology

Influenza A virus (IAV) is an enveloped, single-stranded RNA virus belonging to Orthomyxoviridae family \(^1\). The genome of IAV contains eight negative-sense RNA segments which encode at least 10 proteins, including hemagglutinin (HA), neuraminidase (NA), matrix proteins 1 (M1) and 2 (M2), polymerase acidic (PA), polymerase basic 1 (PB1), polymerase basic 2 (PB2), nucleoprotein (NP), non-structural proteins 1 (NS1), and 2 (NS2) \(^1,2\). Each RNA segment is encapsidated by a helical coil of NP and one copy of the viral polymerase, consisting of PA, PB1, and PB2. This structure is known as the ribonucleoprotein (RNP) complex \(^3\). Electron microscopy of isolated RNPs displayed that each viral RNA segment forms the circular or supercoil structure \(^4\), and the RNA polymerase is located at or very close to the end of each RNP \(^5\), the remainder of the segment is wrapped by NP \(^6\). In the virion, each RNP interacts with the M1 protein which lies under the lipid envelope \(^7-9\). The lipid bilayer, which is derived from the host’s cell membrane during the viral budding, is studded with HA and NA glycoproteins and traversed by the ion channel M2 protein. HA is a homotrimer and the rod-shaped spike
through an electron microscope, while NA is a homotetramer and the mushroom-shaped spike 10. Morphologically, the IAV virion is 80–120 nm in size with shapes varying from small spherical to long filamentous 11.

When reaching a potential host cell, IAV initiates the infection by binding HA to the sialic acid (SA) moieties of cell surface receptors 12-14. The linkage of the terminal SA is fundamental to species specificity. HAs from human IAV recognize receptors with the α-2,6-linked SA, whereas HAs of avian IAV prefer the α-2,3 linkage 15. And those from swine recognize both, and therefore pigs have been regarded as a mixing vessel 16. Then, receptor-mediated endocytosis occurs, and IAV enters the host endosome. The low pH environment of the endosome triggers the fusion of virus and endosome membranes which is mediated by HA2 fusion peptide. HA is cleaved by cellular proteases into HA1 and HA2 subunits, and the low pH induces a conformational change exposing the HA2 fusion peptide, which inserts itself into the endosome membrane 17, 18. The acidic environment of the endosome also opens the M2 ion channel acidifying the inside virion, which results in the uncoating of the vRNPs 19, 20. vRNPs are subsequently released to the cytoplasm through the fusion pore and translocated into the nucleus. Inside the nucleus, IAV RNA-dependent RNA polymerase carries out transcription and replication internally on viral RNA 21. Viral RNAs are transcribed into mRNAs which are exported to the cytoplasm for translation. The replication of viral RNAs is that the negative-sense viral RNAs are transcribed into complementary RNAs (cRNA), followed by the transcription of
new vRNA copies using cRNAs as templates. The newly synthesized viral proteins NP, PA, PB1, and PB2 are imported into the nucleus to assist in viral mRNA transcription and vRNA replication. For example, cRNAs associate with these proteins to form cRNPs which generate multiple new vRNA copies. New vRNAs assemble into new vRNPs to produce additional viral mRNAs and cRNAs. Progeny vRNPs are then exported to the cytoplasm with the aid of M1 and nuclear export protein (NEP) and packaged as viral particles together with HA, NA, M1, and M2 under the plasma membrane of host cells. Finally, viral particles bud from the apical side of polarized cells and are released from the host cells by NA-mediated cleavage of the sialic acid (Fig. 1).
Figure 1 The IAV life cycle.

The initial stage of IAV infection is that viral HA attaches to cell surface sialic acid receptors, viruses from different species recognize different linkages. Then receptor-mediated endocytosis occurs and the virus enters the endosome. Low pH environment of endosome not only triggers fusion of viral and endosome membrane mediated by HA2 fusion peptide but also opens the M2 ion channel, acidifying the inside of the virion. Consequently, viral RNPs are released to the cytoplasm and translocated into nucleus. In the nucleus, viral RNAs are transcribed into mRNAs and replicated into more copies. Translated viral proteins required in these two steps (such as PB1, PB2, PA, NP) are back to the nucleus, and then new vRNPs are formed and exported to the cytoplasm assisted with M1 and NS2 proteins. Together with other viral proteins, the viral particle is assembled. Budding occurs
and viral particle is released from the host cells.

1.1.2. Epidemiology

IAV is a highly contagious respiratory pathogen that causes influenza or flu. Symptoms of influenza vary from typical symptoms characterized by sore throat, cough, runny nose, fever, headache, muscle pain, and general weakness to severe or lethal pneumonia. IAV infection can also result in multiple non-respiratory complications affecting the heart, central nervous and other organ systems. IAV infection associated with influenza occurs as annual seasonal epidemics and occasionally pandemics. Seasonal epidemics often happen in winter in the northern and southern hemispheres in temperate regions while they can occur all seasons in tropical areas. The transmission of IAV among persons primarily through droplets expelled from infected individuals by coughing, sneezing, or talking. The other route is via touching surfaces or objects contaminated with droplets from infected persons, followed by touching one’s mouth or nose. It is estimated that each year seasonal influenza leads to between 0.3 million and 0.6 million deaths worldwide and approximately 9 million – 41 million illnesses, 12000-52000 deaths in the United States alone. IAV infection occurs in people in any age group, but individuals with young (<1 year) and elderly (>65 years) ages are the most vulnerable populations to IAV infection and mortality. About 100 children die each year from
influenza in America, and this number has been stable since 2010. In addition, pregnant women and people with certain health conditions, like cancer, HIV, heart disease, diabetes, and asthma, are at higher risk of serious flu complications.

IAV can be subtyped based on the genetic and antigenic differences in HA and NA glycoproteins. To date, 18 antigenically distinct HA and 11 antigenic variants of NA have been identified, including 2 HA subtypes (H17, H18) and 2 NA subtypes (N10, N11) spread only in bats. The natural hosts of IAV are aquatic birds. All 1-16 HA subtypes and 1-9 NA subtypes are circulating in their natural reservoirs. And these IAV subtypes have adapted to a wide range of other host species, like poultry, swine, horses, dogs, cats, and humans. Human-adapted IAV subtypes are H1N1, H3N2, and H2N2. Among them, the first two subtypes are current circulating ones, while the H2N2 IAV spread only from 1957 to 1968. The same three subtypes have also circulated in pigs. Although H5, H6, H7, and H9 subtypes of avian influenza viruses (AIV) are nonpathogenic in their aquatic bird reservoir species but become highly pathogenic in domestic poultry currently do not transmit between humans, humans can get infected after exposure to these subtypes infected poultry and manifest a more systematic disease when the subtypes are H5 and H7.

Since IAV RNA-dependent RNA polymerase is error-prone, circulating IAV strains are constantly mutating. The continuous process due to the accumulation of point mutations in the HA and NA genes is antigenic drift. This process helps IAV escape immune
responses induced by prior infection or vaccination, thus causing seasonal influenza epidemics. In contrast to antigenic drift, antigenic shift refers to drastic changes in the antigenicity of the HA of circulating IAV strains which occurs either by reassortment or induction of a novel HA from the aquatic bird reservoir. The IAV genome is segmented, allowing for the reassortment of gene segments in host cells which are simultaneously infected with IAVs from two different species. Considering the wide tropism of IAV and the potential zoonotic transmission, there is a great chance that mammal cells coinfect with at least two different IAVs, therefore generating a totally new strain with a novel HA. The majority of the human populations do not have immunity to the antigenic shift produced novel viruses, and when these viruses have the ability to spread efficiently from person to person, an influenza pandemic can occur. There have been four influenza pandemics in the past 100 years, the 1918 Spanish H1N1 pandemic, followed by 1957 Asian H2N2 pandemic, the 1968 Hong Kong H3N2 pandemic, and 2009 swine-origin H1N1 pandemic. Among them, the Spanish influenza pandemic in 1918 was the most catastrophic, with at least 50 million deaths worldwide. Its viral strain H1N1 disappeared from humans in 1957 and reemerged in 1977. Each pandemic IAV acquired gene segments from the avian reservoir by reassortment, and the 2009 pandemic swine virus is the result of the reassortment of Eurasian avian-like swine virus (H1N1) with swine triple reassortant viruses H3N2 and H1N2 (of North American avian, human H3N2, and classical swine H1N1 origin).
1.1.3. Prevention and treatment

The mainstay of influenza prevention in human is vaccination. Currently, there are three categories of influenza vaccines approved by the Food and Drug Administration (FDA), inactivated influenza vaccine (IIV), recombinant influenza vaccine (RIV), and live attenuated influenza vaccine (LAIV). The detergent-split IIV is the most common annual vaccine and consists of three or four candidate vaccine viruses (CVVs), including an influenza A H1N1 and H3N2 virus as well as either one or two influenza B viruses (Victoria or Yamagata lineage) \(^{48}\). CVVs for IIV can be propagated in both embryonated chicken eggs and Madin-Darby Canine Kidney (MDCK) cells, followed by inactivation, purification, and detergent-split \(^{49}\). But most current IIVs are produced in eggs. IIV is administered intramuscularly and induces the antibody responses, which are predominantly serum IgG against the head of the HA protein \(^{50}\). Some examples of the IIV are Fluzone®, Fluarix™, and Flucelvax®. IIVs are safe for anyone older than 6 months \(^{51}\). Unlike IIV, the second kind of influenza vaccine RIV, such as Flublok®, completely avoids using influenza viruses and embryonated systems. Instead, RIV only contains the HA protein of CVVs for the particular year expressed by baculoviruses in insect cells \(^{52}\). A quadrivalent RIV is recommended for individuals over 18 years old, especially for people 65 years old and older \(^{53}\), and administered intramuscularly. Lastly, the LAIV, known as FluMist®, contains the same CVVs as the IIV, but the viruses are cold-adapted to replicating at temperatures
below 25 °C, which fits the temperature in the upper airway and restricts the replication there 49. LAIV is delivered intranasally and can elicit both antibody and cellular immunity, including a mucosal IgA response 54. Since it is composed of live virus, a quadrivalent LAIV is approved to use in healthy persons aged 2–49 years old, but high-risk groups, such as pregnant women and immunocompromised individuals, are excluded 55.

Since IAV can occur antigenic drift and antigenic shift, continual surveillance of circulating influenza viruses is required for the production of annual strain-specific influenza vaccines 48. This task is conducted by more than 100 designated national influenza centers around the world. Twice a year, representatives from five World Health Organization (WHO) Collaborating Centers for Reference and Research on Influenza review year-round surveillance data and make recommendations for CVVs in the upcoming influenza season. Each country then decides on appropriate strains for inclusion in its own country. As the vast majority of influenza vaccines are produced in eggs, the entire process, from strain selection to vaccine availability, usually takes 6-8 months 50. Although this whole process is both time and efforts consuming, the effectiveness of influenza vaccines in the United States only ranged from 10% to 60% in the past years, which is comparatively lower than that of many other vaccines for common infectious diseases, for example, the effectiveness of measles vaccine is 97% 44. There are several limitations of influenza vaccines that may contribute to the low effectiveness of influenza vaccines. First, egg-based vaccine manufacturing requires CVVs to be selected at least 6
months before vaccines are available. Since IAV constantly occurs antigenic drift, it is with a great chance that circulating IAV strains drift dramatically within 6 months, leading to the antigenic mismatch between CVVs and the circulating IAV strains. In addition, this timeline determines that in the first wave of an influenza pandemic, there will be no available vaccines because pandemic strains are totally novel and unpredictable. Strain-specific seasonal influenza vaccines cannot provide protection, and the production of new vaccines requires at least 6 months. Second, mutations occur when human influenza viruses are grown in eggs with the reason that human IAV HA binds to α-2,6 linked sialic acids, while allantoic cavities of embryonated chicken eggs only contain α-2,3 linkages. Selective pressure drives the human IAV to occur mutations. This kind of mutation may not affect the match between vaccine viruses and circulating strains but may impair the induction of a protective immune response. For example, vaccine strains of the 2016–2017 flu season were found to lack a glycosylation site on H3N2 HA antigenic site B, which is crucial to induce neutralizing antibodies. Third, influenza vaccine production relies closely on the steady supply of sterile embryonated eggs. Although cell-based vaccine production and the RIV avoid these drawbacks, their high expenses prevent them from being popular in the vaccine market. As for LAIV, it seems to induce mucosal and more broadly protective immune responses than inactivated influenza vaccines. However, it has been reported that LAIVs have poor efficacy against the influenza A H1N1 component. Thus, while vaccines are the most effective method to prevent influenza, they can be improved. Better vaccine manufacturing and universal coverage of viral strains are
required.

Apart from vaccines, antivirals play a critical role in the prevention and treatment of influenza infection. In general, antiviral drugs are mainly used in severe hospitalized influenza cases and patients with a high risk of severe infection or complications during a normal influenza season. While in a pandemic, especially in the period when vaccines are unavailable, antivirals are utilized not only in the treatment of infected individuals but also to provide protection for persons who have been exposed. Up to date, there are only four FDA-approved antiviral drugs recommended for influenza treatment and chemoprophylaxis: oseltamivir (Tamiflu), zanamivir (Relenza), peramivir (Rapivab) and baloxavir marboxil (Xofluza). Among them, three (oseltamivir, zanamivir, peramivir) are NA inhibitors (NAIs) that block the enzyme activity of NA, thus, IAV cannot be released from infected cells. While baloxavir marboxil is a cap-dependent endonuclease inhibitor that blocks the endonuclease activity of the polymerase acidic (PA), then further interferes with the viral RNA transcription and ultimately inhibits IAV replication. Only oseltamivir and zanamivir are approved for the prophylaxis, and peramivir is usually given intravenously in the hospital.

The major challenge for antivirals is the emergence of drug-resistant viruses. Adamantanes previously functioned as antivirals that inhibit the M2 ion channel of influenza viruses. In 2008, adamantane-resistant strains of IAV became more common. Both the 2009 pandemic H1N1 IAV and the seasonal H1N1 that followed are also resistant
to adamantane. Thus, since 2009, adamantanes are no longer recommended for influenza treatment \(^68\). In addition, current circulating IAVs contain NAI-resistant strains. Studies found that mutations that occurred in several NA active sites, like R292K, N294S, I222V, H274Y, H275Y, and E119V, confer resistance to NAIs on IAV. Moreover, the mutant H274Y in NA of the H5N1 and H1N1 is the most common mutation that is resistant to oseltamivir and peramivir \(^69\). Oseltamivir-resistant H1N1 IAV emerged in 2007 and became dominant by the 2008-2009 season. However, the 2009 pandemic introduced the oseltamivir-sensitive, which replaced most of the oseltamivir-resistant H1N1 IAV. Currently, there is still a high prevalence of resistance to oseltamivir in children, immunocompromised individuals, and hospitalized patients \(^70\). And the rise of oseltamivir resistance can occur either with drug-selection pressure or natural infection \(^71\). Although there is limited data on baloxavir marboxil resistance which is a newer antiviral, recent research revealed that about 10% of influenza-infected healthy patients developed a specific mutation (PA/I38X) after baloxavir marboxil treatment and the PA/I38X variants were related to higher viral loads and long-term symptoms \(^72\).

Another concern for current antivirals is that both NAIs and endonuclease inhibitors merely pause the progression of IAV infection rather than eliminate or greatly reduce the viral burden. Thus, to effectively treat IAV infection, antivirals should be administered within 48 hours to 2 days after the onset of symptoms. This narrow timing window of therapeutic efficacy is a significant challenge for the treatment in many countries \(^73\).
The limitations of currently available therapeutics for influenza prevention and treatment illustrate the need for new antiviral therapies.

1.2. Innate immune responses to IAV

1.2.1. Overview of antiviral innate immunity

When the host is invaded by pathogens, innate immunity acts as the first line to rapidly detect and defend them within minutes. Innate immunity is conferred by both non-inducible and inducible mechanisms. The non-inducible defense refers to the anatomical and physiological barriers that function as the first line to prevent the host from invasions of various pathogens. These anatomic and physiologic barriers include the intact skin, mucosae of internal body tracts, mechanical actions of body structures (such as coughing, cilia movement) or chemicals produced by tissues (such as lysozyme), the hostile physiological environment (such as low stomach pH) as well as the microbiome. Once foreign pathogens penetrate these anatomical and physiological barriers, the inducible innate mechanism, which is characterized by pattern recognition, is in charge of protecting the host. This category of innate immunity is performed by various immune cells, including macrophages, dendritic cells, mast cells, neutrophils, eosinophils, natural killer (NK) cells, as well as nonhematopoietic cells like epithelial cells lining the respiratory, gastrointestinal, and genitourinary tracts. In contrast to the massive, randomly generated repertoire of receptors expressed by T and B cells, these immune cells express a limited number of
germline-encoded pattern-recognition receptors (PRRs) which recognize highly conserved components of microbes, known as pathogen-associated molecular patterns (PAMPs) \(^76\). The model of pattern recognition was first proposed by Charles Janeway Jr. and illustrates two features of innate immunity: one is to discriminate infectious nonself molecules and self-molecules, and the other is to activate the adaptive immune immunity \(^77\).

