Genome-Wide Screen Identifies RTF2 as a Host Restriction Factor That Restricts Influenza A Virus Replication

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Genome-wide Screen Identifies RTF2 as a Novel Host Restriction Factor that Restricts Influenza A Virus Infection

Abstract

Viral infection triggers the secretion of type I interferons, which in turn induce expression of hundreds of genes. However, the roles and molecular mechanisms of these induced genes in the context of antiviral immunity remain largely unknown. This has limited our ability to develop host-based antiviral therapeutics against pathogenic viruses such as influenza virus, which causes annual epidemics and recurring pandemics. While recent work has identified antiviral factors that are sufficient to restrict viruses when these factors are overexpressed, the question of whether these factors are indeed inhibiting viruses in a physiological context remains unanswered.

Here, we performed a loss-of-function genetic CRISPR screen in cells pre-stimulated with type I interferon to identify antiviral genes that restrict influenza A virus replication. In addition to the key components of the interferon signaling pathway, we found a new factor, Replication Termination Factor 2 (RTF2). Our data reveal that RTF2 restricts influenza, at least, at the nuclear stage of the viral life cycle based on several lines of evidence. First, a deficiency in RTF2 leads to higher levels of viral primary transcription, in the presence of cycloheximide to block genome replication and secondary transcription. Second, cells that lack RTF2 have enhanced activity of a viral reporter that depends solely on four viral proteins that carry out replication and transcription in the nucleus. Third, when RTF2 protein is mislocalized outside the

nucleus, it is not able to restrict replication. In addition to restricting transcription, the absence of RTF2 reduces expression of antiviral factors in response to interferon. RTF2 thus inhibits influenza primary transcription, likely acts in the nucleus, and contributes to upregulation of antiviral effectors in response to type I interferons.

Influenza A virus remains a global health threat, with an estimated 3-5 million cases of severe illness, and 0.29-0.65 million deaths worldwide annually. This work contributes to the field of antiviral immunology by discovering and characterizing a novel restriction factor of influenza, and may ultimately be useful for understanding how to control a virus that causes significant morbidity and mortality worldwide.

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LIST OF ABBREVIATIONS

25HC	25-hydroxycholesterol
ADAR	adenosine deaminase acting on RNA
AP-MS	affinity purification-mass spectrometry
ATR	ataxia telangiectasia and Rad3-related protein
cDNA	complementary DNA
Ch25h	cholesterol 25-hydroxylase
CHX	cycloheximide
CRISPR	clustered regularly interspaced short palindromic repeats
CRISPR-SAM	CRISPR-synergistic activation mediator
CRM1	chromosomal maintenance 1
cRNA	complementary RNA
DENV	Dengue virus
dsRNA	double-stranded RNA
EMCV	encephalomyocarditis virus
FACS	fluorescence-activated cell sorting
FPPS	farnesyl diphosphate synthase
GalNAc	N-acetylgalactosamine
GEF	guanine nucleotide exchange factor
GOF	gain-of-function
HA	hemagglutinin
HAT	human airway trypsin-like protease
HCV	hepatitis C virus
HIV	human immunodeficiency virus
HSF	HEPES-Sucrose-Ficoll
IAV	influenza A virus
IBV	influenza B virus
IFIT	interferon-induced protein with tetratricopeptide repeats
IFITM	interferon-induced transmembrane
IFN	interferon
IFNAR	interferon-α/β receptor
IMPα	importin-α
IMPβ	importin-β
IRES	internal ribosome entry sites
IRF	interferon regulatory factor
ISG	interferon-stimulated gene
ISGF3	interferon-stimulated gene factor 3
JAK	Janus kinase
LOF	loss-of-function
MDA5	melanoma differentiation-associated protein 5
MEF	mouse embryonic fibroblast
MOI	multiplicity of infection
mRNA	messenger RNA
NA	neuraminidase
NES	nuclear export signal

NLS	nuclear localization signal
NP	nucleoprotein
NPC	nuclear pore complex
NS1	non-structural protein 1
NXF1	nuclear RNA export factor 1
OAS	2’-5’-oligoadenylate synthase
PA	polymerase acidic protein
PABII	polyadenylate binding protein 2
PAI-1	plasminogen activator inhibitor 1
PAMP	pathogen-associated molecular pattern
PAP	polyadenylate polymerase
PB1	polymerase basic protein 1
PB2	polymerase basic protein 2
PCA	principal component analysis
PKR	protein kinase R
PML	promyelocytic leukemia
pppApG	triphosphorylated AG dinucleotide
PR8	influenza A/Puerto Rico/8/1934
PRR	pattern recognition receptor
RAN	ras-related nuclear protein
RdDM	RNA-dependent DNA methylation
RIG-I	retinoic acid-inducible gene
RING	Really Interesting New Gene
RLR	RIG-I-like receptor
RNAi	RNA interference
RTF2	Replication Termination Factor 2
RTS1	Replication Termination Site 1
ssDNA	single-stranded DNA
TLR	Toll-like receptor
TMPRSS2	transmembrane protease serine S1 member 2
TYK2	non-receptor tyrosine-protein kinase 2
vATPase	vacuolar ATPase
vRNA	viral RNA
vRNP	viral ribonucleoprotein
VSR	virus-encoded suppressor of RNAi
VSV	vesicular stomatitis virus
Y2H	yeast two-hybrid
ZAP	zinc finger antiviral protein
ZAPL	long isoform of ZAP
ZAPS	short isoform of ZAP
ZMPSTE24	zinc metallopeptidase STE24

CHAPTER 1: Introduction

A major goal of antiviral research is to harness knowledge to prevent and combat viral infections. By studying how viruses replicate themselves, how the host sets up restrictive barriers, and what happens when such restriction fails, we might be able to design appropriate interventions to either favor resistance against infection, or recovery if we do get infected. The field of virology involves many different disciplines, including, but not limited to, structural biology, molecular biology, cellular biology, and immunology. Another useful way to frame our thinking of the vast virology research space is to consider the scale. Researchers could focus on the atomic study of how proteins carry out enzymatic reactions, how an individual cell mobilize its intracellular resources to detect and/or interfere with a virus infection, or even the complex interplay amongst different cell types in a host organism to cope with an infection.

Since the discovery of interferons (IFN) in 1957[1], an important cell-to-cell signaling molecule that confers resistance against viruses, many restriction factors have been identified. Many are interferon-stimulated, i.e. upregulated in the presence of IFN signaling. However, even though a handful of restriction factors have been extensively studied, many more remain unknown in terms of their biological roles and mechanisms of action in the cell[2]. This knowledge gap has vastly limited our ability to develop host-based antiviral therapeutics against pathogenic viruses.

Currently, most of the FDA-approved antiviral therapeutics are small molecule drugs that are virus-oriented[3]. In other words, these antivirals tend to target a specific step in the life cycle

of a specific virus. Given the high replication and mutation rates of viruses, and the emergence of resistance towards existing antiviral drugs, this approach of developing antivirals is not sustainable. In addition, with the prospect of newly emerging viruses such as SARS-CoV2, and their pandemic potential (due to increasing connectedness and the lack of pre-existing immunity), we desperately need host-directed strategies that are rapidly scalable and effective against a broad spectrum of viruses. Indeed, the administration of IFNs (and their modified formulations) to manage HCV infections, though probably not developed with these considerations, could be thought of as an early draft of host-based therapeutics. However, IFN exposure upregulates hundreds of genes[4,5]. In fact, a recent study using a statistical approach, rather than an arbitrary cut-off, reports that approximately 10% of the human genome is upregulated upon IFN exposure, and that many of these genes have annotated roles beyond the antiviral context[5]. This inadvertently implies that severe side effects and toxicities could arise from the use of IFNs, as have been observed with HCV patients undergoing IFN treatment[6]. Hence, a better understanding of how individual restriction factors function in the cell could reveal avenues for developing more targeted therapies that inhibit multiple viruses, without the off-target toxicities seen with non-specific upregulation of genes. Perhaps drugs that mimic how innate restriction factors function could be developed. In addition, given the recent advancements in gene therapy, both in terms of gene silencing and gene replacement, perhaps upregulation of restriction factors as a form of antiviral therapy may become a possibility in the distant future.

Influenza A Virus – Epidemiology and Impact on Human Health

Despite medical advances in vaccine development and antiviral therapeutics, Influenza A virus (IAV) remains a major human pathogen that causes respiratory illnesses. Within the last century, several strains of IAV have been responsible for major pandemics. These include the 1918 Spanish flu (H1N1), 1957 Asian flu (H2N2), 1968 Hong Kong flu (H3N2), and the relatively recent 2009 A(H1N1) pandemic flu. The 1918 pandemic itself is estimated to cause approximately 50 million deaths worldwide[7], while 3.9 million and 1.4 million deaths are attributed to the 1957 and 1968 pandemics respectively[8].

Aside from causing pandemics, influenza viruses are also responsible for seasonal epidemics, particularly in the winter months. Currently, IAV with subtypes H1N1pdm09 (short for H1N1 pandemic 2009) and H3N2, along with influenza B viruses, are the dominant circulating strains responsible for the seasonal epidemics. It is estimated that influenza viruses still cause approximately 3-5 million cases of severe illness, and 0.29-0.65 million deaths worldwide annually[9,10].

As IAV is a segmented RNA virus, it could escape immunity through either antigenic drift or antigenic shift. Antigenic shift occurs when influenza viruses swap gene segments. This could occur if two or more IAV strains co-infect the same cell. New reassortant strains gaining new hemagglutinin (HA) subtypes that the humans are naïve to could result in pandemic outbreaks. Examples of such pandemics include those that occurred in 1957 and 1968, when the H2N2 and H3N2 viruses emerged. In addition, even if the reassortant virus' HA belongs to the

same subtype as a circulating strain, the reassortant virus could still cause a pandemic if its HA is antigenically distinct enough. For instance, although the 2009 H1N1 pandemic strain is of the same subtype as the then-circulating human seasonal flu strain, it was sufficiently antigenically different to cause widespread transmission[11]. On the other hand, antigenic drift occurs when the virus acquires point mutations in the HA and neuraminidase (NA) genomic segments due to the low fidelity of the RNA polymerase, resulting in changes in the surface HA and NA proteins. Due to high mutation rates, the flu vaccine has to be updated every season. The World Health Organization convenes a meeting in February every year to select the strains to be included in the vaccine for the upcoming season for the northern hemisphere (September for the southern hemisphere). The decision of which strains to include in the vaccine is made based on analyzing data collected from over 100 national influenza centers conducting year-round surveillance in over 100 countries.

Besides vaccines, there are also antivirals available against influenza viruses. These include the M2 proton channel inhibitor (adamantanes such as Amantadine and Rimantadine), PA cap-dependent endonuclease inhibitor (baloxavir marboxil, tradename Xofluza) and neuraminidase inhibitors (Oseltamivir, tradename Tamiflu; Zanamivir, tradename Relenza; Peramivir, tradename Rapivab), and a nucleoside analog that is currently only approved in Japan (Favipiravir, tradename Avigen). Although most of the circulating IAV H1N1 and H3N2 strains remain susceptible to neuraminidase inhibitors[12], most circulating human IAVs are now resistant to adamantanes[12,13]. The emergence of resistant strains underscore the importance of developing new vaccine and therapeutics.

Biology of Influenza A Virus

Influenza viruses are enveloped, segmented, single-stranded negative-sense RNA viruses belonging to the *Orthomyxoviridae* family. IAV and influenza B virus (IBV) have eight unique genomic segments encoding ten essential viral proteins and several accessory proteins[14,15], while influenza C virus, and the recently identified influenza D virus contain seven segments encoding nine proteins. The first three types can infect humans and cause respiratory illness[16,17]. IAV is further subdivided into subtypes based on the antigenic properties of HA and NA proteins. With the isolation of a novel H18N11 IAV in fruit bats[18], 18 different HAs and 11 different NAs have been reported across various animal hosts[16], including aquatic birds and bats. Each of the genomic segment consists of a negative-sense RNA molecule that wraps around nucleoproteins (NP) to form a twisted antiparallel double helix, and a trimeric polymerase subunit complex comprising PA, PB1 and PB2, that is attached to the end of the viral ribonucleoprotein (vRNP) opposite of the hairpin loop structure[19].

IAV Entry

Airway epithelial cells are the primary target cells of influenza, although macrophages and dendritic cells are also susceptible. Despite being susceptible, macrophages and DCs tend to result in abortive infection, and infectious progeny virions are not released[20]. Airway epithelial cells, on the other hand, are both susceptible and permissive to influenza virus replication.

The first step of virus entry involves binding to receptors present on cell plasma membrane. The receptors of IAV are sialic acid residues that are linked to the penultimate galactose residues on plasma membrane-resident glycoproteins or glycolipids. Human and other mammalian influenza viruses HAs prefer binding to sialic acids that are attached to the galactose via a α-2,6 linkage, while avian influenza viruses HAs prefer binding to sialic acids that have a α-2,3 linkage; these differences in binding receptor specificity could potentially account for viral tropism and transmissibility[21–24].

Upon binding to the sialic acid receptors, the viruses could induce clathrin-dependent endocytosis[25,26], as well as macropinocytosis[27] for internalization. Following that, the virus is trafficked from the plasma membrane to the late endosome. As pH of the endosome drops, viral HA undergoes a conformational change[28], moving aside to expose HA2's N-terminal fusion peptide. The fusion peptide gets inserted into the endosomal membrane to initiate fusion between the viral membrane and the endosomal membrane[29].

Prior to the pH-induced conformational change, IAV HA has to be proteolytically pre-cleaved[30,31] by cellular proteases such as transmembrane protease serine S1 member 2 (TMPRSS2), or by human airway trypsin-like protease (HAT) into HA1 and HA2[32]. This pre-activation cleavage could occur in the trans-Golgi network[33] prior to budding from the virus-producing cell (by TMPRSS) or at the plasma membrane of the target cell being infected[34] (by HAT). Besides TMPRSS2 and HAT, other proteases such as furin may also play a role in cleaving HAs that contain a multibasic cleavage site[35], as seen in highly pathogenic avian IAVs.

In addition to triggering fusion between viral and endosomal membranes, the low pH also causes the interior of the viral particle to get acidified as protons enter through the M2 proton channel on the viral envelope. This causes M1 to depolymerize and the vRNPs to dissociate from the matrix[36,37]. The vRNPs escape into the cytoplasm as the fusion pore forms.

Transcription

Cytoplasmic vRNPs are then imported into the nucleus via the classical importin-α (IMPα)-importin-β1(IMPβ1)-dependent nuclear import pathway[19]. First, the adaptor protein IMPα bind to the vRNPs via recognition of basic (arginine- and lysine-rich) nuclear localization signals (NLSs). The vRNP-IMPα complex then interacts with the transport receptor IMPβ before the ternary complex translocates through the nuclear pore complex (NPC)[36]. Although all four protein components of vRNP contain NLS motifs, nuclear import is probably mainly mediated by NP, which has two NLSs – a non-classical NLS within its first 13 amino acids that binds to IMPα[38], and a classical bipartite NLS in the middle. Although the relevance of the second classical bipartite NLS in NP has been questioned, because crystallography shows that the bipartite motifs are too close together to be functional[39], subsequent work using NLS-mimicking peptides and anti-NLS antibodies have demonstrated that both NLSs contribute to the nuclear import of vRNPs[40].

Once the vRNPs enter the nucleus, activated ras-related nuclear protein (RAN-GTP) promotes the dissociation of both importins from vRNPs. The released vRNPs then undergo primary transcription mediated by the viral polymerase complex, independently of *de novo* viral

protein synthesis[41]. Transcription is primer-dependent and relies on cellular capped pre-mRNAs. IAV PB2 first binds to cellular pre-mRNAs that have a 7-methylguanosine cap, before PA cleaves the pre-mRNAs about 10-13 nucleotides downstream of the cap, using its endonuclease domain located in the N-terminus[42]. The resulting 3'-hydroxyl group on the cleaved pre-mRNA is used to initiate transcription by the polymerase complex in *cis*[43] from the vRNP template.

During elongation of the viral mRNA, the RNA polymerase complex remains associated with the 5' end of the genomic viral RNA (vRNA) template, as the template gets threaded into the PB1 active site in a 3' to 5' direction. Upon reaching the conserved 5' end of the vRNA, the polymerase encounters a poly-U motif and stutters due to template slipping. The repeated copying of the U sequence results in a polyA tail[44,45] and termination of transcription. Some segments, such as segments 7 (M) and 8 (NS) undergo alternative splicing to generate multiple proteins from a single segment, while other segments utilize different strategies such as alternative translation initiation sites and ribosomal frameshifting. After primary transcription, the nascent 5' capped and 3' polyadenylated mRNAs are exported from the nucleus to the cytoplasm, in a NXF1-dependent (but not CRM1-dependent) manner[46–49] for translation by cellular ribosomes.

Although the precise mechanisms by which viral mRNAs are exported remains unclear, the mechanisms of how IAV exhibits selective inhibition of cellular mRNAs nuclear export is more well-characterized. For instance, IAV non-structural protein 1 (NS1) interacts with host proteins NXF1, p15, Rae1 and E1B-AP5 (constituents of mRNA export complex) to promote nuclear retention of polyadenylated mRNAs[50]. NS1 expression also downregulates a nucleoporin

component Nup98, which further reduces mRNA export[50]. In addition, NS1 can also bind to CPSF to inhibit 3' cleavage and polyadenylation of cellular pre-mRNAs, and to polyadenylate binding protein 2 (PABII; mediates processive elongation of polyA tail) to prevent its interaction with polyadenylate polymerase (PAP; catalyzes synthesis of polyA)[51]. The interaction with CPSF and PAP inhibits pre-mRNA polyadenylation, causing these pre-mRNAs to remain trapped in the nucleus and becoming a source of capped RNA primers for IAV transcription[52].