Over the past decades, researchers have discovered that PRRs can detect a wide range of molecules to instigate innate immune responses. PAMPs are components essential for the survival of the microorganism therefore difficult for the microorganism to mutate or microbial products generated during infection \(^74, \, 76\). They may include lipopolysaccharide (LPS), peptidoglycan mannose-containing carbohydrates, CpG dinucleotides from bacteria, chitin from fungal or parasite cell walls, and viral RNA genomes. The innate immune system can not only identify components from invading pathogens but also host molecules generated in danger, known as the damage-associated molecular patterns (DAMPs). DAMPs are endogenous molecules released from damaged or dying cells. Well-characterized DAMPs include high mobility group box 1 protein, heat shock proteins, uric acid, mitochondrial DNA, and genomic DNA \(^78\).

There are five types of PRRs classified by their protein domain homology: Toll-like receptors (TLRs), C-type lectin receptors (CLRs), nucleotide-binding oligomerization domain (NOD-like) receptors (NLRs), RIG-I-like receptors (RLRs), and the Absent in
Melanoma-2 (AlM2)-like receptors (ALRs)\textsuperscript{79}. These receptors can be further divided into membrane-bound receptors and unbound intracellular receptors. TLRs and CLRs belong to membrane-bound receptors located at the plasma membrane or endosomal membrane; thus, they can detect PAMPs or DAMPs in the extracellular space and within endosomes. NLRs, RLRs, and ALRs are unbound intracellular receptors and identify intracellular pathogens\textsuperscript{80}.

The major result of PRR-induced innate immune response is the activation of nuclear transcription factors, leading to the production of type I interferons (IFNs), pro-inflammatory cytokines, and chemokines. These chemical messages support the effector function of innate immune cells and are also crucial to activate adaptive immune responses\textsuperscript{81}. PRR activation also instigates non-transcriptional responses such as phagocytosis, autophagy, cell death, and cytokine processing\textsuperscript{82-84}.

1.2.2. Innate immune sensing of IAV

Once IAV invades the host, viral components or products generated during the infection are recognized as PAMPs by PRRs. The pattern recognition receptor initiates intracellular signaling cascades, which include several different types of enzymes, adaptor proteins acting as scaffolds, and nuclear transcription factors, leading to the production of type I IFNs, proinflammatory cytokines, and chemokines. Type I IFNs bind Type I IFN receptors and stimulate the expression of hundreds of genes, known as IFN-stimulated
genes (ISGs), in both an autocrine and paracrine manner. ISGs serve to control viral infection in the infected cell and induce the antiviral state in neighboring cells. Proinflammatory cytokines and chemokines eliminate viral infection by inducing inflammation and recruiting additional innate and acquired immune cells.

IAV infection can be sensed by at least three classes of PRRs: Toll-like receptors (TLRs), nucleotide-binding oligomerization domain (NOD-like) receptors (NLRs), and RIG-I-like receptors (RLRs).

(1) TLRs

The Toll gene was first discovered in Drosophila in the 1990s with great importance in antifungal responses. To date, 10 TLRs in humans and 12 TLRs in mice have been identified in the TLR family. All TLRs are type I transmembrane proteins sharing a similar domain organization with several leucine-rich repeats (LRRs) in the extracellular domain followed by a single transmembrane domain and a single cytosolic Toll/interleukin-1 (IL-1) receptor (TIR) domain. TLRs can detect various microbial structures and ligands and then initiates innate signaling pathways, which are the adaptor protein myeloid differentiation primary response protein 88 (MyD88)-dependent or TIR-domain-containing adapter-inducing interferon-β (TRIF)-dependent, ultimately resulting in the expression of type I IFNs along with proinflammatory cytokines. Different TLRs recognize different PAMPs. It is known that TLR3, TLR7, and TLR8 sense IAV RNAs. TLR4 and TLR10 are also reported to be related to IAV infection.
TLR3 is widely expressed in immune cells like myeloid dendritic cells (mDCs), macrophages, natural killer cells (NK), and non-immune cells, including fibroblasts, various epithelial cells, and neurocytes. In these cells, TLR3 is localized in both the endosomal membrane and cell surface, with the exception of mDCs where TLR3 is only in the endosome. As first discovered in nucleic acid sensing, TLR3 was identified as a receptor for dsRNA, which is the constitution of the viral genome or a viral replicative intermediate. TLR3 recognizes dsRNA in the endosome during phagocytosis of infected cells and then initiates the signaling via the adaptor protein TRIF which subsequently activates transcription factors interferon regulatory factor 3 (IRF-3), NF-κB, and AP-1, causing the expression of type I IFNs, proinflammatory cytokines. Besides dsRNA, TLR3 has been reported to sense ssRNA viruses, DNA viruses, and the synthetic dsRNA analog, polyriboinosinic:polyribocytidylic acid (poly I:C) is also a TLR3 ligand. Several studies have revealed that TLR3 is involved in IAV recognition. TLR3 in human respiratory epithelial cells senses IAV infection and induces the production of proinflammatory cytokines in an NF-κB-dependent way. In addition, intranasal pre-treatment of mice with TLR3 agonists provided a high level of protection against the lethal dose of a highly lethal avian H5N1 influenza (HPAI) strain and seasonal influenza A/PR/8/34 H1N1 and A/Aichi/2 H3N2 viral strains. However, in the TLR3−/− mice, although higher viral loads in the lung were sustained after lethal IAV infection, knockout mice survived longer than wild-type mice and produced much less amount of inflammatory cytokines. And lethal avian influenza H5N1 infection in both cells and mice preferentially activated TLR3 and triggered
a severe inflammatory response \(^{100}\). These studies indicate that TLR3-mediated innate immune responses to IAV infection might be detrimental to the host.

TLR7 and TLR8 were discovered as receptors of single-stranded RNA (ssRNA) in the exploration of the immunostimulatory potential of ssRNA using ssRNA from human immunodeficiency virus–1 (HIV-1) and Toll-like receptor (TLR)-deficient mice \(^{101}\). Currently, TLR7 and TLR8 have been found to serve as PRRs for ssRNAs of host origins and from a number of viruses, including IAV \(^{102-106}\). TLR7 and TLR8 genes are both located on the X chromosome and with high homology to each other \(^{76}\). The expression of TLR7 is restricted to plasmacytoid DCs (pDCs) and B cells alongside respiratory epithelial cells, while TLR8 is mainly expressed in the myeloid origin cells, including monocytes, myeloid dendritic cells, and macrophages \(^{107}\). Since TLR7 and TLR8 are in the endosomal membrane, they recognize IAV when it enters the endosome through endocytosis. Upon engagement with viral ssRNAs, these two sensors instigate the MyD88-dependent pathway, culminating in the production of type I IFNs and proinflammatory cytokines \(^{90}\). More detailed and recent studies about TLR7/8 and IAV revealed that in human monocytes, although both TLR7 and TLR8 sense IAV infection, TLR8 signaling results in the expression of cytokines involved in CD4\(^+\) T helper 1 (T\(_{H1}\)) cell differentiation while TLR7 induce cytokines for T\(_{H17}\) cell differentiation \(^{108}\). In addition, another study found that TLR7 plays an important role in inducing strong antibody responses to a sublethal dose of IAV infection in mice \(^{109}\). Interestingly, TLR7 was reported to recognize IAV in human
platelet and subsequently cause the C3 release, which leads to neutrophil-DNA release and aggregation. This phenomenon may explain IAV-mediated myocardial infarction \(^{110}\).

TLR4 is mainly expressed in the plasma membrane of myeloid cells, including monocytes, macrophages, mDCs, neutrophils, and also in some non-immune cells, such as endothelial cells \(^{111}\). TLR4 is the first identified human TLR and is thought to be the sensor of lipopolysaccharide (LPS) from Gram-negative bacteria. Current studies have expanded the list of PAMPs sensed by TLR4 to viral pathogens, like respiratory syncytial virus (RSV), vesicular stomatitis virus (VSV), and mouse mammary tumor virus (MMTV) \(^{112}-^{114}\). Once activated, TLR4 initiates either MyD88 dependent or TRIF dependent or both pathways \(^{115}\). TLR4 recruitment of MyD88 mainly activates NF-κB, inducing the production of proinflammatory cytokines, while the TRIF signaling leads to the activation of both IRF3 and NF-κB. Interestingly, studies found that TLR4 doesn’t sense IAV directly. Instead, oxidized phospholipids produced in oxidative stress caused by IAV infection potently activate TLR4 \(^{116}\). In addition, \(TLR4^{-/-}\) mice, the TLR4 antagonist treatment, and anti-TLR4 IgG therapy protect mice from lethal PR8 IAV infection \(^{117,118}\). These studies provide a clue that blocking TLR4 signaling might be a viable therapeutic route to mitigate influenza-induced acute lung injury.

Among TLRs, TLR10 is the only one without a defined ligand or function and is understudied due to lacking mouse models. One study found that in primary human peripheral blood monocyte-derived macrophages and in a human monocytic cell line THP-
1, IAV infection increases the TLR10 expression and knockdown of TLR10 in THP-1 cells considerably impairs cytokines expression after IAV infection 119.

(2) **NOD-, LRR- and pyrin domain-containing protein 3 (NLRP3)**

NLRP3, belonging to the NLRs family, is a tripartite protein that contains three domains: the N-terminal pyrin domain (PYD), central NOD/NACHT domain, and the C-terminal leucine-rich repeat domain (LRR) 120. The NACHT/NOD domain has ATPase activity which is crucial for NLRP3 oligomerization and function. In the rest state, NLRP3 is auto-suppressed in a monomeric form through folding back of the LRR domain onto the NACHT/NOD domain 121. Upon stimulation, NLRP3 is activated in the form of an inflammasome. The NLRP3 inflammasome is a cytosolic multiprotein complex that consists of a sensor (oligomerized NLRP3), an adaptor (ASC; also known as PYCARD), and an effector (caspase 1) 120. NLRP3 is expressed in myeloid cells, like monocytes, DCs, neutrophils, and macrophages, and in human bronchial epithelial cells 81. The activation of NLRP3 inflammasome is tightly regulated and requires two steps: priming of the inflammasome and activation of the inflammasome. The priming step is to upregulate the expression of the inflammasome components NLRP3, ASC, procaspase-1, as well as pro-IL-1β and pro-IL18. The increased expression is achieved through NF-κB activation and subsequent gene transcription induced either by the recognition of various PAMPs and DAMPs by PRRs like TLRs, NOD2 or by cytokines such as tumor necrosis factor (TNF) and IL-1β 122-124. The second step occurs, followed by the stimulation of NLRP3 activators,
and induces full activation and formation of an inflammasome. The assembly of NLRP3 inflammasome is a multiple-step process, including NLRP3 oligomerization, the recruitment of ASC, and the formation of “ASC speck”. ASC is a macromolecule consisting of multiple ASC filaments and the recruitment of pro-caspase1. Finally, pro-caspase1 processes self-cleavage to become the activated form of caspase1, which further proteolytically activates pro-IL-1β, pro-IL-18, and the pyroptotic factor GSDMD. IL-1β and IL-18 subsequently induce the innate and adaptive immune response against pathogens while the activated GSDMD mediates pyroptosis, an inflammatory form of cell death.

Substantial studies have demonstrated that NLRP3 inflammasome activation is involved in IAV infection. Using gene-deficient mice, studies revealed a protective role of NLRP3 inflammasome in IAV infection. For example, mice deficient in caspase-1, ASC, or NLRP3 showed significantly higher mortality and reduced immune responses after PR8 IAV infection. Both NLRP3<sup>−/−</sup> and caspase-1<sup>−/−</sup> mice displayed more prominent lung epithelial necrosis and airway obstruction with a sublethal PR8 IAV infection. But the mechanism remains largely elusive. At least three IAV-associated PAMPs have been identified as activators of NLRP3, which participate in the second step of NLRP3 inflammasome activation. First, ssRNA from PR8 IAV was sufficient to trigger IL-1β secretion in bone-marrow-derived dendritic cells (BMDCs) upon transfection. Neither NLRP3 nor other components of the inflammasome can bind viral RNA. Thus, in this context, NLRP3 inflammasome activation may rely on other viral RNA sensors like RIG-I.
and TLRs. Second, all M2 proteins from different human seasonal and pandemic strains, or avian IAV transduced into LPS primed (step1 active) bone marrow-derived macrophages (BMDMs) and BMDCs resulted in IL-1β and IL-18 secretion. A mutant influenza virus lacking H+ transport activity of M2 protein failed to induce IL-1β and IL-18 secretion in BMDMs and BMDCs, which was restored by ectopic expression of M2. This study revealed that M2 channel–induced inflammasome activation correlates with its Golgi localization and requires an acidified Golgi compartment. Third, PB1-F2, a nonstructural protein of IAV located in the mitochondria, is reported to activate the NLRP3 inflammasome. Mice infected with PB1-F2-deficient PR8 IAV resulted in reduced IL-1β secretion compared to infection with wildtype IAV. In addition, intranasal administration of the PB1-F2 peptide of pathogenic IAV enhanced IL-1β secretion. Furthermore, NLRP3-deficient mice exposed to PB1-F2 peptide or infected with PB1-F2 expressing IAV failed to induce the robust inflammatory response efficiently. Furthermore, high molecular weight aggregated PB1-F2 of IAV in LPS-primed BMDMs induced IL-1β secretion. This NLRP3 inflammasome induced by PB1-F2 requires PB1-F2 aggregation, phagocytosis, and lysosomal acidification. Interestingly, when PB1-F2 is expressed in nonphagocytic HEK293 cells reconstituted with components of NLRP3 inflammasome, it inhibits NLRP3 inflammasome maturation. A recent study found that PB1-F2 of the highly pathogenic H7N9 virus suppresses IL-1β secretion in IAV-infected macrophages. The mechanism appears to be that the H7N9 virus selectively blocks RNA-induced NLRP3 inflammasome activation by preventing NLRP3-MAVS interaction.
Other unveiled mechanisms include the antiviral RNAse L pathway cleaving viral and cellular RNAs during IAV infection. The cleaved products are then recognized by the DExD/H-box helicase, which mediates the activation of NLRP3 inflammasome. In addition, NLPR3 partners with another innate immune sensor protein Z-DNA-binding protein 1 (ZBP1). The latter detects IAV viral proteins NP and PB1, leading to the activation of the NLRP3 inflammasome. Unlike type I IFNs, IL-1β and IL-18 do not induce direct antiviral resistance that is reducing the pathogen burden. Two studies found no difference in viral loads between NLRP3-deficient and wild-type mice until 7 days post-infection. This suggests that NLRP3-mediated host protection involves an increase in disease tolerance, which means reducing the negative impact of infection on host fitness and not antiviral resistance. Specifically, NLRP3 inflammasome activation improves the tolerance of tissues to high-dose influenza virus challenge by inducing tissue repair in the respiratory tract.

(3) RLRs

Retinoic acid-inducible gene I (RIG-I)-like receptors (RLRs) are cytosolic RNA sensors. This family consists of three members: RIG-I, melanoma differentiation-associated protein 5 (MDA5), and laboratory of genetics and physiology 2 (LGP2). All RLRs share a central helicase domain and a C-terminal domain (CTD), which are responsible for binding to the immunostimulatory RNA. RIG-I and MDA5 have two additional N-terminal tandem caspase activation and recruitment domain (CARDs) that
mediate the downstream signal transduction. LGP2 lacks the CARDs domain, making it lack signaling activity. Since RIG-I and MDA5 were discovered to induce type I IFNs during viral infection, it is now clear that RLRs are capable of recognizing viruses from all major viral families. Both RIG-I and MDA5 sense dsRNA, RIG-I recognizes the short dsRNA while the target of MDA5 is longer dsRNA.

IAV infection is strictly recognized by RIG-I. The previous study showed that RNA from IAV-infected cells is incapable of inducing IFNβ when transfected into RIG-I-deficient mouse embryonic fibroblasts while still potently instigating innate immune responses in wild-type or MDA5-deficient mouse embryonic fibroblasts. RIG-I is expressed in almost all nucleated cells and senses viral infection, mainly in epithelial cells, alveolar macrophages, and conventional DCs. Till now, abundant studies have unraveled the structural characteristics of immunostimulatory RNAs required for RIG-I activation. RIG-I recognizes blunt dsRNA terminus bearing a triphosphate (PPP) or diphosphate (PP) group at 5' end. This structure appears either in the viral genome or during replication by binding together the 5' and 3' end of ssRNA viral genome as a panhandle structure. RIG-I has also been reported to recognize DNA viruses such as herpesviruses associated with RNA polymerase III. In the case of IAV infection, although the viral genome is ssRNA, the panhandle structure formed between two ends of each genome fulfills the dsRNA requirement for RIG-I, and the IAV genome serves as the most crucial RIG-I agonist. In addition to the full-length viral genomic RNA, viral genome replication
intermediates, like defective interfering particles (DI) and mini viral RNA (mvRNA), which all contain the panhandle structure, also contribute to the RIG-I activation. At homeostatic state, RIG-I is in a signaling repressed, closed conformation with CARD2 inserted into the helicase domain. When bound to the RNA ligand, the helicase and CTD domains tightly wrap around the RNA and trigger the conformational changes that release the CARDs for interacting with the mitochondrial antiviral signaling protein (MAVS), which is the adaptor protein for RIG-I. RIG-I activation culminates in the activation of transcription factors IRF3/7 and NF-kB, inducing the production of IFNs and other antiviral or immunoregulatory cytokines.

Although RIG-I is primarily localized in the cytoplasm, it has also been reported within the nucleus and specific subcellular compartments, including mitochondria, microsomes, and mitochondria-associated membranes. A recent study discovered the genuine presence of nuclear-resident RIG-I, which exclusively senses IAV replication in the nucleus and induces IFN production. Interestingly, the nuclear RIG-I signals still require MAVS as the cytosolic RIG-I does. The study proposed that the interaction between nuclear RIG-I and MAVS might occur in the perinuclear region, where the greater permeability of the nuclear membrane allows for their intimate contact. Another study revealed that cytoplasmic RIG-I detects viral RNAs in the cytosol and triggers the type I IFNs production in the early time of infection. In contrast, at the late stage of viral infection (after 10 h post-infection of IAV), RIG-I translocates to the nucleus where it interacts with
the DNA repair enzyme Apurinic/Apyrimidinic Endodeoxyribonuclease 1 (APEX1) to induce apoptosis of virus-infected cells.\textsuperscript{159}

1.2.3. **RIG-I signaling and its regulation**

RIG-I is essential for sensing IAV and the process of innate or adaptive immunity, apoptosis, cytokine signaling, and inflammation associated with the host response to different IAV stains.\textsuperscript{160} In addition, mice deficient in the RIG-I-MAVS pathway displayed delayed viral clearance and reduced polyfunctional T-cell responses against IAV.\textsuperscript{161} Furthermore, the NS1 protein of IAV has evolved to suppress RIG-I signaling.\textsuperscript{143,162} These studies indicate that RIG-I plays a key role in innate responses to IAV infection. Thus, our study mainly focuses on the RIC-I pathway during IAV infection (Fig. 2).

While in the inactivated state, RIG-I is auto-repressed by locking the CARDs. Once engaged with RIG-I agonists, conformational changes liberate CARDs, followed by RIG-I oligomerization. RIG-I oligomers are then stabilized by K63-linked ubiquitination, and ubiquitin chains are attached to the outer rim of 2CARD tetramer.\textsuperscript{163} This step is crucial for the downstream binding of the MAVS along with the signal transduction.\textsuperscript{164} It is well known that the tripartite motif containing 25 (TRIM25) mediates K63-linked ubiquitination to RIG-I CARD.\textsuperscript{165} Further studies revealed Riplet also ubiquitinates RIG-I in the K63-linked manner, a prerequisite for TRIM25 to activate RIG-I signaling.\textsuperscript{166} Currently, other E3 ligases TRIM4 and Mex-3 RNA binding family member C (MEX3C) have been reported
to mediate the K63-linked ubiquitination of RIG-I as well \(^{167}\). Then, activated RIG-I relocates to the mitochondria and mitochondrial-associated membranes for interaction with MAVS. A previous study showed that 14-3-3ε protein, one of seven isoforms of the 14-3-3 protein family, forms a complex with RIG-I and TRIM25 and mediates their translocation to MAVs from cytoplasm to mitochondrial \(^{168}\). The binding of RIG-I and MAVS is through their CARDs domains, leading to the activation and oligomerization of MAVS, forming MAVS filaments \(^{169}\). This MAVS assembly acts as a platform for various signal cascades and propagates downstream signaling. Several TNFα-associated recruitment factors (TRAFs), which are a family of 7 proteins containing E3 ligase domains, including TRAF2, TRAF3, TRAF5, and TRAF6 are then recruited to MAVS \(^{170}\). TRAF2/5/6 are essential to recruit and activate the IkB kinase (IKK) complex, while TRAF2/3/5/6 are responsible for TANK-binding kinase 1 (TBK1) in a ubiquitination-dependent manner \(^{170}\). Activated IKK complex and TBK1 subsequently result in activating of transcriptional factors NF-κB and IRF3/7, respectively. Activated transcriptional factors translocate to the nucleus and induce the production of type I IFNs and proinflammatory cytokines (Fig. 2).

RIG-I signaling needs to be precisely regulated to initiate an effective antiviral defense at the right time while staying immune-suppressed in normal physiological conditions. Several mechanisms are involved in controlling the RIG-I activity, including post-transcriptional modifications (PTMs), host binding proteins, cellular non-coding RNAs. First, PTMs regulate the RIG-I signaling. Above mentioned several E3 ligases mediate
K63-linked ubiquitination to RIG-I activation, while deubiquitylating enzymes such as ubiquitin specific peptidase 3 (USP3), USP21, and CYLD lysine 63 deubiquitinase (CYLD) remove K63 polyubiquitin chains from RIG-I \(^{167}\). In addition, RIG-I is degraded by K48-linked ubiquitination via E3 ligases like SHP2/c-Cbl complex, ring finger protein (RNF122), and RNF125 \(^{171-173}\). On the other hand, one study revealed that the E3 ligase FBXW7 translocates from the nucleus to the cytoplasm to stabilize RIG-I by disrupting the SHP2/c-Cbl complex mediated RIG-I degradation during viral infections \(^{174}\). USP4 and USP15 also protect RIG-I and TRIM25, respectively by cleaving K48-linked ubiquitination to these molecules \(^{175, 176}\). Besides ubiquitination, phosphorylation is crucial for regulating the RIG-I pathway. In the autorepressed state, both the CARDs (at S8 and T170) and the CTD (at T770 and S854/855) of RIG-I are kept phosphorylated to avoid premature signaling before infections \(^{177}\). Studies showed conventional isoenzymes of PKC (PKC\(\alpha\) and PKC\(\beta\)) and casein kinase II (CKII) phosphorylate RIG-I at the CARDs and the CTD, respectively \(^{178, 179}\). Following viral infections, RIG-I CARDs must be dephosphorylated before the TRIM25-mediated RIG-I activation. Protein phosphatase 1 (PP1) is responsible for this process \(^{180}\). A recent study further revealed that PP1 specifically dephosphorylates RIG-I is mediated by F-actin-residing PP1-regulatory protein PPP1R12C. Viral infection like IAV induces actin cytoskeleton disturbance which brings PPP1R12C to the cytoplasm and forms a complex together with RIG-I and PP1 \(^{181}\). Other PTMs, like acetylation, are also implicated in the RIG-I regulation. Acetylation of RIG-I in the CTD domain inhibits its binding with viral RNAs and the subsequent oligomerization. Conversely, histone deacetylase 6 (HDAC6)
induces the CTD deacetylation conferring the ability of RIG-I for activation. A new form of PTMs called ufmylation, which is the ubiquitin-like modifier conjugation system, has been reported to be required in RIG-I activation. The E3 ligase of ufmylation UFL1 interacts with both RIG-I and protein 14–3-3ε and induces 14–3-3ε ufmylation following viral infections, which is necessary for the interaction of RIG-I and 14–3-3ε as well as the further binding of RIG-I and MAVS.

RIG-I binding proteins also modulate each step of RIG-I activation. It has been reported that dsRNA binding proteins modulate the RNA-binding ability of RIG-I, including PACT, the zinc-finger protein ZCCHC3, DEAH-box helicase 15 (DHX15), and DExD/H-box helicase 60 (DDX60). These proteins interact with RNA binding domains of RIG-I or RNA ligands to promote RIG-I activation. TRIM25-mediated K63-linked ubiquitination of RIG-I is also under tight regulation. The NDR2 kinase and caspase 12 bind RIG-I and TRIM25, stabilizing this complex and facilitating the K63-linked ubiquitination 185,186. By contrast, one reticulon member RTN3 is upregulated during viral infection, and its interaction with both RIG-I and TRIM25 impairs K63-linked polyubiquitination 187. NLRP12 interacts with TRIM25 to block its ability of ubiquitination, resulting in promoting RNF125-mediated K48-linked ubiquitination and degradation of RIG-I. During viral infection, NLRP12 is downregulated, allowing K63-linked ubiquitination and subsequent activation of RIG-I 188. In addition, long non-coding RNAs (lncRNAs) and miRNAs, mainly produced in viral infection and IFN stimulation, have emerged as
important regulators of RIG-I\textsuperscript{138}. Although mechanisms are diverse, IncRNAs and miRNAs are thought to either promote or suppress the RIG-I activation by regulating the gene expression of RIG-I or other key molecules in the RIG-I pathway\textsuperscript{138}. For example, miR-485 targets the RIG-I mRNA for degradation\textsuperscript{189}. Lnczc3h7a directly binds to TRIM25, promoting K63-linked ubiquitination to RIG-I\textsuperscript{190}. 
IAV RNA can be sensed by RIG-I. Once engaged with viral RNA, RIG-I is activated and oligomerized by K63-linked ubiquitination. E3 ligases such as TRIM25, Riplet mediate K63-linked ubiquitination to RIG-I. Activated RIG-I then relocates to MAVS at the mitochondria assisted with 14-3-3ε protein. The interaction with RIG-I leads to activation
and oligomerization of MAVS, forming MAVS filaments which act as platforms of various signaling cascades. Several TNFα-associated recruitment factors (TRAFs) are then recruited to MAVS including TRAF2, TRAF3, TRAF5 and TRAF6. TRAF2/5/6 are essential to recruit and activate IkB kinase (IKK) complex while TRAF2/3/5/6 are responsible for TANK-binding kinase 1 (TBK1). Activated IKK complex and TBK1 subsequently result in activating of transcriptional factors NF-κB and IRF3/7, respectively. Activated transcriptional factors translocate to the nucleus and induce the production of type I IFNs and proinflammatory cytokines.

1.3. Influenza antagonizes host innate immunity

The innate immune system employs a plethora of mechanisms to subvert IAV infection. However, IAV also extensively interferes with innate immune signaling pathways to successfully establish the infection. The RIG-I signaling is crucial in the innate immune responses against IAV infection. Thus, here we summarize current knowledge regarding how viral proteins NS1, PB2, PB1-F2, and PA-X suppress RIG-I pathways.

1.3.1. NS1

The NS1 protein of IAV is encoded by genome segment eight as a continuous
transcript. Another viral protein from the same genome is non-structural protein 2 (NS2), which is a splicing transcript. As the main non-structural protein of IAV, NS1 is highly expressed in infected cells. NS1 contains 230-237 aa depending on the viral strain, which is divided into two main domains: the N-terminal RNA binding domain (RBD, 1-73aa) and the C-terminal effector domain (ED, 88-202aa). These two domains are linked by 10-15 aa, known as the linker domain, and the ED is followed by a C terminal tail (CTT) which is 11-33aa in length and naturally disordered. The RBD domain mediates NS1 dimerization that is required for dsRNA binding. Interactions between EDs via the 187aa also lead to the oligomerization of NS1. The ED domain is predominantly responsible for interactions with host cell proteins. The conformation of CTT can be changed upon binding to interaction proteins or due to PTMs. NS1 contains one or two nuclear localization sequences (NLS1 and NLS2) and a nuclear export sequence (NES). Viral strains with NLS2 also form a nucleolar localization signal (NoLS). In infected cells, NS1 primarily localizes in the nucleus, and a significant proportion is also found in the cytoplasm, especially at the late stage of the viral infection.

NS1 is a multifunctional protein involved in various viral and cellular processes, including controlling viral RNA synthesis and mRNA splicing, suppressing the host innate immune responses, regulating apoptosis, and engaging with multiple cellular pathways to successfully establish viral infection. Although the functions of NS1 are strain-specific, NS1 is widely regarded as the main antagonist against innate immune responses for all
IAV strains, for IAV unable to express NS1 (delNS1) induces a large amount of IFN \(^{193}\). There are several mechanisms by which NS1 inhibits IFN production and innate immune responses.

(1) NS1 inhibits the IFN mRNA production

As discussed before, the key to the innate immune response is pattern recognition, ultimately inducing the transcription of IFNs and proinflammatory cytokines. Various PRRs sense IAV invading. Among them, RIG-I is the most crucial for IAV-induced IFN production. As the main IFN antagonist, NS1 has been reported to interact with a plethora of components or regulators of the RIG-I signaling pathway to inhibit the IFN transcription.

NS1 directly binds RIG-I or interacts with E3 ligase TRIM25 or Riplet, impairing K63-linked ubiquitination and activation of RIG-I \(^{162, 194-196}\). One of RIG-I activators, PACT, which regulates the dsRNA binding ability of RIG-I as mentioned before, is the target of NS1, thus preventing the interaction between PACT and RIG-I \(^{197, 198}\). Moreover, NS1 interacts with CCAAT/Enhancer binding protein beta (hC/EBPβ) and promotes hC/EBPβ phosphorylation which negatively regulates RIG-I transcription, thus reducing the RIG-I expression \(^{199}\). In addition, NS1 binds 14-3-3ε to prevent the translocation of RIG-I to MAVS \(^{200}\). Furthermore, NS1 regulates the RIG-I pathway by interacting with components downstream of MAVS. For example, NS1 binds TNF receptor-associated factor 3 (TRAF3) as well as IKK complex to inhibit IRF3 and NF-kB activation, respectively \(^{170, 201, 202}\). These NS1-mediated inhibitory activities are all related to the upstream of IFN mRNA
transcription.

(2) NS1 inhibits the IFN mRNA transportation to the cytoplasm

Another common strategy NS1 employed to inhibit IFN production in many IAV strains is preventing nuclear-cytoplasmic transportation of RNA polymerase II transcripts which are host cellular mRNAs. The nuclear retention of host mRNAs could provide cap donors for the use of viral mRNAs. More importantly, blocking cytoplasm processing of host mRNAs is an effective way to suppress various basic cellular activities, including innate immune responses against viral infections.

The inhibitory process is achieved by several mechanisms. First, NS1 interacts with the 30 kDa subunit of cleavage and polyadenylation specificity factor (CPSF30) and poly(A)-binding protein II (PABII). The NS1-CSF30 complex prevents the binding of host pre-mRNAs to CSF30, therefore, suppressing normal cleavage and polyadenylation of the 3’ end of host pre-mRNAs. A/Puerto Rice/8/34 (PR8) IAV fails to interact with CSF30 due to substituting F103 and M106 into other amino acids at these two residues.

Furthermore, the interaction between NS1 and PABII inhibits two functions of PABII which are to stimulate the processive synthesis of long poly (A) tails for the elongation of short poly(A) tails and to export cellular mRNAs. If host pre-mRNAs escape the repression mediated by the NS1-CSF30 complex, the NS1-PABII complex then specifically suppresses their maturation and nuclear export. Second, the direct binding of NS1 to the 3’ poly (A) tail of mRNAs enables the nuclear retention of host mRNAs. Although viral
mRNAs have a poly (A) tail, their activities are not influenced. Third, NS1 forms an inhibitory complex with NXF1/TAP, p15/NXT, Rae1/mrnp41, and E1B-AP5, which are the core constituents of the mRNA export machinery directing mRNAs through the nuclear pore complex. These interactions lead to the blocking of the nuclear export of fully processed host mRNAs.

(3) NS1 inhibits ISGs

2'-5'-oligoadenylate synthetase (OAS) is a cytoplasmic ISG and is activated by dsRNA. Activated OAS induces the production of poly (A) chains which subsequently activate RNase L. RNase L cleaves and degrades viral RNAs, thus inhibiting the virus replication. Furthermore, cleavage products of RNase L bind to and activate RIG-I, leading to the production of IFN. However, NS1 competes with OAS for binding dsRNAs, thus inhibiting the activation of OAS and its antiviral activities.