Nuclear Import of Nascent Viral Proteins, Genome Replication

After translation, some of the proteins, such as NP and the viral polymerase subunits, are imported into the nucleus to facilitate genome replication and secondary transcription, while others such as M2, HA, NA are transported to the plasma membrane. PA and PB1 associate and are imported as a dimer[53] in a manner dependent on PB1's interaction with IMPβ Ran-binding protein 5 (RanBP5)[54,55]. On the other hand, PB2[56,57] and NP[38,58] separately could bind to IMPα and get imported via the classical IMPα/β pathway. Once the PA/PB1 dimer and PB2 are imported into the nucleus, they associate to form the functional polymerase complex[59].

Once the nascent polymerase subunits and NP are imported, genome replication commences. This occurs in two stages, first with positive-sense cRNA being synthesized using the negative-sense vRNA as a template, followed by vRNA synthesis using the newly made cRNA as template. Initiation of cRNA synthesis occurs in a primer-independent fashion where a triphosphorylated AG dinucleotide (pppApG) is stabilized by the PB1 priming loop onto the 3'

end of the vRNA template[60,61]. Once the pppApG dinucleotide is formed, the polymerase can then elongate the cRNA to form a full-length copy of the vRNA without a cap or polyA tail.

In contrast to the terminal initiation of cRNA synthesis, vRNA synthesis from cRNA template occurs via internal initiation independently of the PB1 priming loop. The pppApG is synthesized internally, directed by positions 4 and 5 of the 3' end of the cRNA template, which then gets realigned to positions 1 and 2 before elongation occurs[61]. The resident polymerase complex on the cRNP remains bound to the 5' end of the cRNA, while a trans-activating polymerase complex (composed of the nascent polymerase subunits) binds to the 3' end of the cRNA to carry out the internal initiation and elongation of the vRNA[43,62,63]. The 5' end of the nascent vRNA remains bound to the trans-acting, replicating, polymerase complex, which also promotes NP encapsidation on the nascent vRNA in a 5' to 3' direction. This is in contrast to the transcription process, where the resident cis-acting polymerase carries out the transcription. The newly synthesized vRNPs can then serve as templates for secondary transcription, and eventually get exported out of the nucleus to be assembled into progeny virions.

Nuclear Export, Assembly, and Budding

Besides the viral polymerase subunits and NP proteins, M1 and NEP are also imported to the nucleus. These two have roles for the nuclear export of assembled vRNPs. M1 has been shown to be essential for the export of vRNPs, and could also play a role in preventing nuclear import of assembled vRNPs[36]. NEP mediates the export of vRNPs via the host Crm1 nuclear export pathway[64] by interacting with the cellular CRM1 protein through two distinct N-terminal

nuclear export signal (NES) motifs[65]. While more work needs to be done to clarify how these proteins mediate nuclear export, the current model is that NEP interacts with M1, which binds vRNPs, in order to bridge vRNPs to the nuclear export machinery[64].

Upon nuclear export, the progeny vRNPs are transported across the cytoplasm to the plasma membrane, in a RAB11A- and microtubule-dependent manner[66–69], where assembly of viral particles takes place. It is proposed that vRNPs piggy-back onto RAB11A vesicles through an interaction between RAB11A and the viral polymerase complex[66], possibly due to direct interaction between RAB11A and PB2[68]. Upon reaching the plasma membrane, the eight different vRNPs assemble into viral particles containing HA, NA, M1 and M2. Both 5' and 3' ends of each vRNA segment, including both non-coding and parts of coding regions, form the packaging signal required for assembly into virions[70]. Although two different models were proposed for how packaging occurs, subsequent works using transmission electron microscopy[71] and single-molecule fluorescent in situ hybridization[72] have favored the selective packaging model over the random packaging model .

The HA and NA proteins are targeted to the lipid rafts on the plasma membrane where they cause deformation of the plasma membrane to initiate budding[73]. In addition, other viral proteins also play an important role in virus budding. For instance, M1, besides regulating nuclear export, also assists in bending the membrane presumably through its ability to oligomerize and form curved structures[74–76]. In addition, M2 promotes scission of budding viral particles from the plasma membrane[77], while NA cleaves sialic acids to release these budding particles from the cell surface[78–80].

As viruses have to complete many different steps in the life cycle in order to achieve successful replication, one could hypothesize that, cells, under constant selection pressure, would evolve different strategies to target any of these steps in this never-ending arms race. Indeed, as discussed in the following sections, many restriction factors, acting on various steps in the virus life cycle, have been identified over the years. It is likely that even more factors will be discovered and characterized as novel high throughput methods get developed and the cost of performing large unbiased screens decreases in future.

Review of anti-influenza restriction factors

The first line of defense against a viral infection is the innate immune system, which involves detecting the presence of the invading pathogen within the infected cell, and secreting signaling cytokines to protect itself and prime neighboring cells for action. Such signaling results in both the upregulation and activation of restriction factors, as well as the recruitment of immune cells to the site of infection. One of the earliest cytokines produced during an infection are the type I IFNs, which were first discovered in 1957[1]. Isaacs and Lindenmann noticed that chorioallantoic membranes could resist infection by fresh IAV if the membranes had been pre-exposed to heat-inactivated IAV. They then did a follow-up experiment that showed that such resistance could be transferred to cells that had not been pre-exposed to viruses. This involved washing these virus-exposed membranes before incubating them in fresh media. When this conditioned media was subsequently transferred onto fresh membranes, the fresh membranes became resistant to virus infection too, suggesting that a protective factor (which they named

IFN) was secreted from the original virus-exposed membranes. It took almost two decades before IFN was purified sufficiently from human leukocytes and fibroblasts to be characterized chemically and biologically[81–85]. Around that period, molecular biology techniques had also advanced such that researchers could clone IFN complementary DNA (cDNA)[86,87] to produce IFN from a bacterial expression system. These advancements, along with the discovery that IFNs bind to cell surface receptors[88,89] to induce signaling pathways laid the groundwork for further research and understanding of the molecular mechanisms behind this critical antiviral response[90].

Type I IFNs are secreted when a range of pattern recognition receptors (PRRs) recognize pathogen-associated molecular patterns (PAMPs) that are generated during an infection. Depending on the nature of the pathogen and PAMP, different pathways activate to induce IFNs. RNA viruses could be detected by endosomal Toll-like receptors (TLRs) and RIG-I-like receptors (RLRs). For instance, TLR3 recognizes dsRNA[91], TLR7 and TLR8 recognize U-rich and GU-rich ssRNA respectively[92], melanoma differentiation association gene 5 (MDA5) recognizes long dsRNA[93], and retinoic acid-inducible gene I (RIG-I) recognizes short dsRNA with a 5'-triphosphate[94–97] or diphosphate group[98]. RIG-I is thought to be the main sensor during IAV infections[99,100], although MDA5 may play a supportive role as MDA5-deficient cells show lower IFN upregulation in response to IAV infection[100,101]. Following IFN secretion, IFN can bind to ubiquitously expressed type I IFN receptor and induce upregulation of hundreds of interferon-stimulated genes (ISGs)[4,5] to confer an antiviral state[102–104]. However, even though the existence of ISGs were first reported over 40 years ago[105,106], many of the ISGs that have been identified since then still remain poorly understood in terms of their roles and mechanisms of action[2].

Restriction Factors that Target Viral Entry

One of the earliest ISGs, interferon-inducible transmembrane (IFITM) proteins, had been discovered in 1984[107]. However, despite the recognition that IFITMs are interferon-inducible, it was not until 2009 when two groups separately identified IFITM3 as a virus restriction factor through siRNA screens[108,109]. Although the mechanisms of IFITM1 and IFITM2 have yet to be clearly elucidated, IFITM3, which localizes to endosomal and lysosomal compartments, is much better characterized. IFITM3 is thought to prevent cytosolic entry[110], perhaps by blocking the formation of fusion pores after virus-endosome hemifusion occurs[111], although there was another report suggesting that IFITMs block hemifusion[112]. Recent work has also suggested that IFITM3 may directly interact with incoming virus particles and shuttle them to lysosomes for degradation[113]. Although the exact molecular mechanism warrants further investigation[114], the relevance of IFITM3 *in vivo* has been shown through the use of KO mice[115,116], and in human genetic studies where alleles associated with illness severity have been reported[116,117].

Subsequent characterization of IFITM3 uncovered another restriction factor, zinc metallopeptidase STE24 (ZMPSTE24), through the use of affinity purification-mass spectrometry[118]. ZMPSTE24 is not upregulated by IFN exposure, but is necessary for IFITM3's antiviral activity. Deficiency in mouse embryonic fibroblasts (MEFs) and human tracheal epithelial cells rendered these cells susceptible to IAV and other viruses that utilize the endosomal compartments for entry. Interestingly, the antiviral activity of ZMPSTE24 is independent of its protease activity. In addition, genetic complementation experiments revealed

that ectopic expression of IFITM3 in *zempste24*$^{-/-}$ cells does not restore antiviral function, but ectopically expressed ZMPSTE24 can restore antiviral activity in *ifitmDel*$^{-/-}$ MEFs. Silencing ZMPSTE24 in *ifitmDel*$^{-/-}$ MEFs further decreases antiviral activity, suggesting that ZMPSTE24 is both downstream of IFITM3, and has IFITM-independent antiviral activity. The relevance of ZMPSTE24 *in vivo* was also shown using *zmpste24*$^{-/-}$ mice[118].

In addition to IFITMs, another ISG, B4GALNT2, recently identified through a genome-wide CRISPR synergistic activation mediator (CRISPR-SAM) screen, also targets virus entry[119]. Instead of preventing cytosolic escape, B4GALNT2 adds an extra amino sugar *N*-acetylgalactosamine (GalNAc) residue to the penultimate galactose residue that has an α-2,3-linkage to the terminal sialic acid. This additional of GalNAc inhibits cell-surface attachment of IAV strains that prefers α-2,3-linked sialic acid, i.e. avian IAV strains. Although the mechanism was elucidated using virus binding assays and mass spectrometry analysis of glycans, the functional relevance of B4GALNT2 in a physiological context remains unknown, as the authors did not perform any loss-of-function (LOF) or *in vivo* experiments.

Another restriction factor that might inhibit virus entry is cholesterol 25-hydroxylase (Ch25h). Ch25h forms 25-hydroxycholesterol (25HC) via oxidation of cholesterol[120], and 25HC treatment has been reported to inhibit many enveloped viruses in cell cultures (specifically VSV, HIV, HSV-1, MHV68, Ebola virus, Nipah virus, Russian Spring-Summer Encephalitis virus, and Rift Valley fever virus). The authors proposed that 25HC directly modifies cellular membrane to inhibit virus-cell membrane fusion, based on the limited evidence using artificial liposomes to outcompete 25HC to cause a dose-dependent reversal of antiviral inhibition[121]. In the context of

IAV, 25HC was shown to confer protection to MDCK cells when supplied exogenously, in a dose-dependent fashion[122]. However, the number of plaques at 2 days post-infection of 25HC-treated MDCKs do not differ from untreated cells, even though the plaques are much smaller, suggesting that the block is occurring at a post-entry step[122]. It should be noted that 25HC also affects many other cellular pathways and can even act as an immune mediator by suppressing IgA production in B cells[123], or amplifying inflammatory signaling[124]. In the latter report, Gold *et al.* showed that although immortalized *Ch25h*$^{-/-}$ epithelial cells were more susceptible to IAV infection, and that exogenous 25HC inhibits infection in a dose-dependent manner *in vitro* similar to a previous report[122,124], they could not detect differences in viral RNA load in the lungs of WT and *Ch25h*$^{-/-}$ mice. Instead, they observed lower induction of cytokines and antiviral factors, lower lung pathology, and better survival rates in the *Ch25h*$^{-/-}$ mice after IAV infection[124]. While the relevance of 25HC and Ch25h has been shown *in vivo* via HIV infection of humanized mice and MHV68 infection of *Ch25h-/-* mice[121], the relevance and mechanisms of action of 25HC and Ch25h are less clear during IAV infection. This not only highlights the importance of functional testing *in vivo*, but also the importance of interpreting data in the specific context of individual viruses.

Restriction Factors that Target Nuclear Import

Besides inhibiting virus entry, restriction factors could also target other post-entry steps in the virus life cycle. For instance, myxoma resistance (MX) proteins could inhibit the import of IAV vRNPs into the nucleus, where transcription and genome replication take place. Human MxA, a member of the dynamin superfamily of GTPases, traps vRNPs in the cytoplasm[125], as

evidenced by immunofluorescence studies of IAV-infected A549 cells that overexpressed MxA or had MxA silenced. This restriction has been proposed to occur via MxA forming oligomeric rings around vRNPs[126]. In contrast to human MxA, mouse Mx1 does not block nuclear import of vRNPs[127], but instead interferes with PB2-NP interaction in the nucleus, preventing the assembly of the RNP complex and decreasing polymerase activity[128]. Aside from preventing nuclear entry by retaining incoming vRNPs in the cytoplasm[125], human MxA has also been hypothesized to target IAV at a step after primary transcription based on infecting mouse 3T3 cells that overexpress human MxA in the presence of CHX. These MxA-expressing cells showed the same level of primary transcription as MxA-deficient cells in the presence of CHX, but much lower total viral RNA in the absence of CHX, suggesting that a post-primary transcription step was targeted[129]. Additional groups have tried clarifying the mechanisms of human MxA. For instance, by using a minigenome luciferase assay, Turan *et al.* reported that engineered nuclear MxA could inhibit IAV transcription in mouse 3T3 cells[130]. However, the relevance of this proposed mechanism in human cells remains questionable, as the MxA used in their study was specifically engineered to localize to the nucleus. Regardless of the exact molecular mechanism, the ability of MxA to restrict IAV *in vivo* has been demonstrated with a transgenic mouse model overexpressing human MxA[131].

Recent work by Lee *et al.* provided evidence of a new role of MxA during IAV infection[132]. They proposed that MxA could sense IAV infection, via an unknown mechanism, and trigger inflammasome activation to secrete IL-1β in a caspase-dependent manner in respiratory epithelial cells. MxA-mediated inflammasome activation leads to a more rapid inflammatory response in the bronchioles of transgenic mice, and promotes viral clearance and

survival during IAV infection. In addition, the authors also provided *in vitro* evidence that MxA expression could reduce IAV NP protein levels in the absence of the key inflammasome adaptor protein ASC, if MxA had been upregulated prior to IAV infection. In agreement with the earlier studies described above, their data suggest that MxA has a direct antiviral effector function independent of its inflammasome activation role. MxA could have multiple roles throughout the course of IAV infection.

Restriction Factors that Target Transcription, Translation and Genome Replication

As IAV successfully invades the nucleus and begins transcription and replication, more viral ligands are generated. These could be recognized by RLRs and other antiviral restriction factors such as the protein kinase R (PKR), which is a serine/threonine kinase that recognizes dsRNA. PKR is expressed at a basal level in all kinds of cells, but can be upregulated in response to environment stress, apoptosis, cellular growth arrest, and autophagy to regulate protein synthesis. Type I and III IFNs also upregulate PKR expression. Upon binding to dsRNA (such as the panhandle secondary structure formed by IAV vRNA termini), the latent PKR dimerizes and phosphorylates eIF2α[133], as well as IκB[134]. Phosphorylating IκB turns on the NFκB signaling pathway, which could boost IFN production[135]. On the other hand, phosphorylation of eIF2α results in a global translational shutoff. Phosphorylated eIF2α has increased affinity for eIF2B, and this increased binding leads to decreased guanine nucleotide exchange factor (GEF) activity of eIF2B. Without eIF2B GEF activity, eIF2-GTP complexes are not regenerated after eIF2-GDP is released from the ribosome. Without eIF2-GTP to bring Met-tRNA to 40S ribosomes, translation initiation is inhibited[136]. The importance of PKR *in vivo* has been demonstrated

through the use of PKR-deficient mice, as $PKR^{-/-}$ mice have higher viral yields in the lungs and lower survival rates[137]. However, an earlier study using the same mouse strain reported only slightly lower LD_{50} in the $PKR^{-/-}$ mice, and found no significant differences in viral titer in the lungs of WT and $PKR^{-/-}$ mice[138]. Differences in infectious dose and virus strains used may account for the contradicting observations[137,138].

Besides PKR, another dsRNA sensor is the 2'-5'-oligoadenylate synthase (OAS) family of proteins. Upon binding to dsRNA, human OAS1, OAS2, and OAS3 could convert ATP to 2-5-oligoadenylate (2'-5'A)[139–142]. The 2'-5'A is then recognized by latent RNAseL, which dimerizes and cleaves single-stranded RNAs (both viral and cellular) in U-rich sequences, typically after UU or UA[143,144]. Besides limiting viral replication within the infected cells, RNAseL cleavage may also induce apoptosis as part of the broader antiviral program[145,146]. Degradation products may also activate RIG-I[144], and MDA5[147] and enhance IFN production. In particular, the functional relevance of OAS-RNAseL system in restricting IAV infection has been demonstrated *in vitro* through the use of OAS3-knockout A549 cells[148], RNAseL-silenced A549 cells and RNAseL-knockout mouse cells[149]. The importance of OAS-RNAseL has also been demonstrated *in vivo* with *Rnasel*$^{-/-}$ mice[150]. However, as implied above, destroying viral RNA may not be full picture. In the same study, Chakrabarti *et al.* showed that RNAseL generates cleaved RNAs with 2',3'-cyclic phosphate termini that activate NLRP3 inflammasome, which has previously been implicated to be important for controlling IAV *in vivo*[151,152].

Another class of restriction factors that might act as viral PAMP sensors during IAV infection is the interferon-induced tetratricopeptide (IFIT) proteins, which have no known enzymatic activity. Several mechanisms of action have been proposed for the IFITs. First, IFIT1 can bind to uncapped 5'-ppp-RNA in infected cells and sequesters them from the actively replicating pool of RNAs[153]. Second, IFITs may also inhibit translation via two different mechanisms. IFIT1 could bind to 5' ends of mRNAs whose caps lack 2'-*O*-methylation on the first ribose (Cap 0 structure) to impair translation [154–156]. As such methylation pattern is present in viral mRNAs, but not cellular mRNAs, IFIT1 thus competes with eIF4E for cap binding and prevents cap-dependent translation initiation on viral mRNAs[156,157]. In addition, IFITs could also inhibit translation initiation by binding to eIF3[158,159], leading to an inhibition of overall cellular protein synthesis during infection. Moreover, since eIF3 is also involved in cap-independent translation initiation on internal ribosome entry sites (IRES), IFIT1 can potently restrict viruses that undergo cap-independent translation, such as HCV[160].

However, the relevance of IFITs during IAV infection remains controversial. Previously, Pichlmair *et al.* showed that IFIT1 can bind to 5'-ppp-RNA in IAV-infected HEK cells and possibly impair replication via a minigenome luciferase assay in HeLa cells[153]. On the other hand, evidence of IFITs directly inhibiting IAV translation is lacking. First, there has been no evidence that IFIT1's recognition of Cap 0 mRNAs applies to IAV, probably because IAV performs cap snatching from cellular pre-mRNAs. Although recent studies reported that IAV also snatches caps from noncoding RNAs such as U1 and U2 snRNAs[161,162], an even more recent study demonstrated that IFITs do not bind 5'-trimethylguanosine caps[163], which are found on U1 and U2 snRNAs[163–165], suggesting that IAV mRNAs might not be targeted by IFITs after all.

Second, the relevance of IFIT1 in inhibiting IAV translation via eIF3 remains unclear too because IAV does not encode an IRES and might not be as susceptible as viruses that depend on IRES for translation. On the other hand, eIF3 is involved in both cap-dependent and cap-independent translation initiation, and unsurprisingly, has been identified as an important host dependency factor for IAV replication in siRNA screens[166,167]. Most importantly, a recent study by Pinto *et al.* showed that human IFIT1 has no functional relevance in restricting IAV through both overexpression and knockout of IFIT1 in HEK293 and A549 cells respectively[168]. They also tested the role of murine Ifit1 in mouse tracheal epithelial cells *in vitro*, and *in vivo* with wildtype and *Ifit1*$^{-/-}$ mice, and found no evidence that Ifit1 restricts IAV. Perhaps IFIT1's activity and expression differ in different cell types under different experimental conditions, but the contribution of IFITs to restricting IAV, if any, warrants further investigation.

Besides sensing dsRNA products, restriction factors could also direct recognize single-strand RNA products produced during IAV replication. An example would be the zinc finger antiviral protein (ZAP), which has two different isoforms arising from alternative splicing (leading to different C-termini), each of which having distinct molecular mechanisms of action. The short isoform of ZAP (ZAPS), which lacks a C-terminal PARP domain, can bind specifically to viral mRNAs[169], and repress translation initiation[170]. It could also recruit degradation machinery to promote mRNA decay[171,172]. Further detailed characterization has revealed that ZAP binds to CG-dinucleotide-rich RNA to recruit RNA degradation machinery[173]. Although the above mechanisms were worked out using different viruses, and that some viruses, such as HSV-1, YFV and VSV, are unaffected by ZAP[174], Tang *et al.* have shown that ZAPS reduces IAV mRNAs and hence represses translation of PA, PB2 and NA[175]. The long isoform

(ZAPL) on the other hand, can bind to poly-ADP-ribosylated and ubiquitinated PA and PB2 via its C-terminal PARP domain, and targets these proteins for proteasomal degradation[176].

ISG20, first identified as an IFN-induced protein associated with promyelocytic leukemia (PML) nuclear bodies[177], also displays antiviral properties against IAV[178,179]. It has been thought that ISG20 exerts antiviral effect via RNA degradation. This is based on the following observations: ISG20 exhibits higher RNA but much lower DNA exonuclease activity[180]; restricts several RNA viruses (VSV, IAV, EMCV) but not a DNA virus (adenovirus)[178]; and virus inhibition is dependent on ISG20's exonuclease activity[178,179]. However, a recent study found that ISG20 could cause a drastic reduction in VSV protein levels with only minimal viral mRNA degradation[181], suggesting that ISG20 might act by blocking translation instead. Although the exact mechanism of ISG20 remains to be worked out, its relevance in restricting VSV has been demonstrated with the use of knockout mice[181]. The relevance of ISG20 in restricting IAV infection *in vivo*, however, remains to be investigated.

Adenosine deaminase acting on RNA (ADAR) 1 is another restriction factor that could target IAV RNA[182]. However, unlike PKR and OAS, which act as sensors for dsRNA, or ZAPS, which binds to mRNAs, ADAR1 modifies the viral RNAs by first converting adenosine to inosine by deamination. As inosine is structurally similar to guanosine, similar rounds of RNA replication would lead to the adenosine being replaced by a guanosine residue, resulting in A-to-G transition mutations. This has been observed in the stem-loop structure present in the viral matrix M1 mRNA[183]. As the p150 isoform of ADAR1 protects MEFs from IAV-induced cytopathic effects during IAV WSN33 infection[184], this suggests that ADAR1 may confer

protection against IAV, although the mechanism has yet to be fully elucidated. However, silencing ADAR1 has also been found to impair IAV protein expression, suggesting that ADAR1 might be a proviral factor[185]. More work is required to reconcile these conflicting observations, and to clarify the role and mechanism of ADAR1 in IAV infection.

Besides targeting IAV RNAs during transcription, translation, or replication, host restriction factors could also directly target viral proteins through various mechanisms such as sequestration, adding post-translational modifications, targeting for proteasomal degradation, and targeting for autophagic degradation.

ISG15, an IFN-induced ubiquitin-like protein that gets conjugated to target proteins, could exert antiviral effects. This is likely because ISGylation of viral proteins could alter their ability to bind to other target proteins. For instance, Tang *et al.* reported that ISGylation of IAV PR8 NS1 interferes with NS1 ability to bind to PKR[186]. Since NS1 can bind to PKR to inhibit PKR activation[187], ISGylation of NS1 could possibly prevent NS1 antagonism of PKR, which in turns leads to IAV restriction. Separately, Zhao *et al*. showed that ISGylation of NS1 inhibits NS1's interaction with IMPα[188]. As NS1 interferes with the proper processing of cellular pre-mRNAs[189,190] including those of IFN-β and antiviral genes like ISG15, IFIT1 and MxA[191,192], preventing NS1's nuclear entry could potentially release NS1's inhibition of antiviral responses and result in a heightened antiviral cell state. Furthermore, the relevance of ISG15 in IAV infection has been shown *in vivo* using ISG15$^{-/-}$ mice[186,193]. However, while the above mechanisms seem plausible, in reality, the roles of ISG15 are far from being fully understood,

since hundreds of cellular proteins can get ISGylated to achieve different outcomes, and that conjugated and unconjugated ISG15 can also have distinct functions[194].

TRIM proteins, characterized by the N-terminal Really Interesting New Gene (RING) domain, one or two B-boxes, and a coiled-coil domain, are important restriction factors. Most of the 80 known family members are E3 ubiquitin ligases. Besides regulating signaling cascades such as TRIM25 ubiquitinating RIG-I[195], TRIM65 ubiquitinating MDA5[196], TRIM31 promoting aggregation and activation of MAVS[197], and TRIM32 and TRIM56 promoting K63-linked ubiquitination and activation of STING[198,199], TRIM proteins can also function as direct restriction factors. Specifically, in the case of IAV, TRIM22 targets NP for proteasomal degradation[200], TRIM32 promotes proteasomal degradation of PB1[201], and TRIM56 reduces IAV RNAs through its C-terminal domain in a still-unknown E3 ligase activity-independent fashion[202]. It is also noteworthy that some TRIMs could have dual roles in regulating signaling pathway, and acting as a direct restriction effector, depending on its localization. An example would be TRIM25, which has also been reported to bind to vRNPs in the nucleus to prevent RNA chain elongation post-cap snatching, independent of its E3 ligase activity[203].

Additional examples of restriction factors targeting viral proteins include the following: MOV10 binds NP to interfere with interaction with IMPα[204]; DDX21 binds PB1 to interfere with PB2 and PA association and decreases RNA synthesis[205]; PKP2 competes with PB2 for PB1 binding and lowers polymerase activity and subsequent viral replication[206]; CypA interacts with M1 and promotes its degradation via the proteasomal degradation pathway[207]; and CypE binds to NP and interferes with vRNP formation[208].

Restriction Factors that Target Assembly, Budding and Maturation

Beyond entry, transcription, translation and genome replication, restriction factors could also act on later stages in the IAV life cycle, such as assembly and budding. For instance, cyclin D3, independent of its cellular function of regulating cell cycle G0/G1 phase progression, can inhibit IAV release. Cyclin D3 binds to M2, and inhibits M1-M2 interaction, preventing the proper assembly of progeny virions[209].

Another restriction factor that has been well studied is tetherin, which has an N-terminal transmembrane domain, a middle coiled-coil ectodomain, and a C-terminal GPI anchor. Working as a parallel homodimer, it prevents release of progeny enveloped virions by tethering the particles onto cell surface of infected cells. This was first identified as a restriction factor against HIV-1[210]. The role of tetherin in restricting IAV is disputed, with some studies suggesting that tetherin can restrict IAV virus-like particles[211,212] and infectious IAV[213–216], but others disputing this[211,217–219]. Subsequently, it was suggested that the sensitivity of IAV to tetherin is strain-specific and depends on the HA and NA[216]. Interestingly, it has been found that IAV M2 can downregulate cell-surface tetherin via proteasomal degradation[220], and that IAV NA can counteract tetherin possibly via desialylation[212–214], suggesting that perhaps tetherin does restrict IAV but that viruses have evolved mechanisms to counter its restriction. However, the relevance of tetherin *in vivo* has yet to be demonstrated as Londrigan *et al.* found no difference in weight loss or viral titers between IAV-infected wildtype and tetherin-knockout mice[218].

RSAD2 encodes for viperin, which is another restriction factor that acts on a late step in the IAV life cycle. Through an unknown mechanism involving farnesyl diphosphate synthase (FPPS), an enzyme in the isoprenoid synthesis pathway, viperin causes disruption of lipid rafts (detergent-resistant membrane microdomains that are enriched in sphingolipids and cholesterol) and enhances membrane fluidity in the plasma membrane[221]. Because IAV buds from lipid rafts[222], viperin expression disrupts budding and the production of infectious particles. Viperin-mediated restriction is proposed to involve FPPS because viperin interacts with FPPS and inhibits FPPS' enzymatic ability to synthesize FPP. Furthermore, overexpression of FPPS in viperin-expressing cells rescues virus release, while silencing FPPS reduces virus release. However, because protein isoprenylation does not appear to be altered by viperin overexpression, the mechanisms of how viperin inhibits FPPS, and how FPPS overexpression rescues IAV release, remain unknown. The relevance of viperin restricting IAV *in vivo* is unclear too, as viperin-deficient mice did not show increased viral load or lung damage compared to wildtype mice during a lethal challenge[223]. While this may suggest that endogenous viperin does not play a significant role in restricting IAV, perhaps due to the redundancy of other restriction factors and/or potential IAV antagonism of viperin, the high challenge dose might have also saturated viperin's ability to restrict viral replication. Besides affecting budding and release, viperin has also been reported to function through other mechanisms. For instance, viperin could bind to viral proteins such as HCV NS5A[224] and DENV NS3[225], or catalyze the production of an antiviral ribonucleotide (ddhCTP) that acts as a chain terminator to cause premature chain termination during flaviviral RNA elongation[226]. However, evidence of similar mechanisms occurring during IAV infection is lacking.

Last but not least, plasminogen activator inhibitor 1 (PAI-1), a protease inhibitor, has also been reported to inhibit IAV too. In 2015, Dittmann *et al.* provided the first evidence of an extracellular direct-acting restriction factor, by showing how PAI-1 can inhibit TMPRSS2 and other extracellular proteases. Inhibition of proteases by PAI-I causes HA of budded virions to not undergo proteolytic cleavage and maturation, resulting in lower infectivity and spread[215]. The *in vivo* relevance was also demonstrated, as *Serpine1*$^{-/-}$ mice succumb to lethal IAV challenges earlier than wildtype mice and display higher pulmonary viral titers.

Non-IFN-based Intrinsic Antiviral Restriction

Beyond protein-based direct-acting IFN-stimulated restriction factors, RNA-based restriction in mammalian cells has also been gaining attention recently. Early experiments suggested that antiviral RNAi might restrict viruses in mammalian cells, as silencing Dicer increased IAV titers and cell death in Vero cells, which lack type I IFNs[227]. However, it was years later before additional evidence of functional antiviral RNAi in mammalian cells inhibiting Nodamura virus and EMCV was published by two separate groups[228,229]. Maillard *et al.* provided evidence that small RNAs of about 22 nucleotides, generated in a Dicer-dependent manner, are loaded onto AGO2 in virus-infected mouse embryonic stem cells[228]. Furthermore, depletion of AGO2 results in higher accumulation of viral genomic RNA. On the other hand, Li *et al.* took advantage of the fact that Nodamura virus has a virus-encoded suppressor of RNAi (VSR) to show functional relevance of RNAi in suckling mice, without genetically disrupting RNAi machinery in the host[229]. Suckling mice displayed higher RNAi activity (as measured by presence of viral siRNAs) and survived the lethal challenge when infected with viruses without

VSR or with a mutated VSR protein, compared to wildtype Nodamura virus. A subsequent follow-up study demonstrated the relevance of antiviral RNAi in mammalian cells during IAV infection, where it was shown that viral siRNAs could be isolated in 293T, Vero and A549 cells during ΔNS1 IAV infection, and is dependent on Dicer[230]. Furthermore, RNAi activity is dependent on a catalytically active AGO2, but is independent of IFN signaling. It would be interesting for future studies to address if RNAi contributes to flu restriction *in vivo*, and if so, the relative contributions between RNAi and the IFN system in conferring antiviral protection.

Recurring Themes and Study Rationale

Several important themes arise from looking at previous works. First, although many restriction factors have been identified[4,5], not all restriction factors are effective against all viruses. For example, although APOBEC3G, a well-characterized antiviral restriction factor against HIV-1[231], is induced during IAV infection, it does not restrict IAV[232]. This was also shown in a more systematic fashion in the studies by Schoggins *et al.*[2,233], where they showed that different ISGs have different range in their antiviral specificities, with some displaying a broad specificity but others displaying much narrower specificities. In addition, Richardson *et al.* also reported that although IFI6 can restrict several flaviviruses, IFI6 has no effect against the related *Flaviviridae* family member HCV[234]. Unless a protein is involved in the regulation of many other restriction factors (for instance, regulating the IFN signaling pathway), it would be unwise to conclude whether a particular virus is susceptible to a particular restriction factor without prior testing.

Second, a restriction factor could have multiple mechanisms of action and/or act on different steps in the virus life cycle. For instance, IFITs could sequester RNAs from replication[153], and block translation initiation[154–156]. ZAP, depending on its isoforms, could either repress mRNA translation (ZAPS)[175], or promote viral protein degradation (ZAPL)[176]. Viperin prevents budding and release of IAV[221], but can also synthesize antiviral ribonucleotides that prematurely terminate RNA elongation in flaviviruses[226], further underscoring the fact that restriction factors may have different functional relevance during infections with different viruses. Moreover, restriction factors can also have both direct effector function through interacting with viral proteins or nucleic acids, and indirect regulatory roles affecting the general antiviral state of the cell. For instance, depending on its subcellular location, TRIM25 can prevent viral RNA chain elongation in the nucleus[203], and regulate RIG-I activation in the cytoplasm[195]. Beyond the context of a single cell, restriction factors may also affect the antiviral response at the organism level through regulation of inflammatory signaling, such as MxA inducing the secretion of IL-1β[132], or CH25H catalyzing the formation of 25HC[124]. Hence, it is imperative to investigate the functional relevance of any restriction factor *in vivo*. Although *in vivo* studies using knockout mouse models have validated the importance of some restrictions factors such as IFITM3[115,116], PKR[137], RNAseL[150] and ISG15[193], the picture is less clear for other restriction factors, such as viperin[223], IFIT1[168], and tetherin[218], which have shown inconsistencies between *in vitro* and *in vivo* data. This could be due to various reasons such as ectopic overexpression altering the properties and/or localization of the restriction factors; presence and absence of required cofactors; or differences in virus strains, infectious doses and cell types. Furthermore, this is also complicated by the fact that many viruses have evolved

countermeasures to overcome these restriction factors, or even hijack these factors to promote their own replication.

Despite the tremendous amount of progress in antiviral research since 1950s, we still do not have a good grasp of how antiviral protection is achieved. Advancements in experimental techniques have allowed for cellular manipulation such as ectopic overexpression and gene silencing, and more recently, high-throughput genome-wide screens. However, the identification of the many ISGs[4,5] is only the first step. Probably many other genes, without being upregulated by IFN, also play important roles in antiviral defense. The functions and molecular mechanisms of both IFN-stimulated and non-IFN-stimulated restriction factors remain poorly characterized.

To address this, we performed a genome-wide CRISPR-KO screen to identify potential novel factors that could exhibit antiviral properties against IAV in A549 cells. The specific aims of this study is to conduct a genome-wide pooled CRISPR screen in A549 cells to identify host factors that restrict IAV, validate candidate hits and elucidate the biology of a novel host restriction factor.

CHAPTER 2: Using a Screening Approach to Identify Antiviral Factors

Introduction

The use of high throughput screens to identify factors required for virus growth and/or restrict virus growth is a relatively recent advancement. One approach would be genetic screens. These include genome-wide LOF screens such as RNAi screens using siRNAs or shRNAs, haploid genetic screens involving insertional mutagenesis, and more recently CRISPR-based knockout screens. These screens reveal genes that are necessary for a particular phenotype, such as viral replication or inhibition of viral replication. On the other hand, genetic screens could also take the complementary approach of gain-of-function (GOF) screens, using tools such as cDNA overexpression and the more recently developed CRISPR-SAM. These screens are effective in identifying factors that are sufficient for a particular phenotype, and especially factors whose overexpression could overcome impairment or inactivation by viral antagonists. Another approach would be the chemical screens, using chemical inhibitors of particular host or viral proteins. However, due to challenges in target identification, chemical screens are usually limited to molecules with known targets, and hence are less unbiased than genetic screens. Integration of data from such screens with other approaches using proteomics tools such as affinity purification-mass spectrometry (AP-MS) and yeast two-hybrid (Y2H) would help narrow down the list of candidate factors and increase confidence in the hits.