Protein Kinases R (PKR) is another antiviral protein activated by dsRNA or PACT, releasing PKR from auto-inhibition and leading to phosphorylation of its substrates, including the eukaryotic translation initiation factor 2a (eIF2a). Phosphorylated eIF2a inhibits protein synthesis of both cellular and viral proteins. NS1 binds to a linker region of PKR which prevents the conformational change required for PKR activation, thus escaping the PKR-mediated translation inhibition.
1.3.2. PB2

PB2 is a component of IAV viral RNA-dependent RNA polymerase (RdRp), which is responsible for viral transcription and genome replication. The other two subunits are PB1 and PA. As a component of the polymerase complex, PB2 is essential for binding the m7G cap structure of the host mRNA in the cap-snatching process. Cap snatching refers to the process in which IAV steals caps from host mRNAs to prime the viral transcription, for it not having a cap-synthesizing machinery. Cap snatching involves four steps: first, PA associates with the C-terminal domain (CTD) of a serine-5 phosphorylated cellular RNA polymerase II (Pol II); second, PB2 binds 5'-methylated caps of nascent host transcripts through the cap-binding domain; third, PA endonuclease domain cleaves 10-13 nucleotides downstream of the 5' cap; lastly, the PB2 cap-binding domain shifts the conformation to position capped RNA primer to the PB1 catalytic center where it is elongated using vRNA as the template. In addition, IAV PB2 is a key determinant of the host range. Host adaption requires viruses not only to bind to the specific cell receptors but also to adapt to host machinery for genome replication and protein synthesis. The 627 site of PB2 is a dominant adaption marker facilitating the IAV replication in specific host species, a lysine present in mammalian isolates, while the glutamic acid in avian influenza virus, indicates that PB2 is an important viral virulence. Recent studies reveal that PB2 also antagonizes host innate immune signaling.
pathways, thereby promoting IAV infection. For example, PB2 interacts with the mitochondrial antiviral signaling protein (MAVS) and inhibits MAVS-mediated beta interferon (IFNβ) production \(^{225-227}\). PB2 also interferes with K63-linked ubiquitination of TNF receptor-associated factor 3 (TRAF3) and the formation of MAVS-TRAF3 complex, thus, suppressing IFNβ production \(^{228}\). Moreover, PB2 is recently reported to be involved in the janus kinase/signal transducer and activator of transcription (JAK/STAT) signal transduction pathway, which mediates the functions of IFNs. The binding of IFNs to the cognate IFN receptor (IFNAR) induces JAK1 and tyrosine kinase 2 (Tyk2) to phosphorylate STAT1/STAT2. The activation and translocation of STAT1/STAT2 into the nucleus further trigger the transcription of various IFN-stimulated genes (ISGs). This study reveals that PB2 directly ubiquitinates and degrades JAK1 thus negatively regulating IFNs response \(^{229}\).

1.3.3. PB1-F2

PB1-F2 is a small nonstructural protein encoded by an alternate (+1) reading frame within the PB1 gene \(^{230, 231}\). The PB1-F2 protein is not in all IAV strains, and the length, amino acid sequence, as well as subcellular localization, vary in different strains, contributing to the strain-specific pathogenicity. PB1-F2 was first found to localize in the mitochondria and induce cell death \(^{230}\). Interestingly, the mitochondrial localization of PB1-F2 is strain-dependent \(^{232}\). Recent works further found that PB1-F2 blocks the RIG-I
signaling pathway, modulates NLRP3 inflammasome activation, and enhances viral polymerase activity.

The first ascribed function of PB1-F2 is inducing apoptotic cell death in immune cells \(^{230}\). Current studies have further confirmed that the property of PB1-F2 to induce apoptosis is exclusive for IAV strains with the mitochondrial localization and mainly sensitive in cell types like macrophages and monocytic U937 or RAW264.7 cells \(^{232, 233}\). PB1-F2 translocates into the mitochondria inner membrane through Tom40, the import channel of the mitochondrial outer membrane \(^{131}\). The proapoptotic function of PB1-F2 in immune cells is proposed to be the result of its interaction with two mitochondrial membrane proteins, voltage-dependent anion channel po1 (VDAC-1) and adenine nucleotide translocator 3 (ANT3) \(^{231}\).

Besides inducing apoptosis, PB1-F2 is found to regulate innate immune responses. The outer membrane of the mitochondria is the signaling platform for MAVS, the downstream effector of RIG-I. Recent studies show that the mitochondrial PB1-F2 suppresses viral RNA-elicited innate immunity by modulating MAVS in the mitochondria. PB1-F2 localizes to the mitochondria’s inner membrane space and causes the attenuation of membrane potential, which results in the suppression of RIG-I signaling \(^{131}\). PB1-F2 is also found to bind the transmembrane domain of MAVS and dissipate the mitochondrial membrane’s potential \(^{234, 235}\). However, it is not clear whether there is a correlation between MAVS binding and membrane potential. Additionally, PB1-F2 interacts with IKKβ and
inhibits NF-kB activation. PB1-F2 also interacts with CALCOCO2, a TBK1-binding protein, and inhibits TRAF2/3-mediated IFN induction. Although PB1-F2 subverts host IFN production signaling pathways, PB1-F2 modulates NF-kB signaling and promotes the expression of pro-inflammatory cytokines through TRAF6. PB1-F2 not only regulates the RIG-I signaling but also modulates the inflammasome activation, as discussed in the NLRP3 part of section 1.2.2. The comparison of wild type and PB1-F2 deletion IAV found that PB1-F2 expression induces the expression of genes linked to cell death, inflammatory response, and neutrophil chemotaxis. The aberrant activation of innate immunity, such as pro-inflammatory cytokine, is an indicator of severe infection with high pathogenic IAVs. Therefore, it will be of great interest to elucidate the pathogenic role of PB1-F2 in the aberrant innate immune responses and the underlying mechanisms in the future.

PB1-F2 of PR8 IAV is also reported to have a nuclear localization and interact with PB1, resulting in an increased PB1 retention in the nucleus and enhanced polymerase activity. However, many natural isolates of IAV, such as the 2009 H1N1pdm, completely lack a PB1-F2 open reading frame (ORF) due to multiple stop codons. Furthermore, recent studies found a minimal effect of PB1-F2 on viral replication in cultured cells and in mice, suggesting that PB1-F2 is not essential for viral polymerase activity.
1.3.4. PA-X

PA-X is an accessory viral protein that is translated as a +1 frameshift ORF from the PA segment\textsuperscript{244}. During translation, the ribosome shifts to a uracil (U)-rich stretch, followed by a rare codon in the PA mRNA\textsuperscript{245}. The rare codon is decoded more slowly, thereby promoting ribosomal frameshifting. The one nucleotide frameshift results in the generation of PA-X that comprises the same first N-terminal 191 amino acids of PA protein and a unique short C-terminus extension containing 41 or 61 amino acids. The frameshift motif is highly conserved, and most IAV PA-X proteins have a 61 amino acid extension\textsuperscript{246}. Of note, the PA-X of 2009 H1N1pdm only encodes a 41 amino acids extension\textsuperscript{246}.

An important function of PA-X promoting the IAV infection is to shut off host translation\textsuperscript{244, 247}. With the N-terminal endonuclease domain of PA, PA-X possesses endonucleolytic activity and the ability to degrade RNA\textsuperscript{244, 247}. Interestingly, PA-X selectively degrades host RNA Pol II transcripts and non-coding RNAs, while it has no effect on the production of pol I and pol III and viral mRNA\textsuperscript{248, 249}. What determines the selectivity is not very clear, and it may be dependent on cellular factors. A study found that the complete degradation of host mRNAs following PA-X-mediated endonucleolytic cleavage is also dependent on the cellular 5'->3'-exonuclease Xrn1\textsuperscript{248}. Moreover, a recent study further showed that PA-X usurps RNA splicing machinery to selectively target host nascent mRNA for destruction\textsuperscript{250}.

As the shutoff of host translation is a viral evasion strategy, it is reasonable to predict
that PA-X suppresses the expression of host innate immune responses. Indeed, PA-X frameshift mutant viruses induce higher IFNs and pro-inflammatory cytokines than their wild-type counterparts. A recent study showed that the PA-X-deficient virus elevates the expressions of Ifnb1 and Ifna4 in the mouse lungs in a MAVS-dependent manner. However, the loss of PA-X increases IAV virulence in several animal models, including mice, chickens, and ducks. These studies suggest that the expression of PA-X decreases viral pathogenicity. There are a couple of explanations. One is that PA-X shuts off host innate immune responses, especially the expression of pro-inflammatory cytokines. The other one is that PA-X might affect viral replication. Two recent studies showed that PA-X inhibits viral polymerase activity. However, how PA-X modulates viral RNA-dependent RNA polymerase (vRdRp) activity is not clear. In addition, a recent study revealed that PA-X interacts with the host protein ankyrin repeat domain 17 (Ankrd17), a positive regulator of inflammatory responses via the retinoic acid-inducible gene-I (RIG-I)-like receptor (RLR) signaling pathway and inhibits Ankrd17-mediated innate immune responses to IAV infection. It is also worthy of note that a study showed that the loss of PA-X in an avian H9N2 virus results in decreased virulence in mice.
1.4. Perspective

1.4.1. Outstanding questions for IAV NS1

NS1 is the most well-studied viral protein of IAV. Hundreds of studies have revealed its multiple functions and various mechanisms under these functions to establish infections in host cells successfully. For example, besides functions of NS1 in innate immune response as described in section 1.3.1, NS1 interacts with the host protein DEAD (Asp-Glu-Ala-Asp) box helicase 56 (DDX56) to promote IAV replication. In addition, NS1 activates the PI3K/Akt pathway to delay virus-induced apoptosis for sufficient time for virus replication. All functions of NS1 rely on its interaction with host proteins or protein-dsRNA complexes. The discovery of novel binding host factors will delineate a more diverse set of cellular signaling pathways that are potentially associated with viral pathogenicity.

As the well-known IFN antagonist, current studies on the mechanisms by which NS1 inhibits innate immune responses are mainly in two directions: one is pre-transcriptional that NS1 interacts with cytoplasmic components of the RIG-I signaling, thus reducing the mRNA production of antiviral genes while another is post-transcriptional that NS1 suppresses the host mRNA processing and subsequent translations of antiviral genes. However, whether and how NS1 inhibits the exact transcription process of antiviral genes is not well elucidated. One study showed that the C-terminus of the NS1 protein of H3N2 IAV possesses a histone-like sequence-ARSK which is analogous to the first four amino
acids-ARTK of histone H3. The K within the NS1 ARTK sequence is also methylated, similar to the histone H3K4 methylation. The methylated NS1 interacts with the human PAF1 transcription elongation complex (hPAF1C), resulting in the suppression of the hPAF1C-mediated transcriptional elongation of host genes, including antiviral genes. This study sheds light on the role of NS1 in the transcription of antiviral genes. But whether NS1 inhibits the transcription process of IFN and antiviral genes in the RIG-I signaling since it’s the most crucial innate immune response against IAV infection still remains unknown. And NS1 might interact with other host transcriptional-related factors or complexes to regulate the host transcription.

### 1.4.2. Significance for potential antiviral therapies targeting innate immunity

During IAV infection, innate immune responses are the first line of host antiviral defenses. Among them, the activation of the IFN system is the most powerful antiviral response. Innate immunity is considered a promising field to discover new antiviral therapies for the advantage of instigating a broader-spectrum and potent antiviral activity regardless of viral mutations.

RIG-I signaling is the most crucial response for inducing the IFN system against IAV infection. Current studies have reported some therapeutics focused on the RIG-I pathway. One study designed a short double-stranded RNA, named 3p-mNP14690siRNA, which is a siRNA targeting IAV NP gene and possessing a triphosphate group at the 5’ end of the
sense strand and with blunted ends. Thus, this designed RNA exerts dual functions: slicing IAV NP gene and activating the RIG-I mediated IFN production. As expected, the dsRNA potently inhibits IAV infection when transfected into cells and injected into mice. Another study identified small, drug-like molecule compounds which belong to hydroxyquinolines that can activate IRF3 and induce antiviral genes in treated cells against IAV infection. These studies indicate that RIG-I agonists or activators of other components in RIG-I pathways provide insights into developing new therapies targeting the general innate immune responses with little chance of inducing drug-resistant viruses. In this regard, therapies in this direction might be especially beneficial for some immunodeficiency individuals.

Current knowledge of innate immune responses also provides new insights into the IAV vaccine development. As recombinant IAVs with NS1 deletion are attenuated due to more robust host immune responses, they have been used for the development of a live-attenuated influenza vaccine. For instance, an attenuated PR8 virus with a truncated NS1 protects aged mice from a lethal viral challenge. The NS1-deficient influenza vaccine elicits a more robust immune response and has better protective efficacy than an inactivated virus vaccine in aged mice. Recently, a phase I/II clinical trial showed that an influenza virus vaccine lacking the NS1 is safe and induces high levels of antibodies. In addition, RIG-I and TLR7/8 agonists as the combination adjuvant of a licensed inactivated vaccine QIV induce higher antibody titers and enhanced T-cell
responses in mice 269.

The successful establishment of IAV infection relies on extensive interactions of viral proteins and host cellular factors. A comprehensive understanding of host-viral protein interactions will deepen our understanding of the pathogenesis of IAV, thus developing new antiviral therapies. The discovery of novel binding factors helps delineate the integrated map. Searching for drug or compound agonists of host factors that are required for the innate immune responses to IAV infection might be a potential antiviral therapy.
CHAPTER 2. FKBP5 REGulates RIG-I-MEDIATED NF-KB ACTIVATION AND IAV INFECTION.

2.1. Introduction

Influenza A virus (IAV) is a negative, single-stranded RNA virus that belongs to the Orthomyxoviridae family. IAV causes seasonal epidemics and has the potential for global pandemics. IAV first engages with host innate immunity and instigates several innate immune signaling pathways, including the Toll-like receptor 7 (TLR7) and RIG-I. RIG-I is a cytosolic RNA sensor that binds IAV RNA. The binding of RNA induces conformational change and several post-translation modifications, such as K63-linked polyubiquitination, which leads to the oligomerization of RIG-I. The oligomerized RIG-I further induces oligomerization of the mitochondrial antiviral signaling protein (MAVS, also known as CARDIF, IPS1, and VISA). The oligomerized MAVS acts as a signaling platform by the recruitment of IKK and TANK-binding kinase 1 (TBK1). IKK and TBK1 activate the transcription factors, nuclear factor kappa B (NF-kB) and interferon regulatory factor (IRF), respectively. Then, NF-kB and IRF translocate to the nucleus and form transcriptional complexes to activate type I interferon (IFN) expression. The type I IFN acts as a master cytokine that activates hundreds of interferon-stimulated genes, which in turn inhibits IAV infection.
FKBP5 (also known as FKBP51) belongs to the immunophilin family that consists of FKBP and the tetratricopeptide repeat (TPR) domain. The FKBP domain has peptidylprolyl isomerase (PPIase) activity that catalyzes the cis–trans conversion of peptidylprolyl bonds, a reaction that is important for protein folding. The TPR domains at the C terminus mediate protein–protein interactions. FKBP5 is a well-known molecular chaperone of peptidyl steroid hormone receptors, such as progesterone, androgen, and glucocorticoid receptors. In addition, FKBP5 regulates the activity of several kinases, such as AKT1, cyclin-dependent kinase (CDK), and IKK. FKBP5 acts as a scaffolding protein for AKT1 and promotes the dephosphorylation of AKT1, thereby downregulating the AKT signaling pathway. Recent studies found that FKBP5 interacted with CDK4. FKBP5 is required to maintain CDK4 levels in cancer cells. FKBP5 also promotes cis-trans isomerization of the Thr172-Pro173 peptide bond in CDK4 and inhibits phosphorylation of Thr172, an essential step for cell cycle exit and myoblast differentiation. FKBP5 is also found to play a role in NF-kB activation. FKBP5 acts as a scaffold by interacting with several components in the TNFα pathway, including IKKa, IKKb, IKKg, and TNF receptor-associated factor 2 (TRAF2); thus, activating the IKK. However, the role of FKBP5 in the context of RIG-I signaling and IAV infection is not well elucidated.

In this study, we found that knockout of FKBP5 in HEK293 and A549 cells increased susceptibility to IAV infection. Furthermore, FKBP5 preferentially bound IKKa and positively regulated RIG-I-mediated type I IFN activation. Overall, our study suggests that
FKBP5 restricts IAV infection by activation of RIG-I-mediated NF-κB signaling.

2.2. Materials and Methods

2.2.1. Cells and viruses.

HEK293 cells (ATCC, # CRL-1573) and MDCK cells (ATCC, # CCL-34) were maintained in Dulbecco’s Modified Eagle Medium (Life Technologies) containing Penicillin-Streptomycin (Life Technologies) and 10% fetal bovine serum (Life Technologies). A549 cells (ATCC, # CCL-185) were cultured in RPMI Medium 1640 (Life Technologies) plus 10% fetal bovine serum and 1X MEM Non-Essential Amino Acids Solution (Life Technologies).

Influenza A/Puerto Rico/8/34 (H1N1) was purchased from Charles River Laboratories (# 10100374). Influenza PR8-GLuc virus was a generous gift from Dr. Peter Palese and featured a Gaussia luciferase (Gluc) gene inserted downstream of PB2. IAV delNS1 was a gift from Dr. Adolfo Garcia-Sastre (Mount Sinai School of Medicine, NY). IAV was propagated in specific pathogen-free fertilized eggs Premium Plus (Charles River Laboratories, Wilmington, MA), as described by Szretter et al. Nine- to eleven-day-old embryonated chicken eggs are used for the production of influenza virus. 0.2 ml stock influenza virus at 1 × 10^3 TCID50 was injected through the puncture hole into the allantoic cavity. After 72 hr of incubation, allantoic fluid was collected. IAV titers were determined by plaque assay as described by Matrosovich et al. Briefly, 1.2 × 10^6 MDCK cells per
ml were split into 6-well plates. After 2X washes with DMEM, serial dilutions of IAV were adsorbed onto the cells for 1 hr. The cells were covered with DMEM containing 2 × Avicel RC591 NF (FMC Biopolymer, Philadelphia, PA) and 1 μg/ml TPCK-trypsin (Thermo Fisher Scientific, # 20233). Crystal violet staining was performed 48 h.p.i., and visible plaques were counted.

2.2.2. Constructs and Reagents.

FKBP5 was cloned into pCMV-3Tag-8 (Agilent Technologies, # 240203) to generate FKBP5-FLAG. Deletion mutants of FKBP5-FLAG were constructed using a Q5® Site-Directed Mutagenesis Kit (New England Biolabs). Human IKKα was cloned into pCMV-3Tag-8 to generate IKKα-HA and IKKα-Myc.

Anti-β-actin (Abcam, # ab8227), anti-FLAG (Sigma, # F3165), anti-HA epitope (Cell Signaling Technology, # 3724), anti-Myc (Bethyl Laboratories, # A190-105A), anti-IFITM3 (GeneTex, # GTX63349), anti-FKB5 (Abcam, # ab126715), anti-NS1 (Santa Cruz Biotechnology, # sc-130568), anti-NP (GenScript, # A01506-40), anti-IKKα (Cell Signaling Technology, # 11930S). Goat anti-Mouse IgG-HRP (Bethyl Laboratories, # A90-116P), Goat anti-Rabbit IgG-HRP (Bethyl Laboratories, # A120-201P).

2.2.3. Plasmid transfection.
Transfections using Lipofectamine 2000 or Lipofectamine 3000 transfection reagent (Life Technologies) were performed according to the manufacturer’s protocol. For co-immunoprecipitation (co-IP) experiments, a total of 2.5 µg of plasmids was transfected into approximately 1.2 x 10^6 cells. For other experiments, a total of 0.5 µg of plasmids was transfected into approximately 0.2 x 10^5 cells.

2.2.4. Sample preparation, Western blotting, and immunoprecipitation.

Approximately 1 X 10^6 cells were lysed in 500 µl of tandem affinity purification (TAP) lysis buffer [50 mM Tris-HCl (pH 7.5), 10 mM MgCl2, 100 mM NaCl, 0.5% Nonidet P40, 10% glycerol, Complete EDTA-free protease inhibitor cocktail tablets (Roche)] for 30 min on ice. The lysates were then centrifuged for 30 min at 15,000 x g. Supernatants were collected and mixed with 1X Lane Marker Reducing Sample Buffer (Thermo Fisher Scientific). Western blotting and immunoprecipitation were performed as described in a previous study.282

2.2.5. Proteomics analysis of FKBP5 protein complex.

Affinity purification coupled with mass spectrometry (AP-MS) experiments were performed as previously described.282 For protein purification, HEK293 cells stably expressing FLAG-tagged FKBP5 were collected and lysed in 10 ml of the TAP lysis buffer.
Cell lysates were pre-cleared with 50 μl of protein A/G resin before the addition of 20 μl of anti-FLAG resin (Sigma, # F2426) and incubated for 16 h at 4 °C on a rotator. The resin was washed three times and transferred to a spin column with 40 μl of the FLAG peptide for 1 h at 4 °C.

The purified samples were sent for mass spectrometry analysis. Proteins found in the control group were considered non-specific binding proteins. The SAINT algorithm (http://sourceforge.net/projects/saint-apms) was used to evaluate the MS data. Proteins with SAINT score < 0.89 or with < 3 peptide hits are considered non-specific binding proteins.

2.2.6. Real-time PCR.

Total RNA was prepared using the RNeasy Mini Kit (Qiagen, # 74106). 500 ng of RNA was reverse transcribed into cDNA using the ProtoScript First Strand cDNA Synthesis Kit (New England Biolabs, # E6300S). For one real-time reaction, 10 μl of SYBR Green PCR reaction mix (Eurogentec) including 2 mg of the synthesized cDNA plus an appropriate oligonucleotide primer pair, were analyzed on a 7500 Fast Real-time PCR System (Applied Biosystems). The comparative Ct method was used to determine the relative mRNA expression of genes normalized by the housekeeping gene GAPDH. The primer sequences: human GAPDH, forward primer 5’- AGGTGAAGGTCGGAGTCA-3’, reverse primer 5’-GGTCATTGATGGCAACAA-3’; human CXCL10 (IP10), forward primer 5’-
TTCAAGGAGTACCTCTCTCTAG -3’, reverse primer 5’-

CTGGATTACAGACATCTCTTCTC -3’; human CCL5 (RANTES) qPCR primers were purchased from Qiagen (# PPH00703B-200).

2.2.7. RNA sequencing.

Total RNA was prepared using the RNeasy Mini Kit (Qiagen, # 74106). RNA samples were sent to Novogene (Sacramento, CA) for sequencing. Each sample was sequenced to generate a minimum of 20 million reads. The paired-end reads were directionally mapped to the human genome (GRCh38/hg38) using TopHat2. Cufflink and CuffDiff analyses were performed to identify the differentially expressed genes using a fold change of ≥ 2 and an FDR of < 0.05.

2.2.8. CRISPR/Cas9.

The single guide RNA (sgRNA) sequence targeting human FKBP5 is 5’-ATCCGGAGAACAAACGGAA -3’. The sgRNA was cloned into LentiCRISPR v2 (Addgene). 0.5 µg of the lentiviral construct was transfected into HEK293 cells using Lipofectamine 2000. Cells were selected with 10 µg/ml puromycin for 14 days. Single clones were expanded for knockout confirmation by Western blotting and DNA sequencing.
2.2.9. Statistical analysis.

The sample size was sufficient for data analysis using paired two-tailed Student’s t-test. For all statistical analyses, differences were considered to be statistically significant at values of $P < 0.05$.

2.3. Results

2.3.1. Deficiency of FKBP5 increases host susceptibility to IAV infection.

To determine the role of FKBP5 in IAV infection, we first depleted FKBP5 in HEK293 cells by CRISPR. In this regard, a single guide RNA (sgRNA) was cloned into the lentiCRISPR v2 containing Cas9 and transfected into HEK293 cells. After 48 h, cells were selected using puromycin. Single clones were picked and expanded for knockout confirmation by Western blotting. FKBP5 expression was abolished in CRISPR knockout cells as shown by Western blotting (Fig. 3A). First, we infected FKBP5 wild type and knockout HEK293 cells with a PR8-Gaussia luciferase reporter virus, PR8-Gluc. The reporter assay showed that the PR8-GLuc reporter activity increased in FKBP5 knockout cells compared to the wild-type controls (Fig. 3B). In line with these results, the protein expression levels of NS1 and NP were significantly higher in knockout cells (Fig. 3C). To corroborate our observations in HEK293 cells, we further knocked out FKBP5 in human lung epithelial cells A549 by CRISPR (Fig. 3D). Similarly, deficiency of FKBP5 increased PR8-Gluc reporter activity (Fig. 3E) and viral protein expression levels (Fig. 3F). We also
evaluated the impact of FKBP5 deficiency on viral propagation of WSN IAV using the plaque assay. As predicted, FKBP5 knockout cells produced more infectious viral particles than control A549 cells (Fig. 3G). Taken together, these data suggest that endogenous FKBP5 is essential for host restriction to IAV.
Figure 3 Deficiency of FKBP5 increases host susceptibility to IAV infection.

(A) CRISPR knockout of FKBP5 in HEK293 cells. Cell lysates were blotted as indicated.

(B) Wild-type (WT) HEK293 and FKBP5 knockout (KO) cells were infected at MOI of 0.1 or MOI 1 with IAV PR8-Gluc for 24 h. Relative luciferase activities were examined. All
experiments were biologically repeated three times. *, $P < 0.05$. (C) FKBP5 wild type and knockout HEK293 cells were infected with the designated MOI of IAV PR8 for 24 h. Cell lysates were blotted using the indicated antibodies. (D) CRISPR knockout of FKBP5 in A549 cells. Cell lysates were blotted as indicated. (E) FKBP5 wild type and knockout A549 cells were infected at MOI of 1 or 10 with IAV PR8-Gluc for 24 h. Relative luciferase activities were examined. All experiments were biologically repeated three times. *, $P < 0.05$. (F) FKBP5 wild type and knockout A549 cells were infected with the designated MOI of IAV PR8 for 24 h. Cell lysates were blotted using the indicated antibodies. (G) Wild-type and FKBP5 knockout A549 cells were infected at an MOI of 0.1 with WSN IAV. After the designated hour post-infection (h.p.i.), virus titers were determined by plaque assay in MDCK cells. All experiments were biologically repeated three times.

2.3.2. FKBP5 is not an interferon-stimulated gene.

As IAV is intrinsically sensitive to IFN, we speculated that type I IFN might regulate FKBP5 expression. Therefore, we treated A549 lung epithelial cells with IFN-β or infected with a PR8 IAV mutant with an NS1 gene deletion (PR8 ΔNS1). FKBP5 protein expressions were then examined. However, IFN-β stimulation or IAV infection marginally modulated FKBP5 protein levels (Figures 4A and 4B), suggesting that FKBP5 is
constitutively expressed in A549 human lung epithelial cells.

Figure 4 FKBP5 expression is not modulated by IFN and IAV.

(A) A549 cells were treated with the designated amount of IFN-β for 12 h. Cell lysates were blotted with the indicated antibodies. The interferon-induced transmembrane protein 3 (IFITM3) was included as a positive control. (B) A549 cells were infected with 1 MOI of PR8 ΔNS1 IAV for designated times. Cell lysates were blotted with the indicated antibodies.
2.3.3. **FKBP5 interacts with IKKa.**

To discover new FKBP5-interacting proteins, we performed the AP-MS analysis of FKBP5 protein complex. First, FLAG-tagged FKBP5 was transfected into HEK293 cells to generate a stable cell line. After the stable cell line was established by puromycin selection, FKBP5 protein complexes were purified by affinity purification using the anti-FLAG antibody and then were analyzed by mass spectrometry. The AP-MS was biologically repeated twice. To efficiently reduce false positives in AP-MS, we adopted the well-established statistical method SAINT \(^{284}\). Using a stringent statistical SAINT score cutoff of 0.89 (\(P < 0.01\)), we identified 22 high-confidence candidate interacting proteins (HCIPs), including the IKK complex, CDKs, and heat shock proteins (Fig. 5A). IKK has been shown to interact with FKBP5; however, whether the endogenous FKBP5 interacts with IKK is not clear. First, we performed co-IP between FKBP5 and IKKa or IKKb. In this regard, FLAG-tagged FKBP5 was co-transfected with Myc-tagged IKK into HEK293 cells. Interestingly, co-IP found that FKBP5 preferentially interacted with IKKa (Fig. 5B). Furthermore, we examined the endogenous protein interaction between FKBP5 and IKKa. We stimulated HEK 293 cells with the viral RNA mimics, poly(I:C), and then performed IP using an anti-FKBP5 antibody. As shown in Figure 5C, endogenous FKBP5 interacted with endogenous IKKa. Furthermore, poly(I:C) enhanced the IKKa-FKBP5 interaction. Lastly, we determined which FKBP5 domain (Fig. 5D) was required for IKKa interaction. Co-IP demonstrated that the TPR domain was sufficient for the interaction with IKKa (Fig. 5E).
Figure 5 FKBP5 interacts with IKKa.

(A) Map of the FKBP5 protein interaction network. FKBP5 and the high confidence candidate interacting proteins (HCIPs) are shown as square and circles, respectively. (B) FLAG-tagged FKBP5 was co-transfected with Myc-tagged IKKa or IKKb into HEK293 cells. After 48 h, cell lysates were immunoprecipitated and blotted as indicated. (C) HEK 293 cells were stimulated with 1 μg/ml poly(I:C) for 4 h. Cell lysates were immunoprecipitated and blotted as indicated. The same isotype IgG was used as a control for IP. (D) Schematics of FKBP5 mutants. FK stands for FKBP; TPR stands for tetratricopeptide.
repeats. (E) HA-tagged IKKa was transfected with pCMV-3Tag-8 vector, FLAG-tagged FKBP5, or the indicated FLAG-tagged FKBP5 mutant. After 48 h, cell lysates were immunoprecipitated and blotted as indicated.

2.3.4. FKBP5 is required for RIG-I-induced NF-κB activity.

RIG-I is a sensor to IAV RNA and activates IKK and TBK1 signaling pathways to induce type I IFN expression. FKBP5 has been shown to be required for IKK activity; however, to our knowledge, whether FKBP5 is critical for RIG-I-IKK signal axis is unknown. Thus, we first stimulated FKBP5 wild type and knockout HEK 293 cells with poly(I:C) and examined the mRNA expression of two ISGs, IP-10 and RANTES. Real-time PCR assays showed that FKBP5 deficiency impaired poly(I:C)-induced mRNA expression of IP-10 (Fig. 6A) and RANTES (Fig. 6B). RNA sequencing further revealed that mRNA expression of IFNβ and several ISGs were reduced in FKBP5 knockout cells (Fig. 6C). To complement the results in FKBP5 knockout HEK293 cells, we also examined FKBP5 knockout A549 cells. Consistently, poly(I:C)-induced mRNA expression of IP-10 (Fig. 6D) and RANTES (Fig. 6E) was also impaired in FKBP5 knockout A549 cells. Overall, these data suggest that FKBP5 is required for the RIG-I signaling pathway.
Figure 6 FKBP5 is required for RIG-I signaling.

(A-B) FKBP5 wild type and knockout HEK293 cells were stimulated with 1 μg/ml poly(I:C) for indicated times. Real-time PCR was performed to determine the relative mRNA levels of IP-10 (A) and RANTES (B). All experiments were biologically repeated three times. Data represent means ±s.d. of three independent experiments. The P value was calculated (two-tailed Student’s t-test) by comparison with wild type cells. An asterisk indicates $P < 0.05$. (C) Heatmap of the RNA sequencing results of wild type and FKBP5 knockout HEK293 cells stimulated with 1 μg/ml poly(I:C) for 4 h. (D-E) FKBP5 wild type and knockout A549 cells were stimulated with 1 μg/ml poly(I:C) for indicated times. Real-time PCR was performed to determine the relative mRNA levels of IP-10 (D) and RANTES...
(E). All experiments were biologically repeated three times. Data represent means ±s.d. of three independent experiments. The P value was calculated (two-tailed Student’s t-test) by comparison with wild type cells. An asterisk indicates $P < 0.05$.

2.4. Discussion and Future Directions

The pattern recognition receptors (PRRs) are the sentinels of the innate immune system, which discriminate microbial components distinct from the host. RIG-I is a cytosolic PRR and recognizes double-stranded RNA (dsRNA) or 5′ triphosphate RNA in the cytoplasm generated by RNA viruses. Upon the engagement with viral RNA, RIG-I activates signaling cascades that lead to the induction of type I IFN expression. Type I IFN activates the mRNA expression of hundreds of ISGs, Most of ISGs are antiviral proteins, such as IFITM3 and Viperin. In addition to ISGs, a group of antiviral host factors, termed intrinsic immunity factors, are constitutively expressed and IFN-independent. The “pre-existed” expression of these intrinsic host factors guarantees a more rapid response and a direct inhibition of viral infection. For example, TRIM41 targets the nucleoproteins of IAV and vesicular stomatitis virus (VSV) for K48-linked polyubiquitination and protein degradation, thereby impeding IAV and VSV infection. Here, we report that FKBP5 participates in RIG-I-mediated NF-κB signal cascade that is essential for type
I IFN expression.

Our study suggests that FKBP5 regulates NF-kB via the IKK complex. However, FKBP5 might also modulate NF-kB activity indirectly through steroid receptors. For example, glucocorticoids are potent anti-inflammatory agents by activating glucocorticoid receptor (GR) \(^{294}\). A study showed that FKBP5 cooperated with another immunophilin FKBP4 (also known as FKBP52) to modulate GR-mediated NF-kB suppression \(^{295}\). Overexpression of FKBP5 inhibits NF-kB nuclear translocation and NF-kB transcriptional activity. By contrast, FKBP4 counteracts the effects of FKBP5 \(^{295}\). Although their conclusion is mainly based on overexpression, it suggests that optimal expression of FKBP5 might be required for NF-kB activation.

Many genes involved in innate immunity are ISGs; however, we found that FKBP5 expression was not induced by IFN and IAV in A549 cells. Our data suggest that FKBP5 is constitutively expressed. A recent study found that the expression of FKBP5 was induced by glucocorticoid in A549 cells, which facilitates the suppressive effect of glucocorticoid on pro-inflammatory cytokine production \(^{296}\). Further studies will investigate the differential role of FKBP5 in RIG-I and glucocorticoid pathways.

The NS1 of IAV inhibits host innate immune defenses through diverse mechanisms \(^{290}\). First, NS1 inhibits the cellular double-stranded RNA sensors, including RIG-I, protein kinase R, and 2′-5′ oligoadenylate synthetase. Furthermore, NS1 blocks the regulators of these sensors, such as PRKRA, TRIM25, and NF90. A recent study also found that NS1
bound the kinase domain of IKKa/b and impaired their kinase activity, thereby inhibiting NF-κB activity. However, whether NS1 antagonizes the function of FKBP5 is unknown. Future studies will investigate the interactions between NS1 and FKBP5.