Considerations of screens include selecting the gene sets to be tested (genome-wide, specific gene categories based on Gene Ontology, or more restricted gene lists after curating proteomic and/or transcriptomic data), screen type (LOF or GOF), platform (arrayed, pooled, or

selection), infection assay (cell type, virus, experimental readout and quantification), and statistical analyses. Choosing the assay involves deciding which step(s) of the virus life cycle to measure, and the readout. After performing the screen and obtaining a list of candidates, follow-up studies are needed to define the molecular mechanism of the selected factor of interest.

Few large-scale GOF screens have been done, possibly due to technical challenges such as the high costs of cloning all putative open reading frames in the human genome, and the small size limitation of cDNA constructs in expression vectors[119]. In addition, using cDNAs also has an additional caveat of potentially missing isoforms arising from alternative splicing. Despite these challenges, there have been such studies performed. Schoggins *et al.* tested the effects of about 400 different ISGs by overexpressing each cDNA in an arrayed format, and then measuring the effect of each ISG on a panel of about 14 different viruses[233] using a FACS-based assay. More recently, Heaton *et al.* took advantage of the new CRISPR-SAM technology, which modifies Cas9 to recruit transcriptional activators rather than cleave DNA, to overexpress genes. Through this genome-wide CRISPR-SAM approach, they identified a novel restriction factor, B4GALNT2, that restricts avian IAV[119].

On the other hand, several genome-wide LOF screens have been performed to identify proviral and antiviral genes in IAV infection. For example, Hao *et al.* identified a vacuolar ATPase (vATPase) subunit ATP6V0D1, and nuclear export factor NXF1 as IAV host dependency factors in a RNAi screen performed on Drosophila cells[166]. RNAi screens performed in human cells by Karlas *et al.* and König *et al.* identified additional proviral factors, such as SON and CLK1[167], and members of the vATPase and COPI-protein families, nuclear import

components, proteases, and CAMK2B[235]. Besides identifying proviral proteins involved in endosomal acidification, vesicular trafficking, mitochondrial metabolism, and RNA splicing, Brass *et al.* also identified the antiviral proteins IFITMs[108]. A haploid screen has also been done on IAV, identifying CMAS and SLAC35A2 as crucial host dependency factors for IAV infection[236]. Recently, CRISPR-Cas9 has also been used to knock out, rather than knock down, genes in a genome-wide fashion to identify proviral genes during IAV infection[237,238].

In addition to genome-wide LOF screens, combinatorial approaches have been adopted too. For instance, Shapira *et al.* integrated Y2H and gene expression data to identify candidate host factors involved in IAV life cycle before performing siRNA perturbations[109]. Similarly, Watanabe *et al.* first used AP-MS to identify host proteins that co-precipitate with viral proteins before performing siRNA against candidates to identify host proteins involved in IAV replication[239].

While genome-wide LOF screens have identified many antiviral factors that restrict other viruses, such as HIV[240], arboviruses[241], flaviviruses[234], few anti-IAV restriction factors have been identified and validated through such screens. Of the screens that were designed to target host factors involved in IAV infection, only 11 and 4 genes were reported to increase IAV replication and/or gene expression by Hao *et al.* and Brass *et al.* respectively [108,166] (Karlas *et al.* and Konig *et al*. did not provide raw data of genes that, when disrupted, increased viral replication). This could be due to the design of the screens being sub-optimal for the purpose of identifying antiviral factors. For example, in the recent genome-wide CRISPR-KO screen performed by Li *et al.*, a relatively high MOI was used to ensure almost all cells, except those lacking proviral

host dependency factors, got infected before the cells were FACS-sorted on HA expression. As the goal of that study was to identify cells that become resistant to infection upon gene perturbation, cells that had antiviral factors knocked out and became more susceptible would not be distinguishable from cells that still had an intact antiviral repertoire. Furthermore, as these IAV-specific screens were not performed in the presence of IFN treatment, only restriction factors that constitutively have high expression levels and/or exhibit very potent restriction effects would be identifiable.

We thus hypothesize that if we were to tailor the screen conditions specifically to identify antiviral factors, such as pre-treating cells with IFN prior to IAV infection, we could potentially identify novel host factors that restrict IAV infection in a systematic and unbiased way. This would complement the GOF screens, and potentially reveal factors that are not only sufficient, but also necessary, at restricting IAV in a physiological context.

Results

Development of a genome-wide CRISPR screen

To perform a genome-wide CRISPR screen, we generated A549 lung epithelial cells that stably express Cas9, and transduced these A549-Cas9 cells with the AVANA4 lentivirus library[242] containing 74,700 sgRNAs targeting 18,675 protein-coding genes, as well as 1000 non-targeting sgRNAs at a low multiplicity of infection to ensure that most cells only receive a single sgRNA. After 8 days of puromycin selection, we added 200U/ml IFNβ to ~300 million library-transduced cells, and after 24 hours, infected with influenza A/Puerto Rico/8/1934 (PR8) virus at a multiplicity of infection (MOI) of 5. After 16 hours, influenza-infected cells were flow-sorted based on the level of viral HA on the cell surface, and their genomic DNA extracted for sequencing to identify enriched or depleted sgRNAs (**Figure 1A**). In order to avoid complications arising from multiple rounds of virus replication and re-infection, we omitted trypsin from the cell culture media. As A549 cells do not support cleavage of HA with a monobasic cleavage site[32], the progeny virions would not undergo HA cleavage and maturation, and cannot infect cells in subsequent rounds of infection. As a result, our screen assays only a single round of replication up to the point of HA trafficking. To find antiviral factors, we identified sgRNAs that were enriched in cells with the highest 10% amount of viral HA protein on the cell surface (infected, susceptible) versus cells with median viral HA expression in the lower peak (uninfected, protected) (**Figure 1B**).

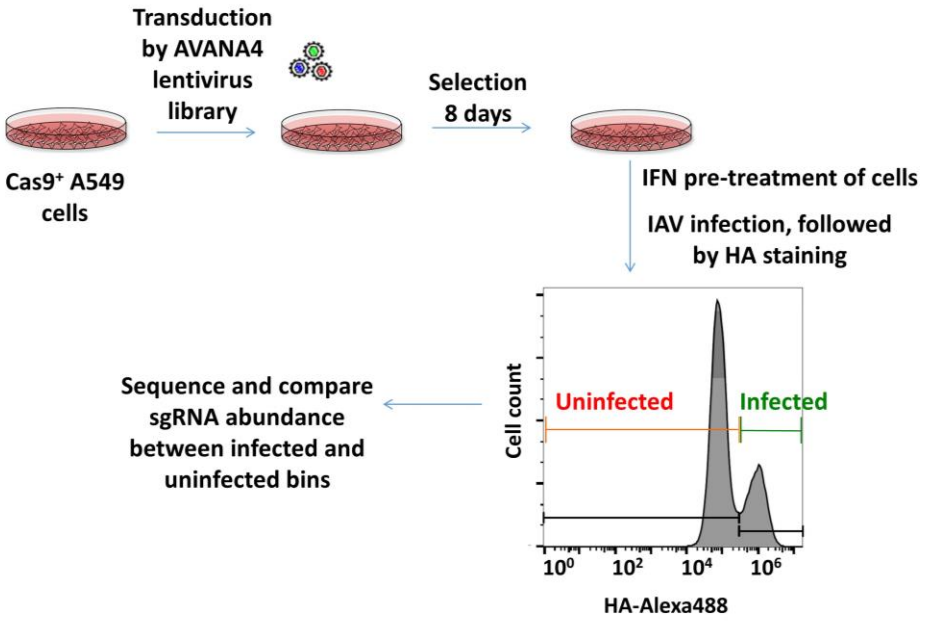


Figure 1A: Schematic overview of the CRISPR/Cas9 screen to identify genes required for blocking of infection in response to interferon.

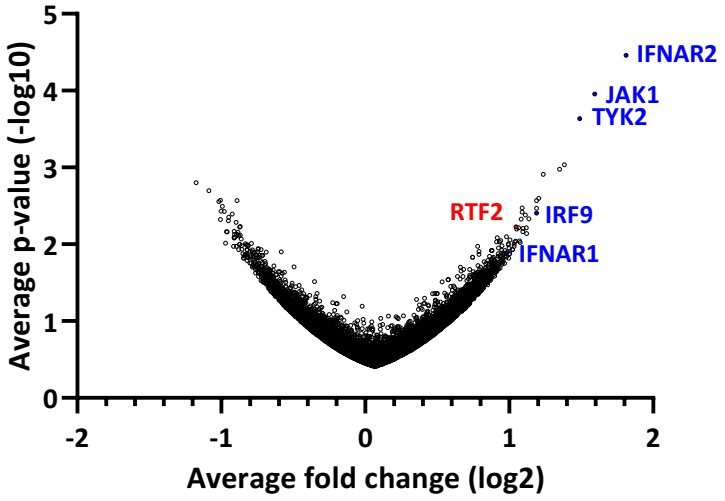


Figure 1B: Volcano plot showing enrichment of sgRNAs in infected versus uninfected cells. Fold change (X axis) and statistical significance (Y axis). Blue text shows known genes of the IFN pathway.

The top three hits were interferon-α/β receptor 2 (IFNAR2), non-receptor tyrosine-protein kinase 2 (TYK2), and Janus kinase 1 (JAK1), and further down the list were interferon-α/β receptor 1 (IFNAR1) and interferon regulatory factor 9 (IRF9), all essential components in the interferon signaling pathway[102], which were further validated using additional single guides. However, we did not recover previously reported antiviral ISGs[104], such as IFITM3[108–111], MxA[128], PKR[137], OAS3[148], RNAseL[149], viperin[221], CH25H[121], TRIM22[200] and ISG15[193], likely because each has a small effect size in the context of many interferon-induced antiviral genes (as well as the expected reduction in effect size due to incomplete editing[243] and potential enrichment for non-edited cells that have higher fitness). In contrast, since the interferon pathway components found in our screen are known to be required for inducing all ISGs, the impact of deleting them is to undo the antiviral effects of interferon.

Validation of RTF2 as an antiviral factor

Using a more sensitive arrayed format to validate some of our primary screen hits, we found that of four well-studied antiviral effectors, IFITM3, MX1, PKR and RSAD2, only IFITM3 showed an increase in infection compared to non-targeting guides (**Figure 1C**) while guides targeting IFN pathway components gave the expected increase in infection.

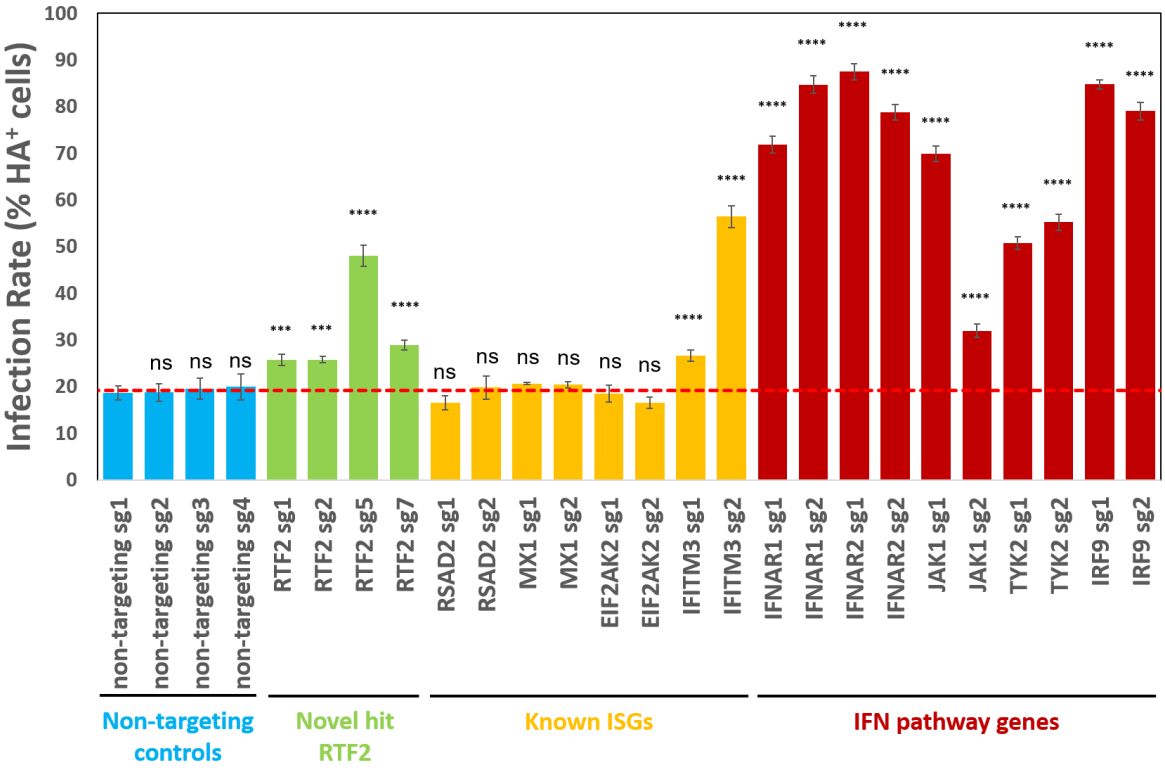


Figure 1C: IAV infection rates based on HA surface protein levels in A549 cells transduced with single sgRNAs and Cas9 lentiviruses (in triplicate). Cells were selected with puromycin (8 days), pre-treated with 200U/ml IFNβ for 24 hours, and infected with IAV at MOI 5 (adjusted for cell counts) for 16 hours. **** $p \leq 0.0001$, *** $p \leq 0.001$ by one-way ANOVA test and Dunnett's multiple comparisons against non-targeting sg1.

Among the top hits in our primary screen, RTF2 was found as an antiviral gene with little known about its cellular function or role in infection[244]. As further validation, we found that

independent guides (**Figure 1D**) led to higher HA levels in infected IFNβ-pretreated A549 cells, with an effect size inversely correlated to RTF2 expression levels (**Figure 1E**).

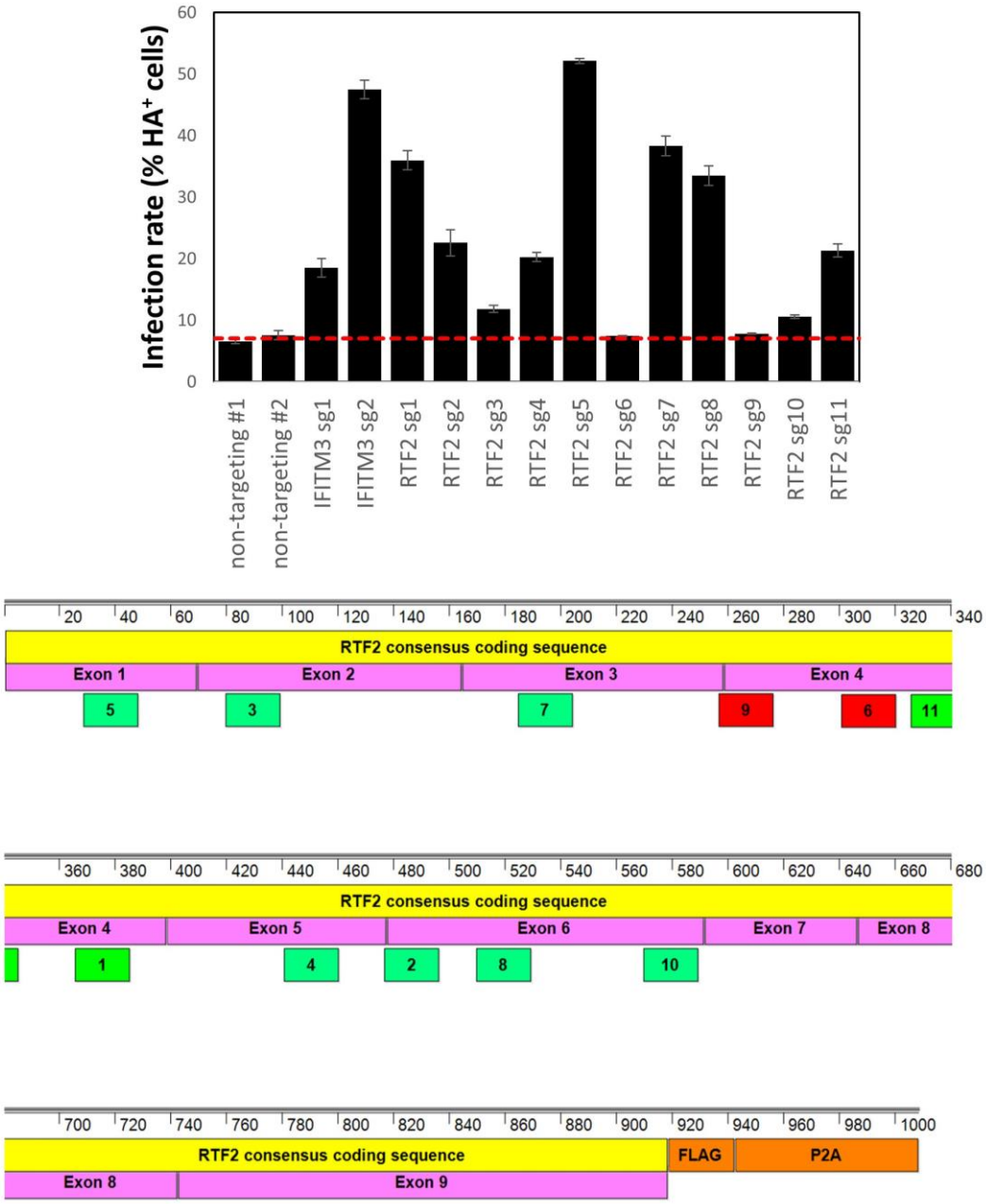


Figure 1D: Different RTF2-targeting sgRNAs, spread out across the gene, lead to different rates of IAV infection. (Top) IAV infection rates based on HA surface protein levels in A549 cells transduced with Cas9 and individual RTF2 sgRNAs. (Bottom) Schematic showing the distribution of the sgRNAs (green boxes) along the consensus coding sequence of RTF2.