On the host side, erratic activation of NF-kB is detrimental due to the overexpression of proinflammatory factors. Thus, feedback control of NF-kB activity is essential. Recently, the IFN-induced protein 44 (IFI44) was found to negatively modulate NF-kB activity induced after viral infections. Interestingly, IFI44 inhibits IKK kinase activity through binding FKBP5. It is worthy to note that NF-kB activity is also required for efficient viral replication of IAV. Blocking of NF-kB activity using inhibitors impaired IAV infection in cells. However, what NF-kB-dependent genes are activated, how they are specifically activated by viruses, and how they facilitate viral infection are still not well elucidated. Nonetheless, virus knows how to skew the balance of NF-kB activity to achieve the ideal environment for viral replication, and at the same time, suppress host innate immune responses. Future work will pinpoint the mechanisms of how IAV gains the control to precisely induce NF-kB-dependent genes required for viral infection.

In conclusion, FKBP5 is not only required for the RIG-I-IKK signaling axis for type I IFN production but also inhibits IAV infection. Our study suggests that FKBP5 is a novel anti-influenza gene. Thus, our study provides a foundation for future development of antiviral therapeutics.
CHAPTER 3. INFLUENZA A VIRUS NS1 PERTURBS SNW1-HOIP TRANSCRIPTIONAL REGULATOR COMPLEX TO ATTENUATE INNATE IMMUNE RESPONSES

3.1. Introduction

IAV is a highly contagious respiratory pathogen and causes seasonal epidemics and occasional worldwide pandemics. The IAV control mainly relies on annual immunizations that are not always effective, making it urgent to discover new anti-IAV strategies. Viruses rely on host proteins to fulfill viral life cycles and perturb host defenses to escape immune surveillance. Thus, the understanding of host-IAV interactions will greatly help reveal IAV pathogenesis and be invaluable in identifying potential therapies by targeting these interactions.

IAV NS1 is a multifunctional protein and is widely regarded as the viral factor to antagonize host innate immune responses. However, the mechanisms by which NS1 suppresses interferon (IFN) production are not fully understood. Our recent study on the IAV NS1-host protein interaction network found the host factor SNW domain-containing protein 1 (SNW1) in the NS1 protein complex. SNW1, also termed NCoA62, SKIP in vertebrates, Prp45 in *Saccharomyces cerevisiae*, and BX42 in *Drosophila melanogaster*, is a nuclear protein containing the highly conserved SNW domain.
is the abbreviation of Ski interacting protein, and NCoA62 means SNW1 is a nuclear receptor coactivator of 62 kDa \(^{306, 307}\). The critical functions of SNW1 are as a spliceosome component and a transcriptional regulator involved in various cellular processes. It is well established that SNW1 cooperates with Notch and nuclear receptors, such as vitamin D receptor and androgen receptor, to mediate transcription \(^{308-312}\). In addition, SNW1 promotes HIV Tat transcription elongation by interacting with p-TEFb elongation factor complex \(^{313}\). Recently, SNW1 has been identified as a novel regulator of the NF-kB pathway in a whole genome RNAi screen. SNW1 binds to the NF-kB-p-TEFb-RNA polymerase II (Pol II) complex to facilitate transcription elongation of NF-kB target genes upon tumor necrosis factor-alpha (TNF-\(\alpha\)) treatment \(^{314}\). Similarly, another study found that increased SNW1 levels result in an increased NF-kB p65 activity and secretion of neuron-derived inflammatory factors \(^{315}\). These studies imply that SNW1 is involved in innate immune responses. Furthermore, SNW1 plays a role in viral restriction. It has been reported that SNW1 interacts with viral interferon regulatory factors 1 and 4 (vIRF-1,4) of Kaposi sarcoma herpesvirus (KSHV), and knockout down of SNW1 enhances KSHV replication due to the reduced IFN\(\beta\) production \(^{316}\). SNW1 also suppresses IAV replication \(^{317}\); however, the mechanism is not fully understood. Thus, the knowledge gap in understanding the role of SNW1 in host defense remains.

Our previous study found that SNW1 is in the HOIP (HOIL1-interacting protein, also known as RNF31) protein complex \(^{318}\). HOIP is the catalytically active component of the
linear ubiquitin assembly complex (LUBAC). The other two subunits in LUBAC are Shank-associated RH domain–interacting protein (SHARPIN) and heme-oxidized IRP2 ubiquitin ligase 1L (HOIL-1L; also known as RBCK1). LUBAC, a 600 kDa complex, is the only E3 ligase that forms Met 1-linked head-to-tail linear ubiquitin chains: the C-terminal Gly76 of one ubiquitin is conjugated to the N-terminal Met1 of the preceding ubiquitin. As the catalytic subunit of LUBAC, HOIP is a RING-between-RING (RBR) type E3, comprising the N-terminal PNGase/UBA or UBX-containing protein (PUB) domain followed by a canonical zinc finger (ZF), two NP14 type ZF domains (NZF1 and NZF2), a middle atypical ubiquitin-associated domain (UBA) and a C-terminal RBR domain together with a unique linear ubiquitin chain determining domain (LDD). The RBR domain associated with the LDD domain is the catalytic core of HOIP and responsible for generating linear ubiquitin chains, while the UBA domain contacts with ubiquitin-like (UBL) domains in both HOIL-IL and SHARPIN. In addition, the LUBAC-tethering motifs (LTMs) in HOIL-1L and SHARPIN interact with each other to form a globular domain. Previous studies have revealed that the binding to either HOIL-1L or SHARPIN releases the auto-inhibited state of HOIP, in which the HOIP UBA domain suppresses the catalytic activity of its RBR-LDD domains. Although HOIL-IL also contains an RBR E3 domain, it is reported to mediate monoubiquitination by forming oxyester bonds between the C terminus of ubiquitin and serine and threonine residues in its substrates instead. A recent study revealed that the HOIL-IL RBR domain conjugates monoubiquitin onto all LUBAC components, which plays a crucial role in regulating LUBAC activity.
The physiological roles of LUBAC were initially discovered to activate the canonical NF-κB pathway \textsuperscript{329}. The molecular mechanism underlying LUBAC-mediated NF-κB activation upon TNFa stimulation has been well characterized. Binding of TNFa to its receptor TNFR1 triggers the assembly of TNFR1 complex I, a signaling complex consisting of multiple adaptor proteins. Among them, E3 ligases, like the cellular inhibitor of apoptosis protein 1 and 2 (cIAP1 and cIAP2), catalyze K63-linked polyubiquitin chains to several components of TNFR1 complex I, through which LUBAC is recruited to the complex. LUBAC then conjugates linear ubiquitin chains to NEMO, the regulatory subunit of IKK complex, and these linear ubiquitin chains are in turn, recognized by NEMO in another IKK complex for NEMO containing a high-affinity linear ubiquitin-binding site. Thus, multiple IKK molecules are accumulated on linear ubiquitin chains, leading to the activation of IKKβ via dimerization and subsequent activating NF-κB \textsuperscript{329-331}. LUBAC also regulates NF-κB activation induced by various other stimulations, like interleukin 1 beta (IL-1β), NOD-like receptors, TLRs, T and B cell receptors \textsuperscript{332,333}. Besides regulating NF-κB pathway, current studies have expanded the functions of LUBAC to innate and acquired immunity, cell death, and cancer \textsuperscript{331,334}. Several mutations in HOIP or HOIL-1L in humans cause multiorgan autoinflammation, combined immunodeficiency, and recurrent viral and bacterial infections \textsuperscript{335-337}, highlighting the pivotal roles of LUBAC or HOIP in the host immunity.

As for innate immunity, LUBAC is involved in type I IFN production. Studies revealed
that either SHARPIN or HOIP is necessary for the TLR3-mediated IRF3 activation. In addition, LPS-, poly (I:C)- and SeV-induced IFN production in the TLR3 signaling is impaired in HOIP-deficient mouse embryonic fibroblasts and human Jurkat T-lymphoblasts. However, the role of LUBAC in the RIG-I signaling pathway is controversial. Previous studies showed that LUBAC suppresses RIG-I activation and the linear ubiquitination activity of LUBAC to NEMO negatively regulates IFN production. But recent studies demonstrated that HOIP and HOIL positively regulate RIG-I signaling and IFN production induced by poly (I:C) and RNA viruses (SeV, Zika) in lung epithelial cells A549 cells. Moreover, the mice model with lung epithelial-specific knockout of HOIP showed reduced inflammatory responses and decreased survival during IAV infection, indicating a positive role of HOIP in the RIG-I pathway and IAV infection. Thus, the role of LUBAC and its components in the RIG-I pathway awaits further investigation. Since HOIP is a potential interactor of SNW1 and its function in the RIG-I signaling during IAV infection is not clear, here, we set out to investigate the role of HOIP in IAV infection and the RIG-I pathway.

In this study, we found that knockout of SNW1 or HOIP increases host susceptibility to IAV infection in A549 cells and reduces RIG-I-mediated IFNβ and ISG expression. However, HOIP enzyme-dead mutant only partially restores innate immune response to IAV in HOIP knockout cells, suggesting that other mechanisms are involved in HOIP-mediated IAV restriction. Because SNW1 and Pol II are in the HOIP protein complex and...
SNW1 belongs to the NF-kB-p-TEFb-Pol II complex to regulate the transcription of NF-kB target genes, we hypothesize that HOIP is within the same transcriptional complex with SNW1 to facilitate the IRF3- and NF-kB-mediated transcription, promoting the production of IFN thus inhibiting the IAV infection. IAV NS1 antagonizes this process by perturbing the formation of the transcriptional complex consisting of HOIP, SNW1, elongation factor p-TEFb, and Pol II. Collectively, our study not only reveals a novel role of SNW1 and HOIP in IAV infection and RIG-I signaling but also might uncover a new mechanism by which NS1 inhibits RIG-I signaling at the transcriptional level.

3.2. Materials and Methods

3.2.1. Cells.

HEK293 cells (ATCC, # CRL-1573) and MDCK cells (ATCC, # CCL-34) were maintained in Dulbecco’s Modified Eagle Medium (Life Technologies) containing Penicillin-Streptomycin (Life Technologies) and 10% fetal bovine serum (Life Technologies). A549 cells (ATCC, # CCL-185) were cultured in RPMI Medium 1640 (Life Technologies) plus 10% fetal bovine serum and 1X MEM Non-Essential Amino Acids Solution (Life Technologies).

3.2.2. Viruses.
Influenza A/Puerto Rico/8/34 (H1N1) was purchased from Charles River Laboratories (# 10100374). Influenza PR8-GLuc virus was a generous gift from Dr. Peter Palese and featured a Gaussia luciferase (Gluc) gene inserted downstream of PB2. IAV delNS1 was a gift from Dr. Adolfo Garcia-Sastre (Mount Sinai School of Medicine, NY). IAV was propagated in specific pathogen-free fertilized eggs Premium Plus (Charles River Laboratories, Wilmington, MA), as described by Szretter et al. Nine- to eleven-day-old embryonated chicken eggs are used for the production of influenza virus. 0.2 ml stock influenza virus at $1 \times 10^3$ TCID50 was injected through the puncture hole into the allantoic cavity. After 72 hr of incubation, allantoic fluid was collected. IAV titers were determined by plaque assay as described by Matrosovich et al. Briefly, $1.2 \times 10^6$ MDCK cells per ml were split into 6-well plates. After 2X washes with DMEM, serial dilutions of IAV were adsorbed onto the cells for 1 hr. The cells were covered with DMEM containing $2 \times$ Avicel RC591 NF (FMC Biopolymer, Philadelphia, PA) and 1 μg/ml TPCK-trypsin (Thermo Fisher Scientific, # 20233). Crystal violet staining was performed 48 h.p.i., and visible plaques were counted.

3.2.3. Plasmids.

Human SNW1, POLR2B were cloned into pCMV-3Tag-8 (Agilent Technologies, # 240203) to generate SNW1-FLAG, POLR2B-3Flag. The generation of HOIP-3Flag and its truncates as well as the mutant has been described before.
3.2.4. Antibodies.

Primary antibodies: Anti-β-actin (Abcam, # ab8227), anti-α-Tubulin (Cell Signaling Technology, # 3873S), anti-FLAG (Sigma, # F3165), anti-HA epitope (Cell Signaling Technology, # 3724), anti-Myc (Bethyl Laboratories, # A190-105A), anti-IFITM3 (GeneTex, # GTX63349), anti-SNW1 (Proteintech, # 25926-1-AP), anti-SNW1 (Santa Cruz Biotechnology, # sc-393856 AC), anti-NS1 (Santa Cruz Biotechnology, # sc-130568), anti-NP (GenScript, # A01506-40), anti-ISG15 (Thermo, # 703132), anti-HOIP (Cell Signaling Technology, # 99633), anti-HOIP (R&D system, # MAB8039SP), anti-RBCK1 (Santa Cruz Biotechnology, # sc-393754), anti-Pol II (Santa Cruz Biotechnology, # sc-56767), anti-POL2B (Santa Cruz Biotechnology, # sc-166803), anti-Rpb1 CTD (Cell Signaling Technology, # 2629S), anti-CDK9 (Cell Signaling Technology, # 2136), anti-NF-kB p65 (Cell Signaling Technology, # 8242S), anti-phospho-p65 (Ser536) (Cell Signaling Technology, # 3033S), anti-TBK1 (Cell Signaling Technology, # 3504S), anti-phospho-TBK1 (Ser172) (Cell Signaling Technology, # 5483S), anti-human IRF3 (Cell Signaling Technology, # 11904S), anti-phospho-IRF3 (Ser386) (ABclonal, # AP0995), anti-IKKα (ABclonal, # A19694), anti- IKKα (Cell Signaling Technology, # 11930S), anti-phospho-IKKα/β (Ser176/180) (Cell Signaling Technology, # 2697S)

Second antibodies: Goat anti-Mouse IgG-HRP [Bethyl Laboratories, # A90-116P, WB (1:10,000)], Goat anti-Rabbit IgG-HRP [Bethyl Laboratories, # A120-201P, WB
Alexa Fluor 594 Goat Anti-Mouse IgG (H+L) [Life Technologies, # A11005, IFA (1:200)], Alexa Fluor 488 Goat Anti-Rabbit IgG (H+L) [Life Technologies, # A11034, IFA (1:200)].

3.2.5. Plasmid transfection.

Transfections using Lipofectamine 2000 or Lipofectamine 3000 transfection reagent (Life Technologies) were performed according to the manufacturer’s protocol. For co-immunoprecipitation (co-IP) experiments, a total of 2.5 µg of plasmids was transfected into approximately 1.2 x 10^6 cells. For other experiments, a total of 0.5 µg of plasmids was transfected into approximately 0.2 x 10^5 cells.

3.2.6. Sample preparation, Western blotting, and immunoprecipitation.

Approximately 1 X 10^6 cells were lysed in 500 µl of tandem affinity purification (TAP) lysis buffer [50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 100 mM NaCl, 0.5% Nonidet P40, 10% glycerol, the Complete EDTA-free protease inhibitor cocktail tablets (Roche, # 11873580001)] for 30 min at 4 °C. The lysates were then centrifuged for 30 min at 15,000 rpm. Supernatants were mixed with the Lane Marker Reducing Sample Buffer (Thermo Fisher Scientific, # 39000) and boiled at 95 °C for 5 minutes.

Western blotting and immunoprecipitation were performed as described in a previous study. Briefly, samples (10–15 µl) were loaded into Mini-Protean TGX Precast Gels, 15
well (Bio-Rad, # 456-103), and run in 1 × Tris/Glycine/SDS Buffer (Bio-Rad, # 161-0732) for 60 min at 140 V. Protein samples were transferred to Immun-Blot PVDF Membranes (Bio-Rad, # 162-0177) in 1 × Tris/Glycine buffer (Bio-Rad, # 161-0734) at 70 V for 60 min. PVDF membranes were blocked in 1 × TBS buffer (Bio-Rad, # 170-6435) containing 5% Blotting-Grade Blocker (Bio-Rad, # 170-6404) for 1 h. After washing with 1 × TBS buffer for a total of 30 min (10 min each time, repeat 3 times), the membrane blot was incubated with the appropriately diluted primary antibody in antibody dilution buffer (1 × TBS, 5% BSA, 0.02% sodium azide) at 4 °C for 16 h. Then, the blot was washed three times with 1 × TBS (each time for 10 min) and incubated with secondary HRP-conjugated antibody in antibody dilution buffer (1:10,000 dilution) at room temperature for 1 h. After three washes with 1 × TBS (each time for 10 min), the blot was incubated with Clarity Western ECL Substrate (Bio-Rad, # 170-5060) for 1-2 min. The membrane was removed from the substrates and then exposed to the Amersham imager 680 (GE Healthcare Life Sciences, Marlborough, MA).