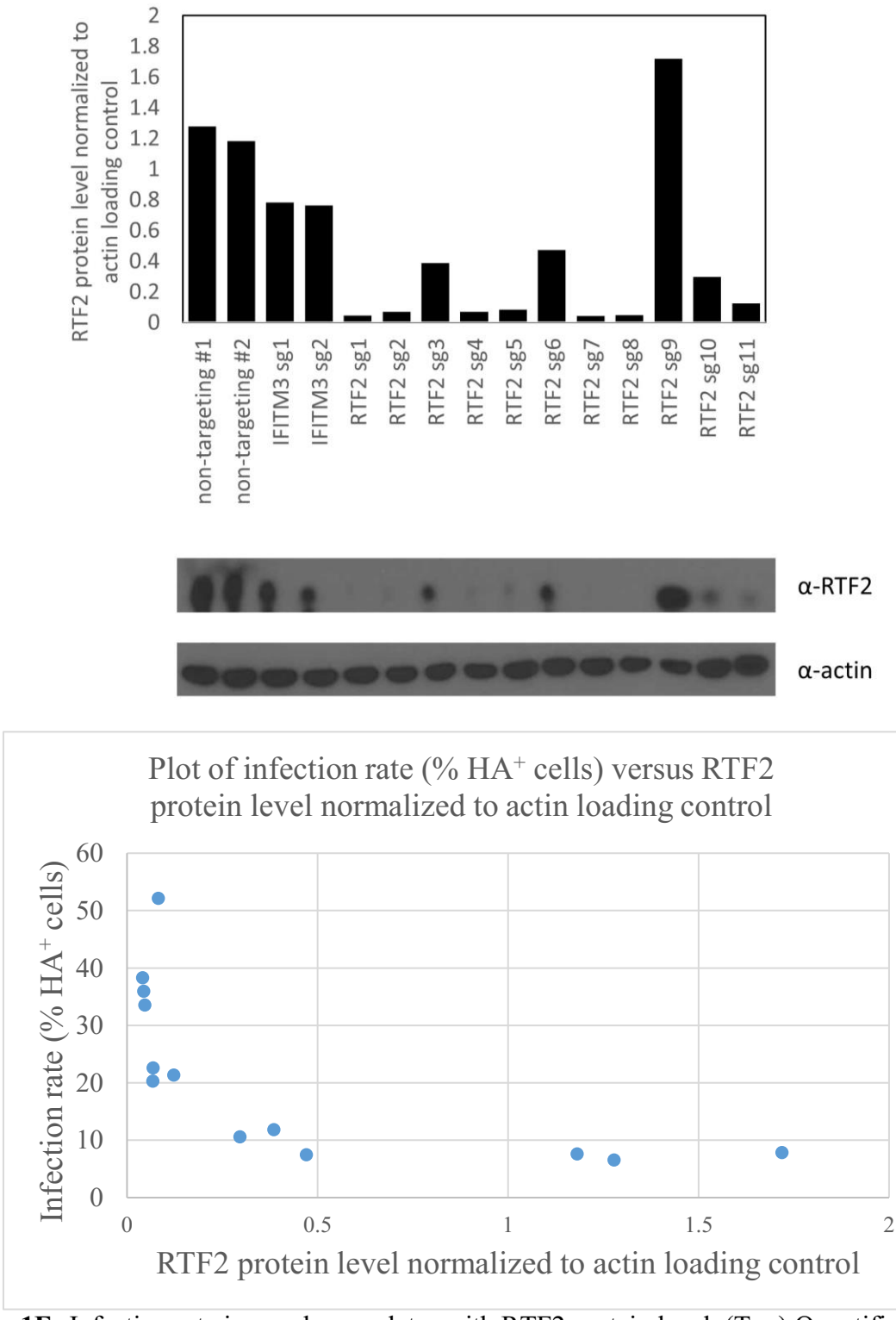


Figure 1E: Infection rate inversely correlates with RTF2 protein level. (Top) Quantification of RTF2 protein levels in RTF2-KO cells generated from different sgRNAs. (Middle) Western blot showing RTF2 protein levels in cells. (Bottom) Pearson correlation analysis gives an r value of -0.66.

In addition, overexpression of sgRNA-resistant RTF2 in RTF2-depleted cells restored protection against IAV infection (**Figure 1F**). Because gene editing with CRISPR-Cas9 does not lead to loss of function in all cells of a polyclonal population, and cells with a wildtype genetic makeup may outcompete cells that have lost RTF2 expression, we isolated individual clones of cells treated with a strong guide against RTF2 to ensure a more homogeneous population and more reproducible data. Indeed, the effect size of knocking out RTF2 was larger in several clonal knockout lines **(Figure 1G)** in which RTF2 protein expression was low or undetectable (**Figure 1H**). Subsequent experiments were conducted using clone 1 ('RTF2-KO' cells) and clone 1 cells expressing sgRNA-resistant RTF2 ('RTF2-rescued' cells). These clonal RTF2-KO cells have no detectable RTF2 proteins and have a 2-bp deletion leading to a frameshift in one allele, and a 24-bp deletion in the other allele (**Figure 1I**). Based on these data, we conclude that RTF2 has an antiviral role during IAV infection.

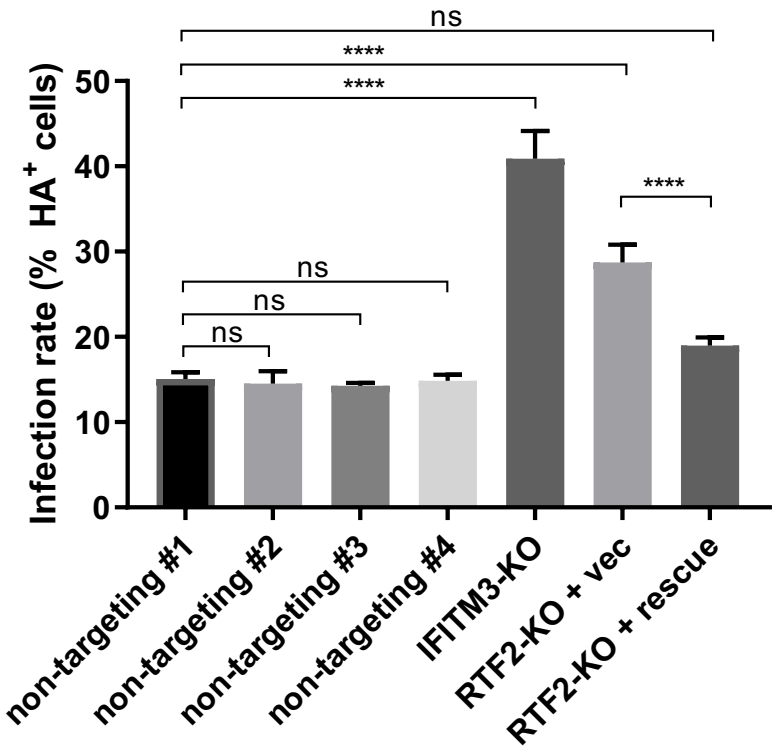


Figure 1F: Rescuing RTF2 expression restores antiviral protection to RTF2-KO cells. IAV infection rates based on HA surface protein levels in RTF2-KO cells and RTF2-rescued cells. RTF2-KO cells were transduced with either an empty vector or a sgRNA-resistant RTF2 cDNA to restore RTF2 expression. **** $p \leq 0.0001$, by one-way ANOVA test and multiple comparisons.

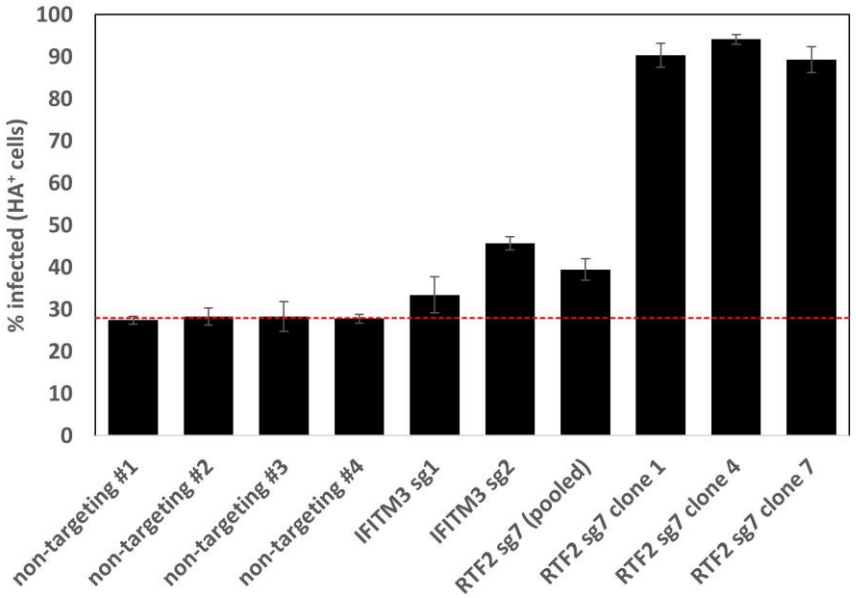


Figure 1G: RTF2-KO clones have a higher infection rate than pooled RTF2-KO cells.

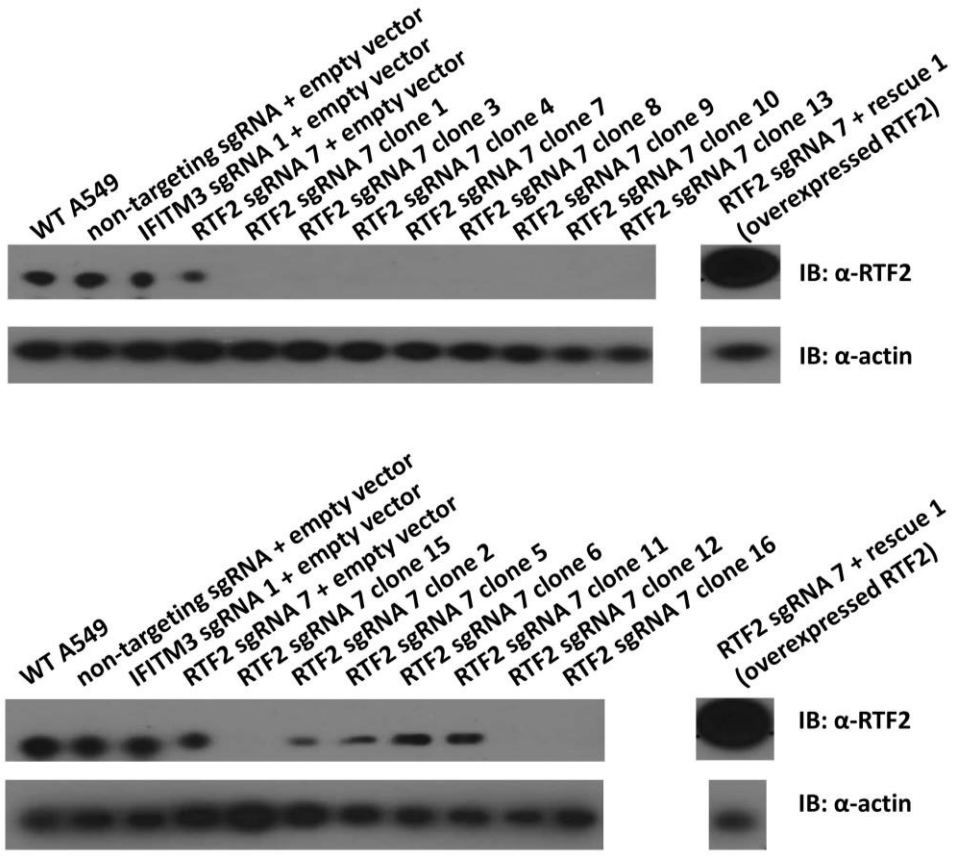
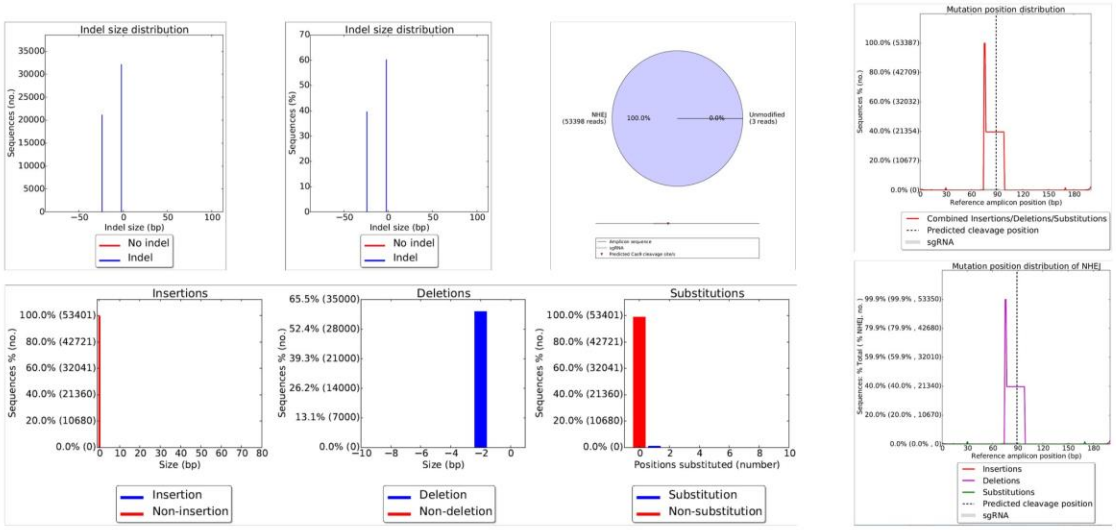


Figure 1H: Western Blot to validate that RTF2 is knocked out in some clones. RTF2 expression is much lower in some of the isolated clones than in the polyclonal cells transduced with RTF2 sgRNA and Cas9.

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CLUSTAL O(1.2.4) multiple sequence alignment

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DF3_CONTIG_177_p2       GGTTATTTGAAAGTCGCTTGTTGACATAATATTCtaatacttactattgcagactttata
                        ************************************************************

NCBI_gi_9581786_201     ACAAAGATGCCGTCATTGAATTTCTCTTGGACAAATCTGCAGAAAAGGCTCTTGGGAAGG
DF3_CONTIG_199_p1       acaaagatgccgtca--gaatttctcttggacaaatctgcagaaaaggctcttgggaagg
DF3_CONTIG_177_p2       acaaagatgccgtc------------------------acagaaaaggctcttgggaagg
                        **************                      **********************

NCBI_gi_9581786_201     CAGCATCTCACATTAAAAGCATTAAGGTAACACGAGTGATTCTGAAGTGCTGTGAGGGTC
DF3_CONTIG_199_p1       cagcatctcacattaaaagcattaAGGTAACACGAGTGATTCTGAAGTGCTGTGAGGGTC
DF3_CONTIG_177_p2       cagcatctcacattaaaagcattaaggtaacacgagtgattctgaaGTGCTGTGAGGGTC
                        ************************************************************

NCBI_gi_9581786_201     TGAACAGTGGCTGAGGTTTAT
DF3_CONTIG_199_p1       TGAACAGTGGCTGAGGTTTAT
DF3_CONTIG_177_p2       TGAACAGTGGCTGAGGTTTAt
                        *********************
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Figure 1I: Specific mutations in RTF2-KO clone 1. Cells were sequenced to determine the exact mutations created during gene editing with CRISPR/Cas9. One allele shows a 2-bp deletion, indicating a frameshift, while the other allele shows a 24-bp deletion.

Besides validating that knocking out RTF2 has an effect based on percentage of HA^+ cells, we wondered if RTF2 affects IAV RNA levels too. To monitor RNA levels, we used qPCR to quantify IAV RNAs from infected cells, and found that the nucleoprotein (NP) mRNA, cRNA, and vRNA levels were higher in RTF2-KO cells compared to wildtype A549 cells or RTF2-rescued cells (**Figure 1J**). We also tracked the kinetics of infection by monitoring NP mRNA levels over time, and noticed that the difference in NP mRNA levels between RTF2-KO cells and RTF2-rescued cells can be detected even at 4 hpi (**Figure 1K**). Our data suggest that depletion of RTF2 leads to increased replication during an early step in the IAV life cycle.

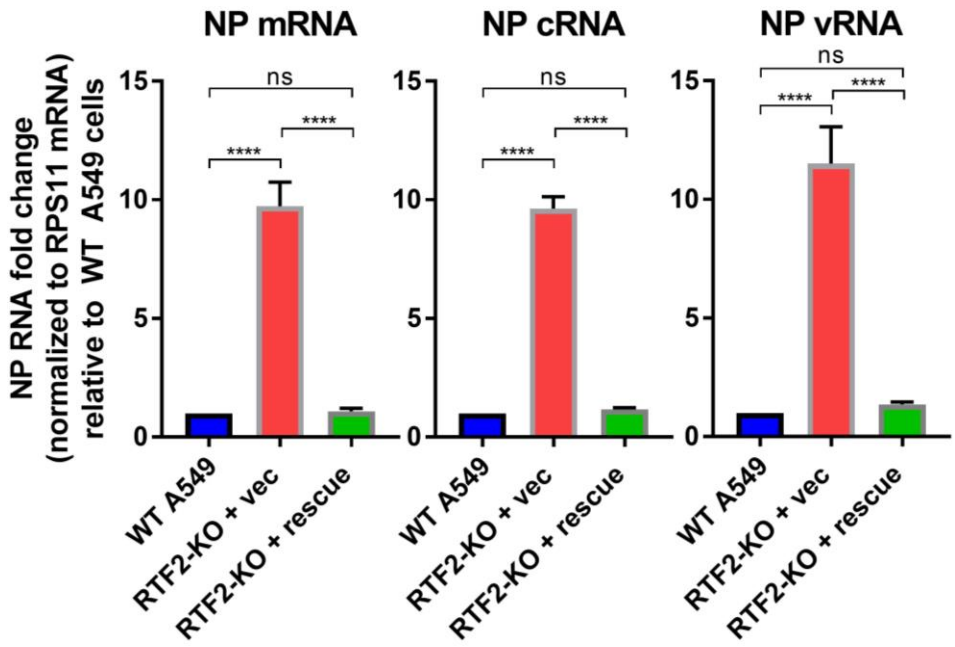

Figure 1J: IAV transcription and replication for WT, RTF2-KO, and RTF2-rescued cells. Cells were pre-treated with 200U/ml IFNβ for 24 hours before IAV infection at MOI 5 for 9 hours. Total RNA was used for strand-specific RT-PCR and qPCR to quantify levels of viral NP mRNA, cRNA, and vRNA. **** $p \leq 0.0001$, by one-way ANOVA test and multiple comparisons.