3.2.7. Real-time PCR.

Total RNA was prepared using the E.Z.N.A.® Total RNA Kit I (Omega, # R6834). 500 ng of RNA was reverse transcribed into cDNA using the ProtoScript First Strand cDNA Synthesis Kit (New England Biolabs, # E6300S). For one real-time reaction, 10 µl of SYBR Green PCR reaction mix (Eurogentec) including 2 µg of the synthesized cDNA plus an
appropriate oligonucleotide primer pair were analyzed on a CFX96 Real-time PCR System (Bio-RAD). The comparative Ct method was used to determine the relative mRNA expression of genes normalized by the housekeeping gene GAPDH. The primer sequences: human GAPDH, forward primer 5’- AGGTGAAGGTGAGGATCA-3’, reverse primer 5’-GGTCATTGATGGCAACAA-3’; human CXCL10 (IP10), forward primer 5’- TTCAAGGAGTACCTCTCTCTAG -3’, reverse primer 5’- CTGGATTCAGACATCTCTCTCTC -3’; human CCL5 (RANTES) qPCR primers were purchased from Qiagen (# PPH00703B-200); human IFNβ1, forward primer 5’- TCATCCTGTTCCTTGGGCAGT-3’, reverse primer 5’- CAGCAATTTCAGTGTGGAAG-3’.

3.2.8. Immunofluorescence assay.

Cells were cultured in the Lab-Tek II CC2 Chamber Slide System 4-well (Thermo Fisher Scientific, # 154917). After the indicated treatment, the cells were fixed and permeabilized in cold methanol for 10 min at -20 °C. Then, the slides were washed with 1 × PBS for 10 min and blocked with Odyssey Blocking Buffer (LI-COR Biosciences, # 927-40000) for 1 h. The slides were incubated in Odyssey Blocking Buffer with appropriately diluted primary antibodies at 4 °C for 16 h. After 3 washes (10 min per wash) with 1 × PBS, the cells were incubated with the corresponding Alexa Fluor conjugated secondary antibodies (Life Technologies) for 1 h at room temperature. The slides were washed three
times (10 min each time) with 1 × PBS and counterstained with 300 nM DAPI for 1 min, followed by washing with 1 × PBS for 1 min. After air-drying, the slides were sealed with Gold Seal Cover Glass (Electron Microscopy Sciences, # 3223) using Fluoro-gel (Electron Microscopy Sciences, # 17985-10). Images were captured and analyzed using a Revolve Microscope (Discover Echo Inc.).

3.2.9. CRISPR/Cas9.

The single guide RNA (sgRNA) sequence targeting human SNW1 is 5’-CCTTTCCGGTATCCGTACGG-3’; human HOIP is 5’-ATGCAAGTTCTCGTACGCC-3’; human RBCK1 is 5’-GCCAGCACCGAGTAGCACAC-3’. The lentiviral construct was transfected with psPAX2 and pMD2G into HEK293T cells using PEI. After 72 h, the media containing lentivirus were collected. The targeted cells were infected with the media containing the lentivirus supplemented with 10 μg/ml polybrene. Cells were selected with 10 μg/ml puromycin for 14 days. Single clones were expanded for knockout confirmation by Western blotting and DNA sequencing.

3.2.10. ChIP-qPCR assay.

The chromatin of A549 cells were extracted according to the manufacturer’s protocol of the Active Motif ChIP-It kit. Then either 2ug of anti-p65, anti-IRF3, anti-POL II, anti-
SNW1, or anti-HOIP antibody were added to the cleared chromatin extracts. After immunoprecipitation, DNA will be extracted and subjected to qPCR. The promoter and elongation regions of p65- and IRF3-targeted genes, IL-8 and IFNβ, will be analyzed by qPCR. The promoter and elongation regions of p65- and IRF3-targeted genes, IL-8 and IFNβ, will be analyzed by qPCR.  

3.2.11. Statistical analysis.

The sample size was sufficient for data analysis using software GraphPad Prism 9. For all statistical analyses, significant differences between the indicated pairs of conditions are shown by asterisks (* $P<0.05$; ** $P<0.01$; *** $P<0.001$; **** $P<0.0001$).

3.3. Results

3.3.1. NS1 interacts with SNW1.

Our recent IAV-host interactome mapping found that SNW1 was in the NS1 protein complex. To validate their interaction, we first examined the interaction between endogenous SNW1 and NS1 in lung epithelial cells A549 after PR8 IAV infection. Endogenous immunoprecipitation (IP) showed that PR8 NS1 bound to SNW1 (Fig. 7A). Next, the subcellular localization of NS1 and SNW1 was determined in mock-treated or PR8 IAV infected A549 cells by immunofluorescence assay (IFA). SNW1 and NS1 were mainly located in the nucleus (Fig. 7B), and SNW1 was co-localized with NS1 in the
nucleus (Fig. 7B). Furthermore, we examined the domains responsible for NS1-SNW1 interaction. NS1 has three different domains, including RBD, ED, and CTT (Fig. 7C). We generated a panel of Flag-tagged NS1 truncates (Fig. 7C) and performed co-immunoprecipitation (co-IP) with HA-tagged SNW1 in HEK 293 cells. Co-IP found that the RBD domain of NS1 was required for SNW1 binding (Fig. 7D). Similarly, we made several Flag-tagged SNW1 mutants, N174, SD, and C340 (Fig. 7E). Co-IP with Myc-tagged NS1 found that the first 174 amino acids in N-terminal of SNW1 were responsible for its interaction with NS1 (Fig. 7F).
Figure 7 NS1 interacts with SNW1.

(A) A549 cells were infected with 1 MOI PR8 IAV for 24h. Cell lysates were immunoprecipitated and blotted as indicated. The same isotype Immunoglobulin (IgG) was used as a control for immunoprecipitation (IP). (B) A549 cells were infected with 1 MOI PR8 IAV for 16h. Cells were fixed and stained with anti-NS1 antibody (Red), anti-SNW1 antibody (Green), and DAPI (Blue). Bar = 10 μM. (C) Schematics of NS1 truncates. RBD stands for RNA binding domain; ED stands for effector domain; CTT stands for C terminal tail. (D) HA-tagged SNW1 was co-transfected with pCMV-3Tag-8 vector, FLAG-tagged PR8 NS1, or the indicated FLAG-tagged NS1 mutant into HEK 293 cells. After 48 h, cell lysates were immunoprecipitated and blotted as indicated. (E) Schematics of SNW1 truncates. SD stands for SNW domain. (F) Myc-tagged NS1 was transfected with pCMV-3Tag-8 vector, or the indicated FLAG-tagged SNW1 mutant into HEK 293 cells. After 48 h, cell lysates were immunoprecipitated and blotted as indicated.

3.3.2. SNW1 is required for RIG-I signaling and IAV inhibition.

The interaction with NS1 suggests a potential role of SNW1 in IAV infection. To determine the function of SNW1 in IAV infection, we generated two SNW1 knockout cell lines in A549 cells by CRISPR (Fig. 8A). Consistent with a previous report, we only
obtained approximately 90% knockout efficiency because a complete knockout might be lethal. We first performed the plaque assay to determine the production of viral particles in SNW1 wild type and knockout A549 cells. As shown in Figure 8A, SNW1 knockout cells produced significantly more infectious viral particles than wild type cells. Next, the expression of viral protein was examined in SNW1 wild type and knockout A549 cells. These cells were infected with different MOI of PR8 IAV for 16 h. In agreement with infectious viral particle production levels, the protein levels of NS1 and NP were much higher in knockout cells than in wild type cells (Fig. 8B). These data suggest that SNW1 is required for host defense to IAV.

Since SNW1 is required for host defense against IAV, we hypothesized that SNW1 regulates the RIG-I pathway, which is the most crucial PRR sensing IAV RNA and inducing type I IFN expression. In this regard, we stimulated SNW1 wild type and knockout A549 cells with viral RNA mimics, poly(I:C). Real-time PCR assays showed that deficiency of SNW1 impaired IFNβ mRNA expression (Fig. 8C). Consistently, the protein expression of two ISGs, IFITM3 and ISG15, was also reduced in SNW1 knockout A549 cells stimulated with poly(I:C) (Fig. 8D). In addition, we infected SNW1 wild type and knockout cells with PR8 IAV mutant with NS1 gene deletion (delNS1) which instigates robust IFN production. In agreement with poly(I:C) experiments, SNW 1 deficiency also impaired IAV delNS1-induced mRNA production of IFNβ (Fig. 8E) and the protein expression of IFITM3 and ISG15 (Fig. 8F). Collectively, these data suggest that SNW1 is required for RIG-I-mediated innate immune responses.
Figure 8 SNW1 is required for RIG-I signaling and inhibits IAV.

(A) Wild type (WT) and SNW1 knockout (KO) A549 cells were infected at a MOI of 0.1 with influenza A/WSN/1933 (WSN). After the designated hour post-infection (h.p.i.), the virus titers were determined by plaque assay in MDCK cells. All experiments were
biologically repeated three times. The p value was calculated by two-way ANOVA followed by Sidak’s multiple comparisons test. ****p<0.0001. Cell lysates of knockout cells were blotted as indicated. (B) SNW1 wildtype and knockout A549 cells were infected with the designated MOI of IAV PR8 for 16 h. Cell lysates were blotted using the indicated antibodies. (C) A549 wildtype and SNW1 knockout cells were transfected with 1 μg/ml poly(I:C) for indicated hours. IFNβ mRNA levels were determined by RT-qPCR. All experiments were biologically repeated three times. The p value was calculated by two-way ANOVA followed by Dunnett’s multiple comparisons test. *p<0.05. (D) A549 wild type and SNW1 knockout cells were transfected with 1 μg/ml poly(I:C) for indicated hours. Cell lysates were blotted with indicated antibodies. (E) A549 wild type and SNW1 knockout cells were infected with PR8 Del NS1 IAV at the MOI 1 for indicated hours. IFNβ mRNA levels were determined by RT-qPCR. All experiments were biologically repeated three times. The p value was calculated by two-way ANOVA followed by Dunnett’s multiple comparisons test. ***p<0.001. (F) A549 wild type and SNW1 knockout cells were infected with PR8 Del NS1 IAV at the MOI 1 for indicated hours. Cell lysates were blotted with indicated antibodies.
3.3.3. **SNW1 interacts with HOIP.**

Our recent LUBAC interactome mapping found that SNW1 was in the HOIP protein complex \(^{347}\), and one recent study showed that the deficiency of LUBAC increased the severity of IAV infection \(^{343}\), suggesting a potential link between SNW1 and LUBAC. To validate the interaction, we first performed a co-IP between SNW1 and each component of LUBAC. Co-IP found that SNW1 interacted with HOIP but not HOIL and SHARPIN (Fig. 9A). Furthermore, endogenous IP in A549 cells confirmed that endogenous SNW1 interacted with HOIP (Fig. 9B). Next, we examined the subcellular localization of SNW1 and HOIP by IFA. Flag-tagged HOIP and HA-tagged SNW1 were transfected into A549 cells. As shown in Figure 9C, HOIP was distributed in the cytoplasm and nucleus, while SNW1 was predominantly in the nucleus. Moreover, SNW1 and HOIP were co-localized in the nucleus (Fig. 9C). To determine which domains of HOIP are responsible for the SNW1 interaction, we further made a panel of HOIP mutants (Fig. 9D). Co-IP revealed that NZF1, NZF2 and UBA domains (amino acids between 349 and 698) of HOIP are required for SNW1 binding (Fig. 9E). Additionally, we also performed co-IP between HOIP and different truncates of SNW1 and found amino acids from 174 to the C-terminal end of SNW1 were involved in the interaction with HOIP (Fig. 9F). Taken together, our data suggest that SNW1 interacts and co-localizes with HOIP.
Figure 9 SNW1 interacts with HOIP.

(A) HA-tagged HOIP, HOIL, or SHARPIN was co-transfected with pCMV-3Tag-8 vector or FLAG-tagged SNW1 into HEK293 cells. After 48 h, cell lysates were immunoprecipitated and blotted as indicated. (B) A549 cell lysates were immunoprecipitated and blotted as indicated. The same isotype IgG was used as a control for immunoprecipitation (IP). (C) A549 cells were transfected into Flag-tagged HOIP, HA-tagged SNW1, or both. After 48h, cells were fixed and stained with anti-Flag antibody (Red), anti-HA antibody (Green), and DAPI (Blue). Bar= 10 μM. (D) Schematics of HOIP truncates. (E) HA-tagged SNW1 was co-transfected with pCMV-3Tag-8 vector, Flag-tagged HOIP, or different Flag-tagged truncates of HOIP into HEK293 cells. After 48 h, cell lysates were immunoprecipitated and blotted as indicated. (F) HEK239 cells were transfected into HA-tagged HOIP with pCMV-3Tag-8 vector, Flag-tagged SNW1, or different Flag-tagged truncates of SNW1. After 48 h, cell lysates were immunoprecipitated and blotted as indicated.

3.3.4. HOIP regulates RIG-I signaling.

Since the HOIP interactor SNW1 inhibits IAV infection, it suggests a potential role of HOIP in IAV infection. In this regard, we examined the effects of HOIP deficiency on IAV infection. We generated two HOIP knockout cell lines in A549 cells by CRISPR (Fig. 10A). HOIP deficiency led to a more than 10-fold increase of viral titers (Fig. 10A) and a higher
expression of NS1 and NP (Fig. 10B). Thus, we hypothesized that HOIP inhibited IAV by
promoting RIG-I signaling because SNW1 suppresses IAV infection by regulating RIG-I
pathway. To this end, we first examined the effects of HOIP deficiency on RIG-I-induced
IFN and ISG expression. Consistent with SNW1 ablation, HOIP deficiency significantly
impaired the poly(I:C)-induced IFNβ mRNA expression (Fig. 10C). Moreover, the protein
expression of IFITM3 and ISG15 was also decreased in HOIP knockout A549 cells (Fig.
10D). Next, we examined the effects on HOIP deficiency on IAV delNS1-induced, RIG-I-
dependent IFNβ and ISG expression. In line with the poly(I:C) treatment, HOIP knockout
cells produced a significantly lower mRNA expression of IFNβ (Fig. 10E) and protein
expression of IFITM3 and ISG 15 (Fig. 10F) in response to IAV PR8 delNS1 infection.
Figure 10 HOIP suppresses IAV infection and regulates RIG-I pathway.

(A) A549 Wild type and HOIP knockout cells were infected at an MOI of 0.1 with influenza A/WSN/1933 (WSN). After the designated hour post-infection (h.p.i.), the virus titers were
determined by plaque assay in MDCK cells. All experiments were biologically repeated three times. The p value was calculated by two-way ANOVA followed by Dunnett’s multiple comparisons test. *p<0.05. Cell lysates of knockout cells were blotted as indicated. (B) Wildtype and HOIP knockout A549 cells were infected with the designated MOI of IAV PR8 for 16 h. Cell lysates were blotted using the indicated antibodies. (C) A549 wildtype and HOIP knockout cells were transfected with 1 μg/ml poly(I:C) for indicated hours. IFNβ mRNA levels were determined by RT-qPCR. All experiments were biologically repeated three times. The p value was calculated by two-way ANOVA followed by Dunnett’s multiple comparisons test. *p<0.05. (D) A549 wild type and HOIP knockout cells were transfected with 1 μg/ml poly(I:C) for indicated hours. Cell lysates were blotted with indicated antibodies. (E) A549 wild type and HOIP knockout cells were infected with PR8 Del NS1 IAV at the MOI 1 for indicated hours. IFNβ mRNA levels were determined by RT-qPCR. All experiments were biologically repeated three times. The p value was calculated by two-way ANOVA followed by Dunnett’s multiple comparisons test. *p<0.05. (F) A549 wild type and HOIP knockout cells were infected with PR8 Del NS1 IAV at the MOI 1 for indicated hours. Cell lysates were blotted with indicated antibodies.
3.3.5. **HOIP enzymatic activity is partially required for IAV inhibition.**

Next, we examined the mechanisms by which SNW1 and HOIP regulate RIG-I pathway and inhibit IAV infection. The first to take into consideration is whether E3 ligase activity of HOIP is required for its role in host defense to IAV infection. To this end, we treated A549 cells with DMSO or HOIPIN8, a chemical compound that specifically inhibits HOIP enzymatic activity, followed by IAV infection. As shown in Figure 11A, HOIPIN-8 increased the production of infectious IAV particles by a small margin, suggesting that HOIP enzymatic activity is partially required for HOIP-mediated IAV inhibition. To further determine the role of HOIP E3 ligase activity, we reconstituted HOIP knockout cells with wild type HOIP and the E3 ligase dead mutant C885A with a single point mutation in the HOIP catalytic active site Cys885. After IAV infection, the reconstitution of wild type HOIP inhibited NP and NS1 expression. Unexpectedly, the catalytic deficient mutant also restricted IAV infection, although its antiviral activity is lower than wild type HOIP (Fig. 11B). These data suggest that HOIP-mediated IAV restriction requires both E3 ligase-dependent and -independent activities. To validate this model, we knocked out HOIL-1L, a component of LUBAC required for HOIP E3 ligase activity. As shown in Figure 11C and 11D, HOIL-1L deficiency only slightly increased the production of IAV particles and expression of IAV viral proteins, indicating that loss of HOIL-1L or HOIP E3 ligase activity cannot fully phenocopy HOIP knockout. These data corroborate that HOIP has an E3 ligase-independent antiviral activity.
Figure 11 HOIP inhibits IAV through E3 ubiquitin ligase-dependent and -independent activities.