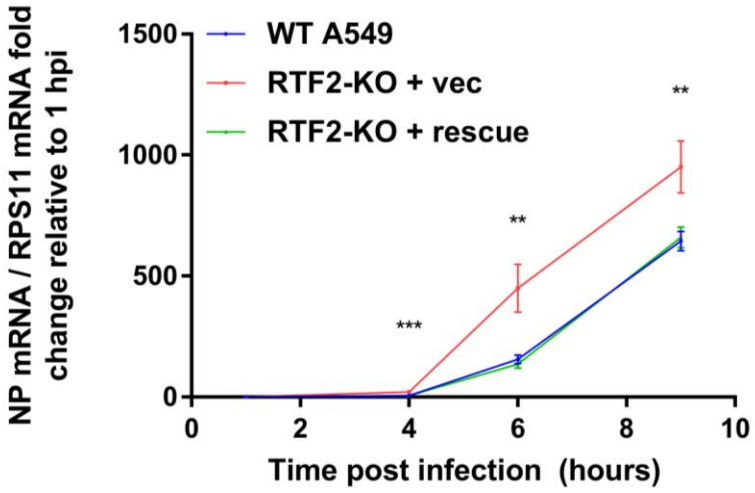

Figure 1K: Time course of IAV NP mRNA in WT, RTF2-KO and RTF2-rescued cells. *** $p \leq 0.001$, ** $p \leq 0.01$, by one-way ANOVA test and multiple comparisons.

Discussion

Our genome-wide CRISPR-Cas9 screen recovered known genes in the IFN signaling pathway (IFNAR1, IFNAR2, JAK1, TYK2, and IRF9). In addition, we also identified a novel antiviral factor RTF2, which we validated by designing new sgRNAs and testing these sgRNAs individually for their effect on IAV infection. Knocking out RTF2 increases IAV infection, as assayed by flow-sorting HA-positive cells, as well as measuring viral RNA levels via qRT-PCR. Rescuing RTF2 expression restores antiviral protection and lowers infection rate back to the same level as wildtype A549 cells. A clonal RTF2-KO line was isolated to ensure homogeneity and reproducibility in follow-up experiments.

In our screen, we used HA expression as a surrogate readout for viral infection and replication. There are inherent caveats to this approach. First, restriction factors that inhibit later stages of the life cycle, such as assembly, budding and release, as well as subsequent rounds of infection, would be missed in our screen. These include antiviral factors such as viperin and tetherin. In addition, restriction factors that inhibit expression of other non-HA late genes, such as M2 and NA, could also be missed. However, any restriction factors that target viral proteins involved in viral gene expression (i.e. polymerase subunits PA, PB1 and PB2, or an indirect regulator such as NS1, which inhibits cellular antiviral response and traps cellular pre-mRNAs) should be captured in our screen. Any restriction factor that inhibits HA expression and/or reduce HA stability should also be recoverable in our screen (since knocking out these hypothetical restriction factors would result in higher HA levels).

To our surprise, we did not recover any of the classical restriction factors, such as IFITM3 and MxA, which have been previously reported to target pre-HA trafficking steps of IAV life cycle. This is in spite of the fact that both IFITM3 and MxA have been reported to be functional in A549 cells[110,125]. Interestingly, this observation of not recovering classical ISGs has also been made in another recently published screen by Richardson *et al.*[234]. Similar to us, their screen mainly recovered genes in the IFN signaling pathway (such as IFNAR1, IFNAR2, JAK1, TYK2, and IRF9) and very few of the classical ISGs. They reported that the only classical ISGs recovered in their screen were IFI6, IRF9 and STAT2, and did not include other classical ISGs such as IFITM3[2,108,113] and TRIM56[202], which have already been shown to inhibit YFV.

Some biological explanations for not recovering classical ISGs in our screen include the small effect size of each individual antiviral effector gene, and the redundancy of the many ISGs upregulated upon IFN exposure. As the effect size of individual ISGs can range from modest to strong, it is likely ISGs work in an additive (and perhaps synergistic) combinatorial manner[2,245]. Hence knocking out a single restriction factor in each cell (as designed in our screen) may not be sufficient for viruses to overcome the IFN-induced protection. Knocking out genes in the IFN signaling pathway, on the other hand, effectively abolishes the entire IFN-induced antiviral response involving many ISGs (although some restriction factors could still be upregulated by IRFs prior to IFN signaling). Perhaps because genome-wide approaches are designed to identify the most potent factors, we only managed to recover IFN signaling pathway genes. Potential solutions to address these caveats will be discussed in the Conclusions and Future Directions chapter.

In addition, there may also be technical reasons for not identifying classical restriction factors in our screen. Although we subjected cells to puromycin selection to eliminate non-transduced cells, some cells may still retain a mutant but functional form of the protein. Such incomplete deletion of genes by CRISPR would further reduce the already small effect size of each ISG, making it even more challenging to observe enrichment of these sgRNAs post-flow sorting. In addition, the design of individual sgRNAs within the library may not have been optimized too. As seen in **Figure 1C** and **1D**, different sgRNAs targeting the same gene could vary in gene editing efficiency and result in different effect sizes. In particular, IFITM3 sgRNA1, which is part of the AVANA4 library, resulted in lower infection rate than sgRNA2, which is designed separately and not included in the library. It is possible that some of the sgRNAs used in the AVANA4 library did not result in efficient gene knockout. However, as the libraries are constantly updated based on new data and improvements in sgRNA prediction, this should become less of a problem for future screens.

Since there are inherent limitations of individual screens depending on the specific context and assays used, complementary approaches using both LOF and GOF screens are an extremely powerful tool and could provide valuable information with high confidence. For example, Richardson *et al.* recovered IFI6 as a restriction factor of YFV in their CRISPR-KO screen done in HUH7.5 cells, while Schoggins *et al.* had identified IFI6 as a restriction factor of YFV in their cDNA overexpression screen done in HUH7 cells. This gives high confidence that IFI6 may be truly functional as an antiviral factor. Although we did not recover classical antiviral ISGs, the fact that we recovered genes in the IFN pathway gave us confidence that our screen had worked, and we proceeded with validating some of the top hits. However, most of the hits

outside of the IFN pathway genes are not ISGs. They also did not validate well when tested with individual sgRNAs in an arrayed format (likely false positives). Fortunately, we managed to validate RTF2 with multiple individual sgRNAs, and in different assays. We then proceeded with further characterization of RTF2's role in restricting IAV, as described in the next chapter.

Methods

Cell culture, Reagents, Virus Strains

A549 and 293T cells (ATCC) were cultured in Dulbecco's Modified Eagle Medium (DMEM; Thermofisher) supplemented with 10% heat-inactivated fetal bovine serum (Avantor), 2mM L-Glutamine (Gibco) and 1% penicillin (Corning). A549-Cas9 cell line was generated by transducing A549 cells with a lentiviral construct (pXPR101) expressing Cas9 and blasticidin deaminase. In order to maintain heterogeneity of the cells, a polyclonal population of the A549-Cas9 cell line was used for the CRISPR screen, and initial validation. For subsequent experiments, a clonal RTF2-KO cell line was used as mentioned above. Human IFNβ used for pre-treating cells before IAV infection was obtained from PBL Assay Science (11410-2). Influenza A virus A/PR/8/34 was grown in Madin-Darby Canine Kidney cells in serum-free DMEM supplemented with 1% BSA and 1μg/ml TPCK trypsin.

Plasmids

The pXPR101 plasmid used to generate A549-Cas9 cells was provided by the Broad Institute Genetic Perturbation Platform. Individual sgRNAs were cloned into pLentiCRISPR-V2 (Addgene #52961) for follow-up studies. For rescue experiments, the Cas9 gene in pXPR101 was replaced by codon-mutated versions of RTF2.

Antibodies

The following antibodies were used: From EMD Millipore, Anti-Influenza A HA (AB1074) (1:200). From ThermoFisher, Alexa Fluor 488 Donkey anti-goat IgG (A11055)

(1:500). From LifeSpan Biosciences, Mouse Anti-RTFDC1 Antibody (clone 1E8) (LS-C340588) (1:2000). From Abcam, Mouse anti-β-actin antibody (ab6276) (1:15000).

Genome-wide CRISPR screen

We transduced ~120 million $Cas9^+$ A549 cells with the AVANA-4 lentiviral library[242] at a low MOI to achieve 30% infection rate and an average of 500-fold coverage of the library after selection. After 24 hours post-transduction, cells were subjected to puromycin selection to remove non-transduced cells. On day 9 post-lentiviral transduction, ~300 million cells were subjected to 200U/ml IFNβ treatment for 24 hours, and then infected with PR8 influenza A virus at MOI 5 for 16 hours. After 16 hours post-infection, the cells were washed with PBS and stained with primary anti-Influenza A HA antibody (AB1074) and secondary Alexa Fluor 488-conjugated anti-goat IgG (A11055) before fixation with 4% formaldehyde. HA-positive and HA-negative cells were sorted by FACS and harvested for genomic DNA using the Qiagen Blood and Tissue extraction kit according to manufacturer protocol. PCR of gDNA was performed in 100 µl reactions to attach sequencing adaptors and barcode samples. Each reaction consisted of 50 µL gDNA plus water, 40 µL PCR master mix and 10 µL of a uniquely barcoded P7 primer (stock at 5 µM concentration). Master mix comprised of 75 µL ExTaq DNA Polymerase (Clontech), 1000 µL of 10x ExTaq buffer, 800 µL of dNTP provided with the enzyme, 50 µL of P5 stagger primer mix (stock at 100 µM concentration), and 2075 µL water. PCR cycling conditions: an initial 1 minute at 95°C; followed by 30 seconds at 94°C, 30 seconds at 52.5°C, 30 seconds at 72°C, for 28 cycles; and a final 10 minute extension at 72°C. Samples were purified with Agencourt AMPure XP SPRI beads according to manufacturer's instructions (Beckman Coulter, A63880) and sequenced on a HiSeq2000 (Illumina). The screen results were

analyzed with the CRISPR Gene Scoring Tool developed at Broad Institute, using both the negative binomial distribution (STARS[14] software version 1.1) and the hypergeometric distribution.

Virus infection

A549 cells were inoculated with 150 µl (12-well plate) or 2 ml (T75 flask) of IAV or vesicular stomatitis virus at MOI 5 for 1 hour at 37ºC in serum-free DMEM. The cells were then rinsed once and replaced with fresh serum-free DMEM supplemented with 1% BSA for the length of the respective experiments. Infection was subsequently monitored by FACS or qRT-PCR.

Rescuing RTF2-KO

To rescue RTF2 expression in RTF2-KO cells, an XPR101_rescue plasmid expressing FLAG-tagged codon-mutated version of RTF2 was used. Cells were selected with 1 µg/µl puromycin and 10 µg/µl blasticidin for 8 days. Expression of rescued RTF2 was confirmed via western blot.

Flow Cytometry

Trypsin was used to lift the adherent cells, before neutralization with DMEM supplemented with 10% FBS. The cells were then rinsed once with cold PBS + 1% BSA before being stained with antibodies in PBS + 1% BSA for 30 minutes on ice. After staining, they were fixed with 2% formaldehyde before being rinsed with PBS. Data were acquired on the BD Accuri (BD Bioscience) and analyzed by FlowJo software (TreeStar).

Western blotting

5 x 10^5 of transduced cells were lysed, after the different treatment conditions as stated in the relevant experiments, in RIPA buffer (Thermofisher) supplemented with EDTA-free Protease inhibitor cocktail (Roche) unless otherwise stated. Cell lysates were centrifuged at 10,000 x g in a microcentrifuge for 10 minutes at 4°C to clear debris. SDS loading buffer and DTT was added to the collected supernatant before heating at 95°C for 5 minutes to denature the proteins. Gel electrophoresis was performed by separating the proteins on a NuPAGE Novex 12% Tris-Glycine gel and transferred to a polyvinylidene difluoride membrane (Millipore). Immunoblotting was performed according to standard protocols using the relevant primary antibodies and HRP-conjugated secondary antibodies.

RNA-extraction and qPCR

Total RNA was extracted from 1x10^5 cells using the RNeasy Mini Kit (Qiagen) according to manufacturer's protocol. First strand cDNA synthesis was performed using 50 ng of total RNA with the Superscript III First-strand Synthesis system with Oligo(dT) (Thermofisher). Quantitative qPCR was performed using the Q5 hot start high fidelity polymerase and SYBR green I Nucleic Acid Gel stain (Thermofisher) on the Roche 480 Light Cycler (Roche). Human RPS11 was used as reference normalization control and expression levels were quantified by the delta delta Ct method. Primer sequences are as follows:

Influenza PR8 NP^{246}

F: 5' – ACCAATCAACAGAGGGCATC– 3'

R: 5’ – TGATTTCGGTCCTCATGTCA – 3’

RPS11

F: 5’ – TACCAAAAGCAGCCGACCAT – 3’

R: 5’ – CCCTCAATAGCCTCCTTGGG – 3’

Primers used in RT for Influenza PR8 NP gene- and sense-specific primers[246]

NP cRNA: 5′-AGTAGAAACAAGGGTATTTTTC-3′

NP vRNA: 5′-AGCAAAAGCAGGGTAGATAATCACTCAC-3′

Statistics

Data were tested for statistical significance via GraphPad Prism. t test, one-way ANOVA, two-way ANOVA were performed with their respective multiple comparisons as indicated in figure legends. All data were represented as mean ± standard deviation.

Chapter 3: Elucidating RTF2's role in restricting IAV infection

Introduction

Having identified RTF2 as an antiviral factor, we decided to investigate its mechanism of action. However, very little has been published about RTF2. The physiological function of RTF2 in the human cell, even outside of the antiviral context, remains unknown. A homolog of RTF2 was first discovered in *Schizosaccharomyces pombe*, where it was reported to be important for efficient DNA replication termination at the site-specific, polar barrier Replication Termination Site 1 (*RTS1)*[247]. *RTS1* terminates replication forks moving in a specific direction in order to regulate mating-type switching. The yeast homolog Rtf2 plays an important role by stabilizing the stalled replication fork at *RTS1* and preventing it from restarting, thereby ensuring that replication forks can only pass in one direction through *RTS1*. Aside from evidence that Rtf2 may interact with PCNA, the molecular mechanism of how Rtf2 stabilizes the stalled fork and prevents restart remains to be elucidated.

A different report of the *Arabidopsis thaliana* plant homolog suggests that *AtRTF2* is important for pre-mRNA splicing[248]. This was a serendipitous finding. Originally, Sasaki *et al.* had set out to look for suppressor mutations that would restore RNA-dependent DNA methylation (RdDM) and transcriptional silencing of GFP in their two-component transgene silencing reporter system. Earlier on, the group had identified mutations that impaired RdDM and rescued GFP expression, and were subsequently looking for suppressor mutations that could restore RdDM. However, on top of finding the desired suppressor mutations that restored RdDM,

Sasaki *et al.* also found genetic mutations that prevented GFP expression independently of RdDM. It turns out that their GFP transcripts did not initiate from the minimal promoter, but from a distal enhancer region, which then underwent splicing using noncanonical donor and acceptor sites to generate productive transcripts. *AtRTF2* turned out to be important for splicing out the cryptic intron to generate a translatable GFP mRNA. RNA sequencing of total RNA extracted from plant seedlings showed increased retention for 13-16% of total introns, suggesting that *AtRTF2* might be important for splicing genome-wide and not just for the GFP reporter. However, the molecular mechanism of how *AtRTF2* regulates splicing remains unknown.

Lastly, a recent study of RTF2 using human cells (U2OS, HeLa and BJ cells) reported that RTF2 has to be removed from stalled replication forks in order to facilitate recovery from replication stress and to maintain genome stability[244]. Kottemann *et al.* provided evidence that the failure to remove RTF2 from stalled replication forks during replication stress would result in failure to resume cell cycle progression and fork restart, eventually resulting in excessive chromosomal damage. In addition, the persistence of RTF2 on stalled forks also promotes ssDNA accumulation and ataxia telangiectasia and Rad3-related protein (ATR) activation. However, as Kottemann *et al.* noted, the physiological role of RTF2 remains unknown. They speculated that RTF2 might facilitate fork pausing at fork barriers, or stimulate ATR signaling after replication forks have stalled. Moreover, the role of RTF2 during normal replication (not under replication stress conditions), if any, remains unknown too.

Based on the above reports, the common themes that arise are: (1) RTF2 remains vastly understudied and its role(s) in the cell under different conditions remains unknown, (2) RTF2

(and its homologs) interacts with some form of nucleic acids either directly or indirectly, (3) some RTF2 proteins have to be present in the nucleus. We decide to start by investigating RTF2's localization in A549 cells.