(A) A549 cells were pretreated with DMSO or 30uM HOIPIN-8 for 4h followed by infection with 0.1 MOI of influenza A/WSN/1933 (WSN). After the designated hour post-infection (h.p.i.), the virus titers were determined by plaque assay in MDCK cells. All experiments were biologically repeated three times. The p value was calculated by two-way ANOVA followed by Sidak’s multiple comparisons test. 

(B) A549 HOIP knockout cells (KO), HOIP knockout cells reconstituted with HOIP-3Flag (KO (HOIP)), or HOIP-C885A-3Flag (KO (C885A)) were infected with the designated MOI of IAV PR8 for 16 h. Cell lysates were
blotted using the indicated antibodies. (C) HOIL-1L wild type and knockout A549 cells were infected at an MOI of 0.1 with influenza A/WSN/1933 (WSN). After the designated hour post-infection (h.p.i.), the viral titers were determined by plaque assay in MDCK cells. All experiments were biologically repeated three times. The p value was calculated by two-way ANOVA followed by Sidak’s multiple comparisons test. Cell lysates of knockout cells were blotted as indicated. (D) A549 wild type and HOIL-1L knockout cells were infected with the designated MOI of IAV PR8 for 16 h. Cell lysates were blotted using the indicated antibodies.

3.3.6. HOIP regulates IKK and TBK1 activation.

We also determined whether the catalytic activity of HOIP is required in its function in RIG-I signaling. The RIG-I activation by various stimuli leads to the activation of both IKK complex and TBK1, which further activates transcriptional factors NF-kB and IRF3, respectively. Thus, we compared the phosphorylation level, the indicator of the active state, of these signature cascades in A549 wildtype vs. HOIP knockout cells, A549 cells pretreated with DMSO vs. HOIPIN-8, and wild type vs. HOIL-1L knockout A549 cells. After poly(I:C) treatment, IKK phosphorylation was dramatically impaired in HOIP knockout cells (Fig. 12A) and HOIPIN-8 treated A549 cells (Fig. 12B), while IKK phosphorylation was
moderately reduced in HOIL-1L knockout cells (Fig. 12C), suggesting that HOIP enzyme activity regulates IKK activation. Consistently, p65 phosphorylation level was impaired in all these three cells. However, there was no significant difference in TBK1 and IRF3 activation. Collectively, our results confirmed that HOIP catalytic activity or LUBAC is required in the IKK and NF-kB activation, the downstream of poly(I:C)-induced RIG-I signaling. Furthermore, these data showed that HOIP has little effect on TBK1 and IRF3 phosphorylation, the other signaling branch of RIG-I, suggesting that other functions of HOIP, independent E3 ligase activity, are also involved in its role against IAV infection.
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**Figure 12** The effect of HOIP and SNW1 deficiency on IKK and TBK1 activation.

(A) A549 wildtype and HOIP knockout cells were transfected with 1 μg/ml poly(I:C) for indicated hours. Cells lysates are blotted with indicated antibodies. (B) A549 cells pretreated DMSO or 30μM HOIPIN-8 for 4h, and then cells were transfected with 1 μg/ml poly(I:C) for indicated hours. Cells lysates are blotted with indicated antibodies. (C) HOIL-IL wild type and knockout cells were transfected with 1 μg/ml poly(I:C) for indicated hours. Cells lysates are blotted with indicated antibodies.

### 3.3.7. SNW1 has little effect on viral mRNA splicing.

Since SNW1 is a component of the splicing complex to facilitate the splicing of certain genes and previous studies showed that the splicing efficiency of the M and NS segments of IAV could be crucial for IAV replication. Thus, it is plausible that SNW1 affects viral mRNA splicing. In this regard, A549 wild type and SNW1 knockout, HOIP knockout cells were infected with PR8 IAV for 0, 12, and 24 h, and then the protein levels of NS1, NS2, M1, and M2 were determined. The ratios of NS2 to NS1 and M2 to M1 in each cell line were calculated. In agreement with our previous results, the expression of viral proteins NS1, NS2, M1, and M2 was higher in both SNW1 knockout cells (Fig. 13A) and HOIP knockout cells (Fig. 13B) compared with wild type cells. However, there was no
obvious difference in ratios of NS2 to NS1, M2 to M1 between A549 wildtype and SNW1 knockout, HOIP knockout cells. Thus, SNW1 and HOIP have little effect on viral mRNA splicing.

Figure 13 SNW1 has little effect on viral mRNA splicing.

(A) A549 wild type and SNW1 knockout cells were infected with PR8 IAV at the MOI 1 for designated hours. Cell lysates were blotted using the indicated antibodies, and the ratios of NS1 to NS2 and M1 to M2 within each cell line were compared. (B) HOIP wild type and knockout cells were infected with PR8 IAV at the MOI 1 for designated hours. Cell lysates were blotted using the indicated antibodies, and the ratios of NS1 to NS2 and M1 to M2
within each cell line were compared.

3.3.8. **HOIP forms a transcriptional complex with SNW1 and NS1 suppresses the formation of this complex.**

Our data suggest that HOIP can inhibit IAV infection independent of its catalytic activity. Our previous study found that both SNW1 and RNA polymerase II subunit B (POLR2B) were in the HOIP protein complex \(^3\)\(^{18}\), indicating a potential role of nuclear HOIP. Furthermore, SNW1 was found in the complex formed by transcriptional elongation factor p-TEFb, p65 and RNA polymerase II (Pol II) to mediate transcription elongation of NF-kB target genes \(^3\)\(^{14}\). Based on these studies, we hypothesized that HOIP was also in the SNW1-p65-p-TEFb-Pol II complex during IAV infection; thus promoting transcription of NF-kB and IRF3 target genes. To test it, we first validated the interaction between HOIP and POLR2B. As shown in Fig. 14A, endogenous HOIP and POLR2B interacted with each other. Moreover, we determined domains of HOIP and POLR2B responsible for their binding. Co-IP revealed the zinc finger domain (amino acids from 1118 to the C-terminus) of POLR2B were necessary for binding to HOIP (Fig. 14B). NZF1, NZF2, and/or UBA domains of HOIP (amino acids from 350 to 698) required for its interaction with POLR2B (Fig. 14C). Next, we examined the formation of SNW1-HOIP-Pol II complex in A549 cells.
Upon poly(I:C) stimulation, the POL II bound to p65, SNW1, HOIP, and cyclin-dependent kinase 9 (CDK9), a component of p-TEFb (Fig. 14D). In addition, PR8 IAV infection promoted the interaction of SNW1 with p65, IRF3, HOIP, CDK9 and POL II (Fig. 14E). Thus, SNW1, HOIP, p65, CDK9, POL II were in the same complex both in poly(I:C) treatment and IAV infection, implying the involvement of HOIP and SNW1 in the transcription of NF-kB and IRF3 target genes. Furthermore, IAV NS1 reduced the formation of this complex since PR8 Del NS1 infection significantly enhanced the binding of SNW1 to p65, IRF3, HOIP, CDK9 and POL II (Fig. 14E), indicating that NS1 might inhibit transcriptional activities by disrupting of this complex. In addition, our result showed even in the basal state, HOIP interacted with Pol II (Fig. 14D) or SNW1 (Fig. 14E), indicating a potential localization of HOIP in the nucleus.
Figure 14 HOIP forms a transcriptional complex with SNW1, and NS1 suppresses the formation of this complex.

(A) A549 cell lysates were immunoprecipitated and blotted as indicated. The same isotype
IgG was used as a control for IP. (B) HA-tagged HOIP was co-transfected with pCMV-3Tag-8 vector, FLAG-tagged POLR2B or the indicated FLAG-tagged truncate of POLR2B into HEK293 cells. After 48 h, cell lysates were immunoprecipitated and blotted as indicated. (C) HA-tagged POLR2B was co-transfected with pCMV-3Tag-8 vector, FLAG-tagged HOIP or different FLAG-tagged truncates of HOIP into HEK293 cells. After 48 h, cell lysates were immunoprecipitated and blotted as indicated. (D) A549 cells were mock-treated or transfected with 1 μg/ml poly(I:C). After 16h, cell lysates were immunoprecipitated and blotted as indicated. The same isotype IgG was used as a control for IP. (E) A549 cells were mock-treated and infected with 1 MOI PR8 IAV or PR8 Del NS1 IAV for 24h. Cell lysates were immunoprecipitated and blotted as indicated.

3.4. Discussion

Innate immune responses instigated by PRRs activating the IFN system are powerful host defenses against viral infections. RIG-I which recognizes dsRNA with a triphosphate (PPP) or diphosphate (PP) group at 5’ end is the pivotal PRR sensing IAV in the cytoplasm and inducing the IFN production. On the other hand, IAV viral protein NS1, the well-known IFN antagonist, has been found to suppress RIG-I signaling. Current unmasked mechanisms by which NS1 inhibits the RIG-I pathway all focus on the
cytoplasm part of RIG-I signaling cascades or activators, thus indirectly reducing the transcription of NF-kB and IRF3. Whether NS1 directly inhibits the transcription of NF-kB and IRF3 in the nucleus remains unknown.

Our previous study of IAV NS1-host protein interactions found that SNW1 interacted with NS1. SNW1 is known as a spliceosome component and a transcriptional regulator of nuclear receptors, such as vitamin D receptor and androgen receptor. The emerging role of SNW1 in regulating the transcription of NF-kB target genes along with p-TEFb and Pol II sheds light on its involvement in innate immunity. Here, our study report novel functions of SNW1 against IAV infection and positively regulating RIG-I signaling.

Furthermore, our previous study showed that HOIP interacted with SNW1 and POLR2B, implying a similar role of HOIP with SNW1 and a novel localization of HOIP in the nucleus. Our study showed that deficiency of HOIP but not HOIL or HOIP catalytic activity abrogated A549 cells dramatically promotes IAV titers (Fig. 10A, 11A, 11C) in host cells, although all three cell lines impaired the poly(I:C) mediated IKK activation (Fig. 12A-C). Moreover, reconstituted HOIP E3 ligase dead mutant into HOIP knockout cells partially rescued the high viral infection (Fig. 11B). Thus, besides catalytic activity of HOIP which is already known to be indispensable for activating IKK upon various stimuli, other functions of HOIP independent of LUBAC are also required in its role against IAV infection.

We next confirmed the interaction between HOIP and Pol II and found SNW1, HOIP and Pol II in a same complex after poly(I:C) treatment and IAV infection (Fig. 14D, 14E). Based
on these results and the mechanism by which SNW1 regulates the transcription of NF-kB target genes, we hypothesize HOIP, SNW1 and Pol II form a transcriptional complex in the nucleus during IAV infection to promote the transcription of NF-kB and IRF3 target genes, which will be further analyzed by CHIP-qPCR assays. On the other hand, NS1 inhibits the formation of this complex (Fig. 14E), thus we speculate NS1 weakens the transcriptional activity of this complex, leading to directly inhibiting the transcription of IFN mRNA in the nucleus.

Our results showed that HOIP is required in regulating RIG-I signaling, since loss of HOIP significantly impaired the poly(I:C) and Del NS1 IAV induced production of IFN (Fig. 10C). However, previous studies reported that LUBAC negatively regulates RIG-I signaling and IFN production. Several reasons might be causing the controversy. First, different components of LUBAC may play different roles in host cellular signals. The positive or negative effects of one component cannot reflect the whole LUBAC complex. Second, these two studies mainly adopted the overexpression of LUBAC but only HOIP and HOIL without SHARPIN into cells to analyze the functions of LUBAC in IFN production. Overexpression of genes might produce conflicting results with complete deletions. And even HOIP and HOIL together might not totally reflect the function of LUBAC. Our results using the complete HOIP knockout cells are consistent with a recent study which revealed knockout HOIP in A549 cells reduced RIG-I mediated innate immune responses and another study showing loss of HOIP in mice lung epithelial cells after IAV infection.
decreased mice survival.

There are still some limitations, and further studies are warranted. First, it needs to determine the dynamic interaction of HOIP and SNW1, Pol II. Although our results suggest the existence of nuclear HOIP, whether a part of cytoplasmic HOIP also translocates to the nucleus to form a complex with SNW1 and Pol II during IAV infection is not clear. Second, precise processes of how the complex SNW1-HOIP-Pol II is formed and regulated are unknown. Third, ChIP-qPCR cannot provide a global view of the SNW1-HOIP-Pol II complex deposition on the chromatin. A ChIP-seq assay might be required to systematically examine genes regulated by the HOIP-SNW1 complex.

In conclusion, our study found that two novel host factors SNW1 and HOIP are required in the host defense to IAV infection and positively regulate RIG-I signaling. Mechanistic analyses revealed that during IAV infection, HOIP functions as the catalytic active component of LUBAC to activate IKK in the cytoplasm. HOIP also forms a complex with SNW1, Pol II independent of E3 ligase activity, thus might promote the transcription of NF-kb and IRF3 target genes. In addition, IAV NS1 reduces the formation of this complex and might further perturb the transcriptional activity of this complex. Overall, we uncover a new mechanism by which NS1 inhibits RIG-I signaling at the transcriptional level.
3.5. Future Directions

Future directions of this study will mainly focus on the role of SNW1 and HOIP on the transcription of NF-kB and IRF3 target genes and examine whether NS1 inhibits the transcriptional activity of the SNW1-HOIP complex. In this regard, we will perform chromatin immunoprecipitation (ChIP)-qPCR assays in SNW1 wild-type vs. knockout cells, HOIP wildtype vs. knockout out A549 cells after stimulation with poly (I:C). Recruitment of Pol II, p65 or IRF3 on the elongation regions of p65- or IRF3-targeted genes, such as IL-8 and IFNβ, will be analyzed. Similarly, A549 cells infected with PR8 IAV or Del NS1 IAV will be analyzed by ChIP-qPCR assays to determine the role of NS1 in the transcription of p65 and IRF3 targeted antiviral genes. We will also transfect wild type NS1 and the NS1 mutant that retains NS1 in the nucleus into HEK 293 cells followed by Poly (I:C) treatment, then perform ChIP-qPCR assays to exclude the interference of cytosolic NS1.
CHAPTER 4. SUMMARY AND FUTURE PERSPECTIVE

The innate immune system acts as the first line of defense against the invasion of viruses. IAV RNA can be sensed by RIG-I, leading to the induction of type I IFN expression. Type I IFN activates the mRNA expression of hundreds of ISGs, which in turn restrict IAV infection. In addition to ISGs, a group of antiviral host factors, constitutively expressed and IFN-independent, guarantee a more rapid response and a direct inhibition of viral infection. Here, we report that FKBP5 is not only required for the RIG-I-IKK signaling axis for type I IFN production but also inhibits IAV infection, indicating FKBP5 is a novel anti-influenza gene by participating in RIG-I-mediated NF-κB signal cascade. On the other hand, viral proteins extensively interact with host proteins to fulfill viral life cycles and perturb host defenses for immune surveillance evasion. IAV NS1 inhibits host innate immune defenses, including the RIG-I pathway. However, whether NS1 antagonizes the function of FKBP5 is unknown. Future studies will investigate the interactions between NS1 and FKBP5.

IAV viral protein NS1 has been found to suppress RIG-I signaling. The mechanisms are not fully understood. Current mechanisms focus on the cytoplasm part of RIG-I signaling cascades or activators, thus indirectly suppressing the transcription of NF-κB and IRF3. Whether NS1 directly inhibits the transcription of NF-κB and IRF3 in the nucleus...
remains unknown. We confirmed that SNW1 interacts with IAV NS1. We are interested in SNW1 because it’s a protein associated with splicing and transcription. Both SNW1 and its interactor HOIP, the catalytic active component of LUBAC, restrict IAV infection and positively regulates RIG-I signaling. Then we set out to explore mechanisms for their role in IAV infection and RIG-I pathway.

Our results revealed that HOIP catalytic activity is required but not sufficient. HOIP functions as the catalytic active component of LUBAC to activate IKK in cytoplasm while a part of HOIP forms a complex with SNW1, Pol II and transcriptional factor p65 or IRF3 during IAV infection independent of its E3 ligase activity. Interestingly, NS1 perturbs the formation of this complex. The transcriptional activity of this complex will be further determined by ChIP-qPCR assays. We hypothesize that the transcriptional complex promotes the transcription of NF-kB and IRF3 target genes. NS1 suppresses the transcriptional activity of this complex, leading to directly inhibiting the transcription of IFN mRNA in the nucleus. Thus, our study not only reveals a novel role of HOIP in transcription but also a new mechanism by which NS1 antagonizes the RIG-I signaling at the transcriptional level.

Taken together, our study found three host factors FKBP5, SNW1, and HOIP that restrict IAV infection and positively regulate the RIG-I pathway. Furthermore, we also revealed mechanisms of how they regulate the RIG-I pathway, thus limiting IAV infection. FKBP5 activates the cytoplasm part of RIG-I signaling, while SNW1 and HOIP might
promote transcription of IRF3 and p65 target genes in the nucleus. A novel mechanism by which NS1 antagonizes the RIG-I signaling at the transcriptional level might also be of profound significance. Our study greatly deepens the understanding of IAV pathogenesis.
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