Results

RTF2 localization is important for its antiviral function

Based on the few published works, we hypothesize that RTF2 functions in the nucleus and that its antiviral function may be tied to its nuclear localization. To test this hypothesis, we first set out to determine the subcellular location of RTF2. Through immunofluorescence (IF) staining of wildtype A549 cells and two different RTF2-KO clones (clones 1 and 7) with the endogenous anti-RTF2 antibody, we found that RTF2 appears to be localized mainly in the nucleus (**Figure 2A**). This was corroborated by biochemical fractionation. Due to the need to maintain macromolecular crowding, RTF2 was extracted in the presence of Ficoll. Digitonin was used, at a low concentration, to disrupt the cholesterol-rich plasma membrane, while leaving the nuclear membrane intact. Indeed, RTF2 separated into the nuclear fraction containing TATA binding protein, but not the cytoplasmic fraction containing tubulin (**Figure 2B**). Besides using both IF and biochemical approaches, we also did live cell imaging of RTF2-KO cells overexpressing mCherry-tagged RTF2. Again, this shows that RTF2 has a clear nuclear localization (**Figure 2C**).

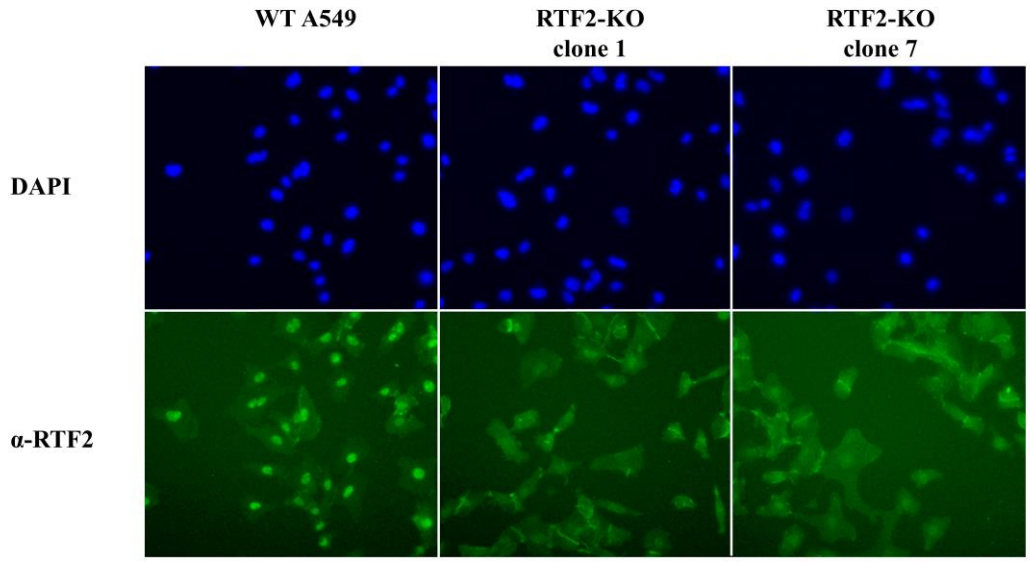

Figure 2A: Immunofluorescence of WT A549 cells and clonal RTF2-KO cells. Two different RTF2-KO clones were imaged here.

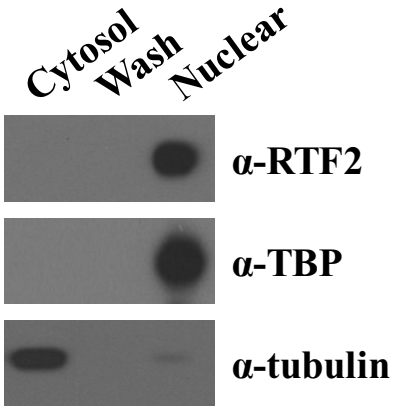

Figure 2B: Biochemical fractionation of WT A549 cells into cytosolic and nuclear fractions. Cells were first suspended in HEPES-Sucrose-Ficoll (HSF) buffer containing digitonin to extract the cytosolic proteins, washed once with HSF buffer, before lysing the nuclear pellet in RIPA buffer to release the nuclear proteins.

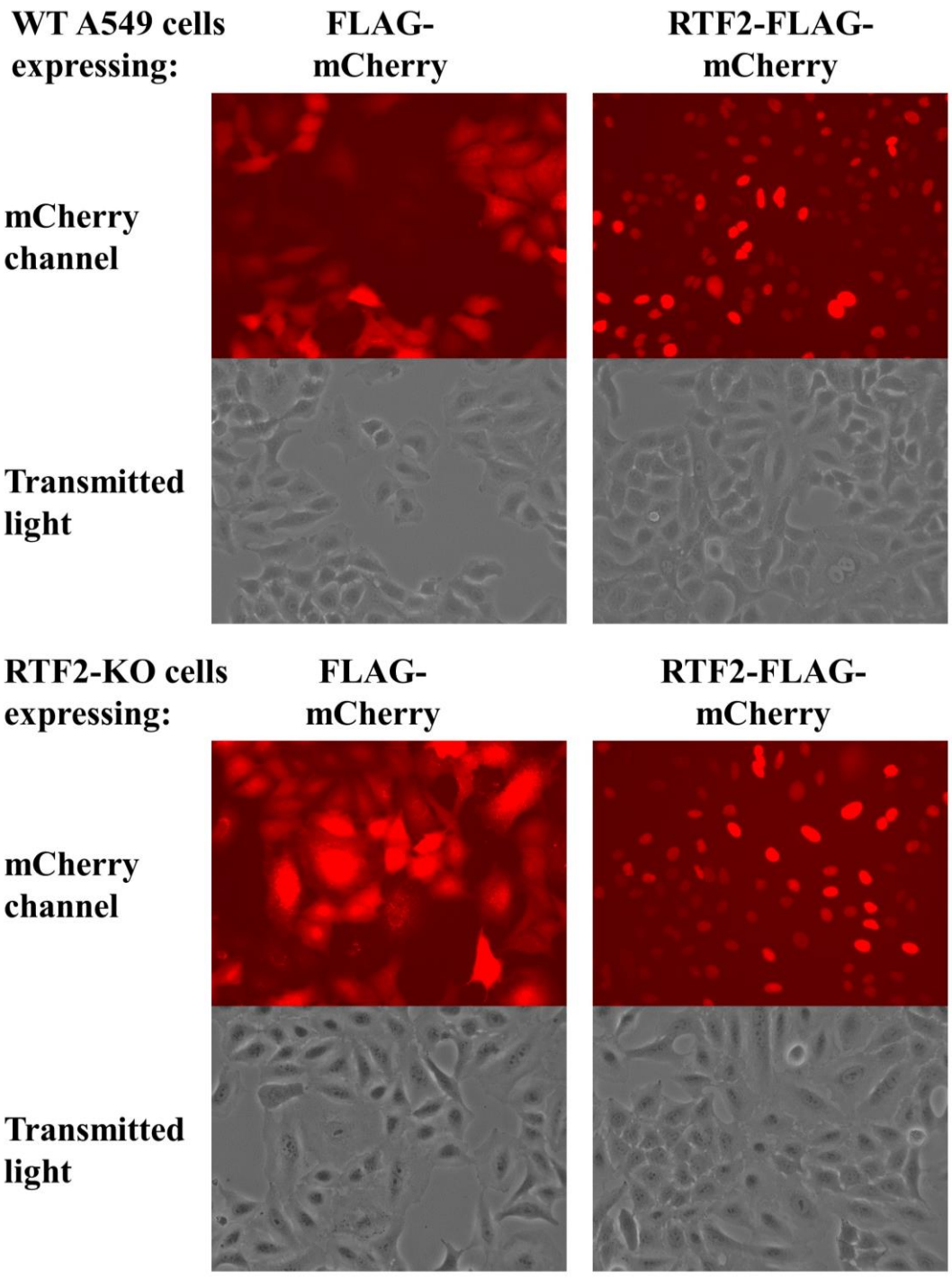

Figure 2C: Live-cell imaging of WT A549 or rescued RTF2-KO cells. RTF2-KO cells were transduced with mCherry vector, or RTF2-mCherry fusion protein, and imaged using a bright field and epifluorescence microscope 8 days after transduction. Overexpressed RTF2 is localized in the nucleus.

We then wondered if the nuclear localization of RTF2 is essential for its antiviral function. To do this, we first used cNLS Mapper[249] to identify potential classical IMPα/β pathway-specific NLSs on RTF2. We found two potential NLSs in RTF2: a monopartite NLS and a bipartite NLS (**Figure 2D**). Site-directed mutagenesis of the monopartite NLS (by either deleting or mutating positively charged residues to a series of alanine residues), had no effect on the location of RTF2, but mutating the predicted bipartite NLS causes the overexpressed protein to be present in the cytosol and nucleus (**Figure 2E**). This is perhaps because RTF2 is a small protein (33 kD) that could still diffuse back into the nucleus upon overexpression despite having both predicted NLSs mutated.

Predicted NLSs in query sequence

```
MGCDGGTIPKRHELVKGPKKVEKVDKDAELVAQWNYCTLSQEILRRPIVA  50
CELGRLYNKDAVIEFLLDKSAEKALGKAASHIKSIKNVTELKLSDNPAWE  100
GDKGNTKGDKHDDLQRARFICPVVGLEMNGRHRFCFLRCCGCVFSERALK  150
EIKAEVCHTCGAAFQEDDVIMLNGTKEDVDVLKTRMEERRLRAKLEKKTK  200
KPKAAESVSKPDVSEEAPGPSKVKTGKPEEASLDSREKKTNLAPKSTAMN  250
ESSSGKAGKPPCGATKRSIADSEESEAYKSLFTTHSSAKRSKESAHWVT  300
HTSYCF                                               306
```

Predicted monopartite NLS

Position	Sequence	Score
195	LEKKTKKPKAA	5.5

Predicted bipartite NLS

Position	Sequence	Score
263	GATKRSIADSEESEAYKSLFTTHSSAKRSKE	5

Figure 2D: cNLS Mapper identifies two potential NLSs in RTF2.

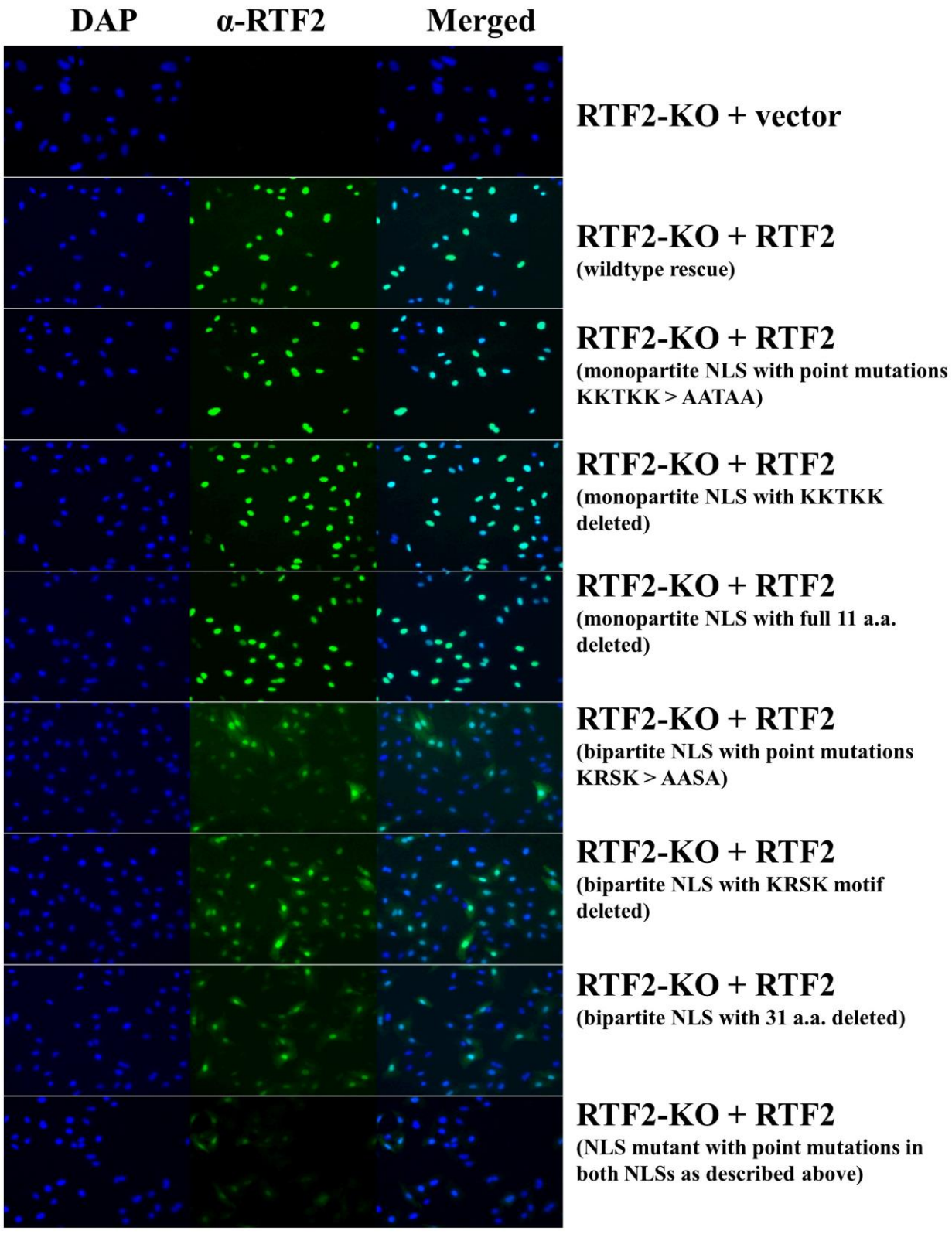
DAP
α-RTF2
Merged
RTF2-KO + vector
RTF2-KO + RTF2
(wildtype rescue)
RTF2-KO + RTF2
(monopartite NLS with point mutations KKTKK > AATAA)
RTF2-KO + RTF2
(monopartite NLS with KKTKK deleted)
RTF2-KO + RTF2
(monopartite NLS with full 11 a.a. deleted)
RTF2-KO + RTF2
(bipartite NLS with point mutations KRSK > AASA)
RTF2-KO + RTF2
(bipartite NLS with KRSK motif deleted)
RTF2-KO + RTF2
(bipartite NLS with 31 a.a. deleted)
RTF2-KO + RTF2
(NLS mutant with point mutations in both NLSs as described above)

Figure 2E (Continued): Predicted bipartite NLS is responsible for nuclear localization of RTF2. Clonal RTF2-KO cells are transduced with lentivirus that encodes empty vector, guide-resistant form of RTF2 or RTF2 with predicted NLSs mutated. Site-directed mutagenesis was performed to either mutate lysine or arginine residues to alanine residues, delete the positively-charged motifs, or delete the entire NLS as predicted by cNLS (Figure 2c). Shown are transduced cells stained with anti-RTF2 antibody. RTF2 with intact NLSs localizes to the nucleus. Mutating the predicted monopartite NLS has no effect on RTF2's nuclear localization. However, mutating the predicted bipartite NLS seems to disperse RTF2 throughout the cell.

To test whether RTF2 loses its antiviral activity if it is mislocalized such that it cannot diffuse back into the nucleus, we generated an ER-retention mutant (KDEL), and a plasma membrane-targeted N-terminus-myristoylated mutant, and showed that the proteins were expressed successfully (**Figure 2F**) and localized away from the nucleus (**Figure 2G**).

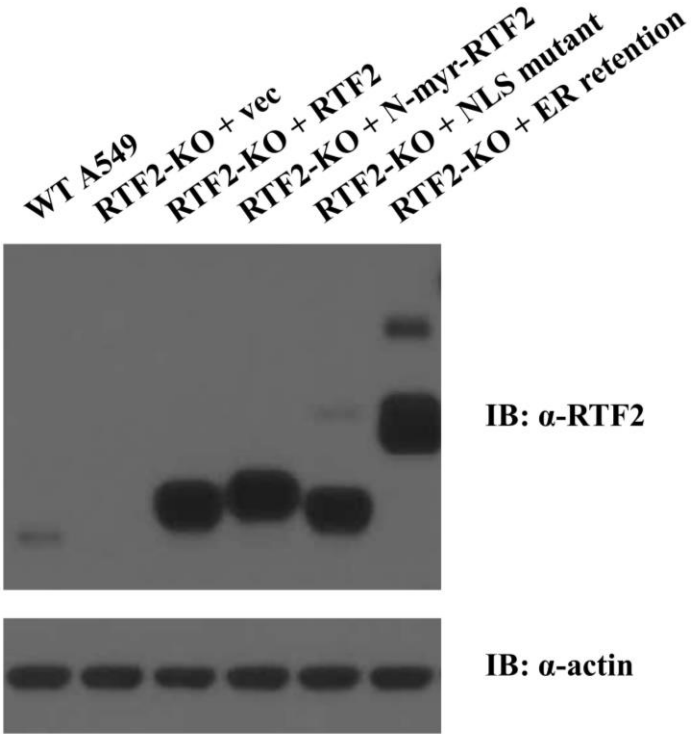

Figure 2F: Western blot showing expression of WT RTF2 and localization mutants. Cell lysates were obtained from WT A549 cells, and RTF2-KO cells rescued with either empty vector or various sgRNA-resistant forms of RTF2: RTF2 (WT RTF2 with intact NLSs), N-myr-RTF2 (RTF2 containing point mutations in both NLSs with an additional plasma membrane-targeting myristoylation signal added to its N-terminus); NLS mutant (RTF2 containing point mutations in both NLSs); ER retention (RTF2 containing point mutations in both NLSs with an additional signal peptide and ER-retaining KDEL motif added).

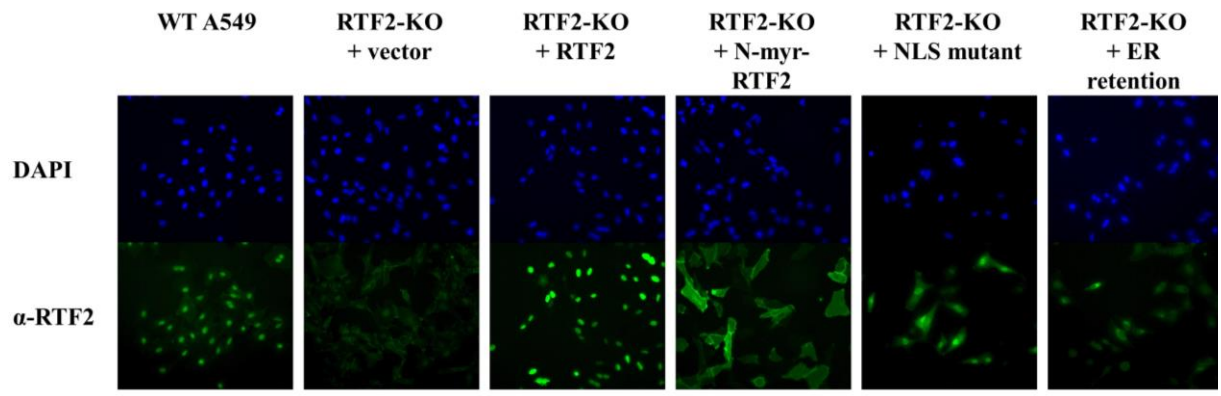

Figure 2G: Immunofluorescence of WT A549, and RTF2-KO cells expressing mislocalized RTF2. Clonal RTF2-KO cells were transduced with empty vector, or sgRNA-resistant cDNA encoding the various constructs described in **Figure 2F**.

To test the effect of mislocalizing RTF2 during IAV infection, we quantified infection based on surface HA expression (**Figure 2H**) and NP mRNA levels (**Figure 2I**). By retaining RTF2 in the endoplasmic reticulum, or by redirecting it to the inner leaflet of the plasma membrane, we disrupted RTF2's ability to confer antiviral protection. On the other hand, point mutations of the NLSs alone had no or weak impact on RTF2 function in rescuing RTF2-KO cells.

Collectively, these data indicate that the RTF2 protein is localized to the nucleus, and that re-localization to the plasma membrane or ER impairs its antiviral function.

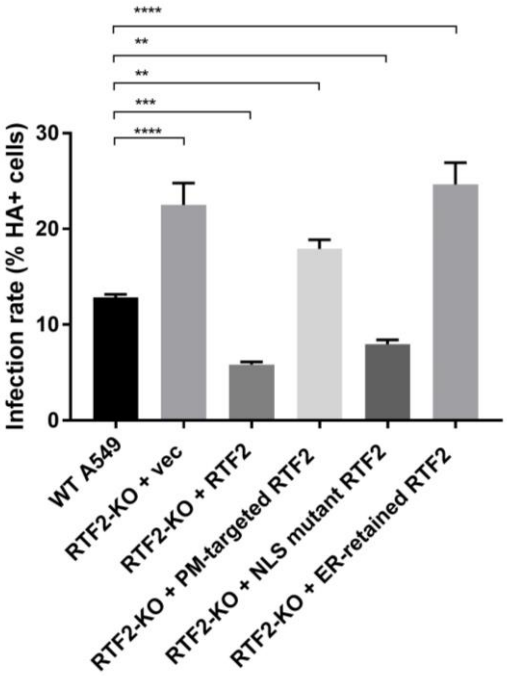

Figure 2H: Effect of mislocalizing RTF2 on IAV infection as assayed by HA staining. Cells were pre-treated with 200 U/ml IFNβ before IAV infection at MOI 5. Flow cytometry based on cell surface HA was performed at 16 hpi. **** $p \leq 0.0001$, *** $p \leq 0.001$, ** $p \leq 0.01$, by one-way ANOVA test.

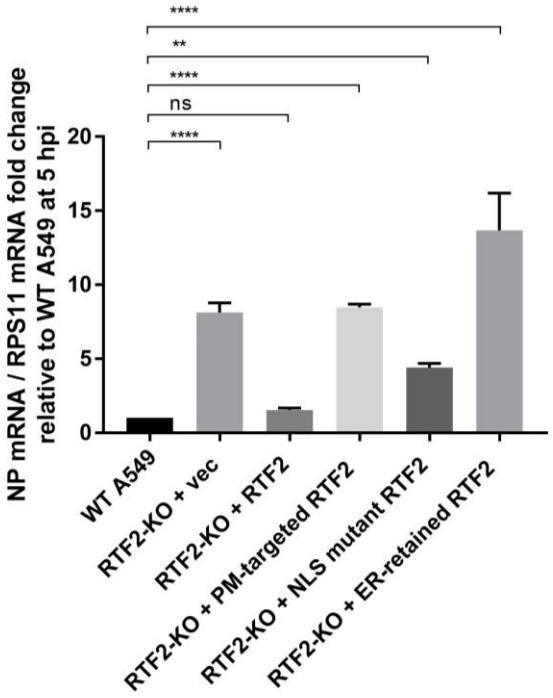

Figure 2I: Effect of mislocalizing RTF2 on IAV infection as assayed by qPCR measurement. Cells were pre-treated with 200 U/ml IFNβ before IAV infection at MOI 5 for 5 hours before RNA was isolated for qPCR. **** $p \leq 0.0001$, ** $p \leq 0.01$, by one-way ANOVA test.

RTF2 restricts influenza transcription

Given the requirement of nuclear localization for the antiviral function of RTF2, and since IAV, unlike most RNA viruses, transcribes and replicates its genome in the nucleus, we wondered if RTF2 impairs IAV transcription and/or genome replication. To test this, we transfected wildtype A549, RTF2-KO and RTF2-rescued cells with a plasmid encoding firefly luciferase reporter driven by vRNA backbone, along with plasmids encoding PR8 polymerase subunits (PA, PB1, PB2) and NP^{250} (**Figure 2J**). This allows us to bypass viral entry, and specifically assay polymerase activity in the nucleus.

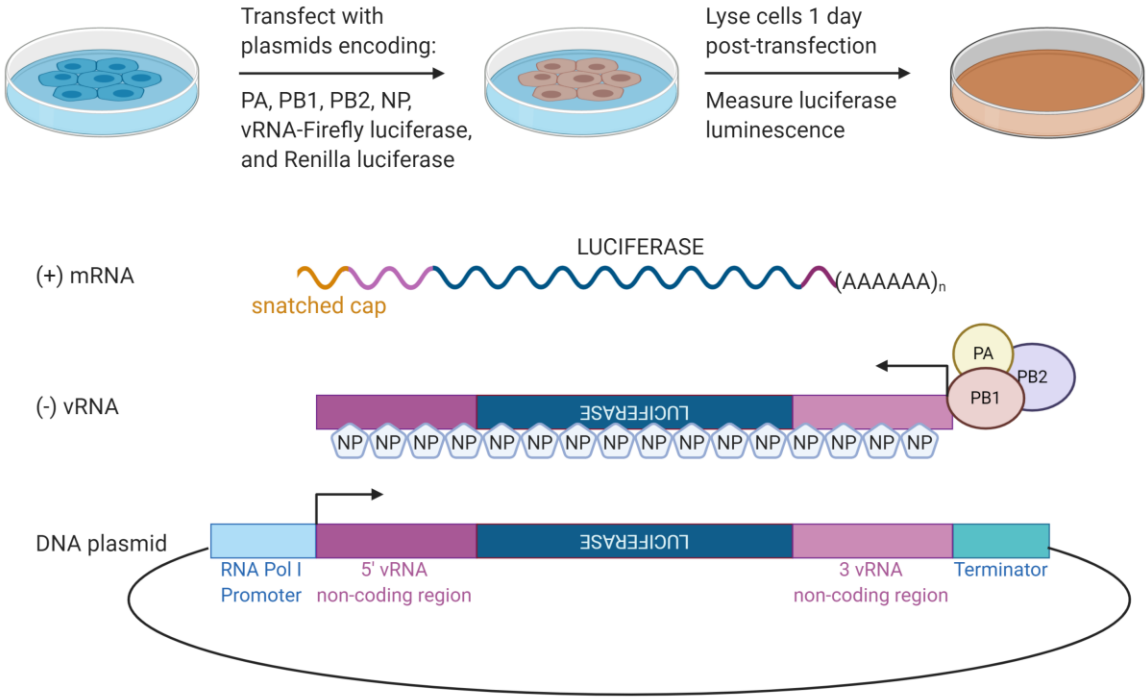

Figure 2J: Schematic of the minigenome luciferase reporter assay. (Top) Workflow describing the luciferase reporter assay. (Bottom) Firefly luciferase is engineered into the vRNA backbone in the reverse orientation. Production of vRNA-luciferase is driven by RNA Pol I promoter. In the presence of the four viral proteins (PA, PB1, PB2, and NP), the flu polymerase can transcribe mRNAs encoding luciferase in the correct orientation. In addition to transcription, the flu polymerase complex can also generate cRNA to be used as templates for further amplification of vRNA (not shown). Renilla luciferase is included as transfection control.

We found that RTF2-KO cells have a higher luciferase activity, suggesting that cells that lack RTF2 are less able to restrict influenza transcription/replication (**Figure 2K**). We then tested whether rescuing RTF2-KO cells with the mislocalized mutants described above would result in lower or higher luciferase activity compared to rescuing with the NLS-intact form of RTF2. We found that overexpressing mislocalized RTF2 in the RTF2-KO cells does not reduce the higher luciferase signal seen in RTF2-KO cells relative to wildtype or RTF2-rescued cells (**Figure 2L**), once again indicating that nuclear localization of RTF2 is important for its function.

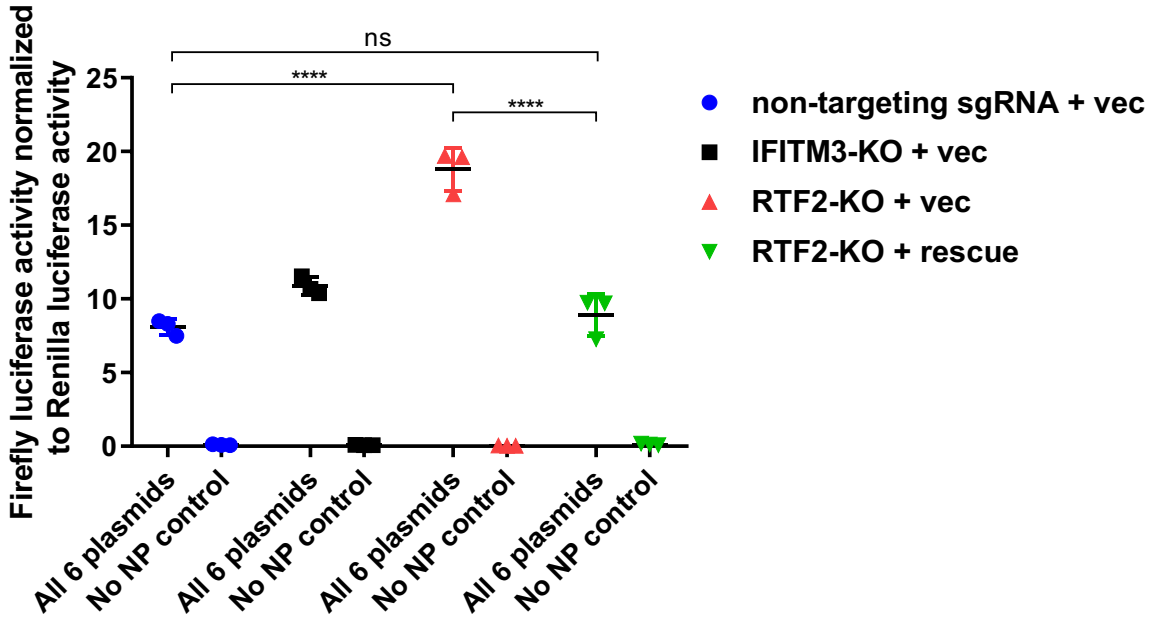

Figure 2K: IAV polymerase activity is enhanced in the absence of RTF2. IAV polymerase activity was measured via a minigenome luciferase reporter assay. Cells were transfected with IAV polymerase subunits and NP, firefly luciferase reporter construct and Renilla luciferase. Luciferase activity was measured at 24 hours post-transfection. **** $p \leq 0.0001$, by one-way ANOVA test.

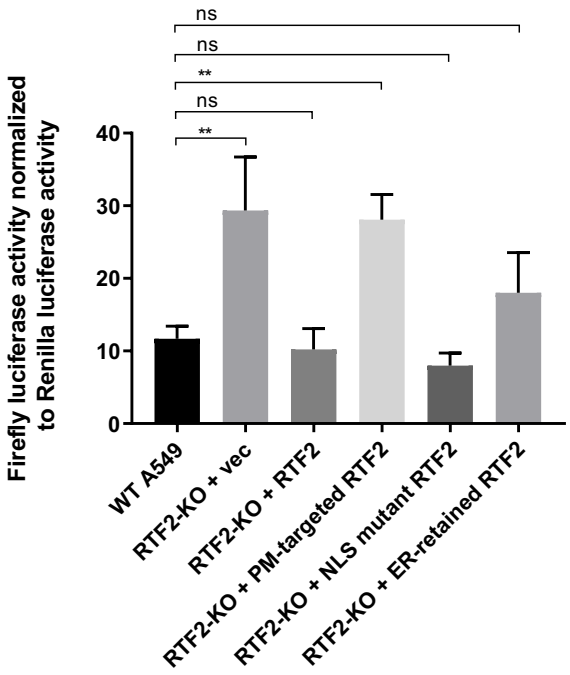

Figure 2L: Effect of mislocalizing RTF2 on IAV polymerase activity. IAV polymerase activity was assayed in a minigenome luciferase reporter assay. Cells were transfected with plasmids encoding IAV polymerase subunits and NP, a reverse-orientation luciferase reporter on a vRNA backbone, and a Renilla luciferase as transfection control. Cells were lysed 24 hours post-transfection to measure luciferase activity. ** $p \leq 0.01$, by one-way ANOVA test.

To further dissect RTF2's role in controlling IAV infection, we tested whether RTF2 specifically affects primary transcription, by employing cycloheximide (CHX) to block protein translation. We tracked NP mRNA, cRNA, and vRNA levels after infecting CHX- and IFN-pretreated cells with IAV. As CHX treatment blocks synthesis of new viral proteins, and hence halts genome replication and secondary transcription, the levels of NP cRNA and vRNA did not increase after infection in CHX-treated cells (**Figure 2M**). However, IAV NP mRNA levels (resulting from primary transcription) did go up slightly over time in the presence of CHX (**Figure 2N**). In RTF2-KO cells, NP mRNA was higher than in RTF2-rescued cells, suggesting that RTF2 affects primary transcription. Altogether, these findings are consistent with the model that RTF2-deficient cells allow more primary transcription of the virus.

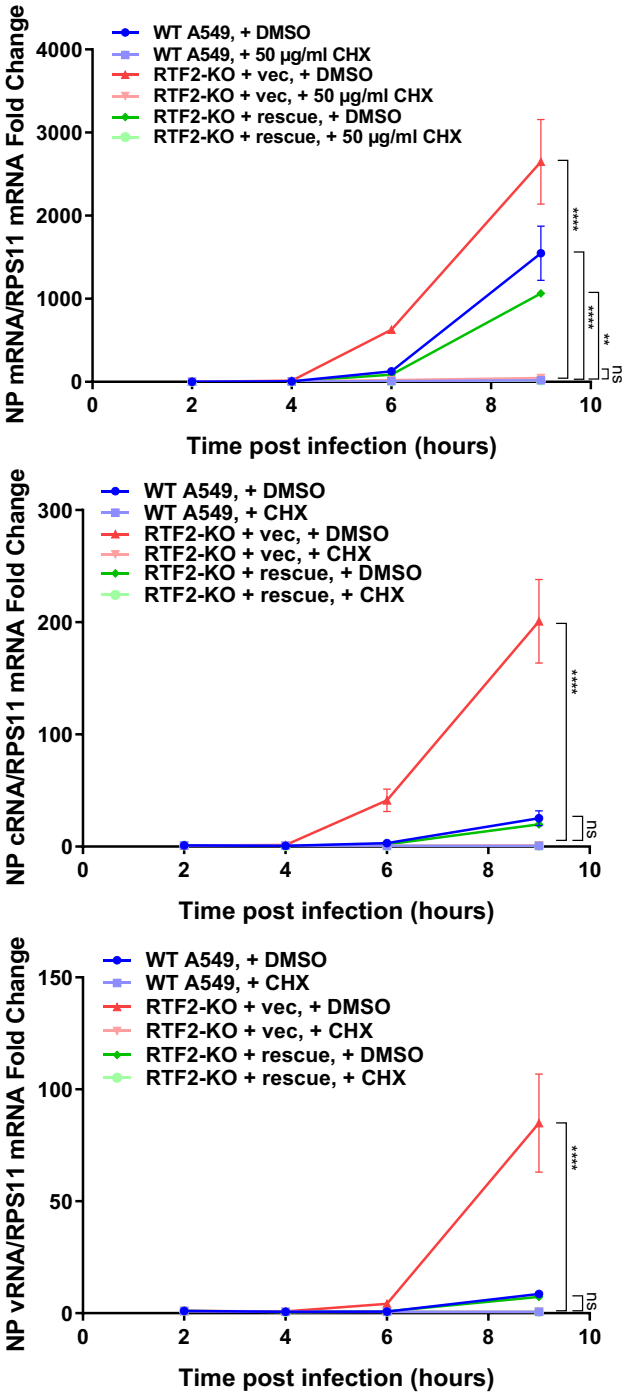

Figure 2M: Time course of IAV NP RNA levels in cells infected in the presence or absence of CHX treatment. IAV NP mRNA (top), cRNA (middle), and vRNA (bottom) levels of infected cells were monitored. WT A549, RTF2-KO and RTF2-rescued cells were pre-treated with 200U/ml IFNβ for 24 hours, followed by either CHX or DMSO for 2 hours, and then infected with IAV at MOI 5 for 9 hours. **** $p \leq 0.0001$, by 2-way ANOVA and Tukey's multiple comparison test comparing each of the 9 hpi samples against CHX-treated WT A549 cells.

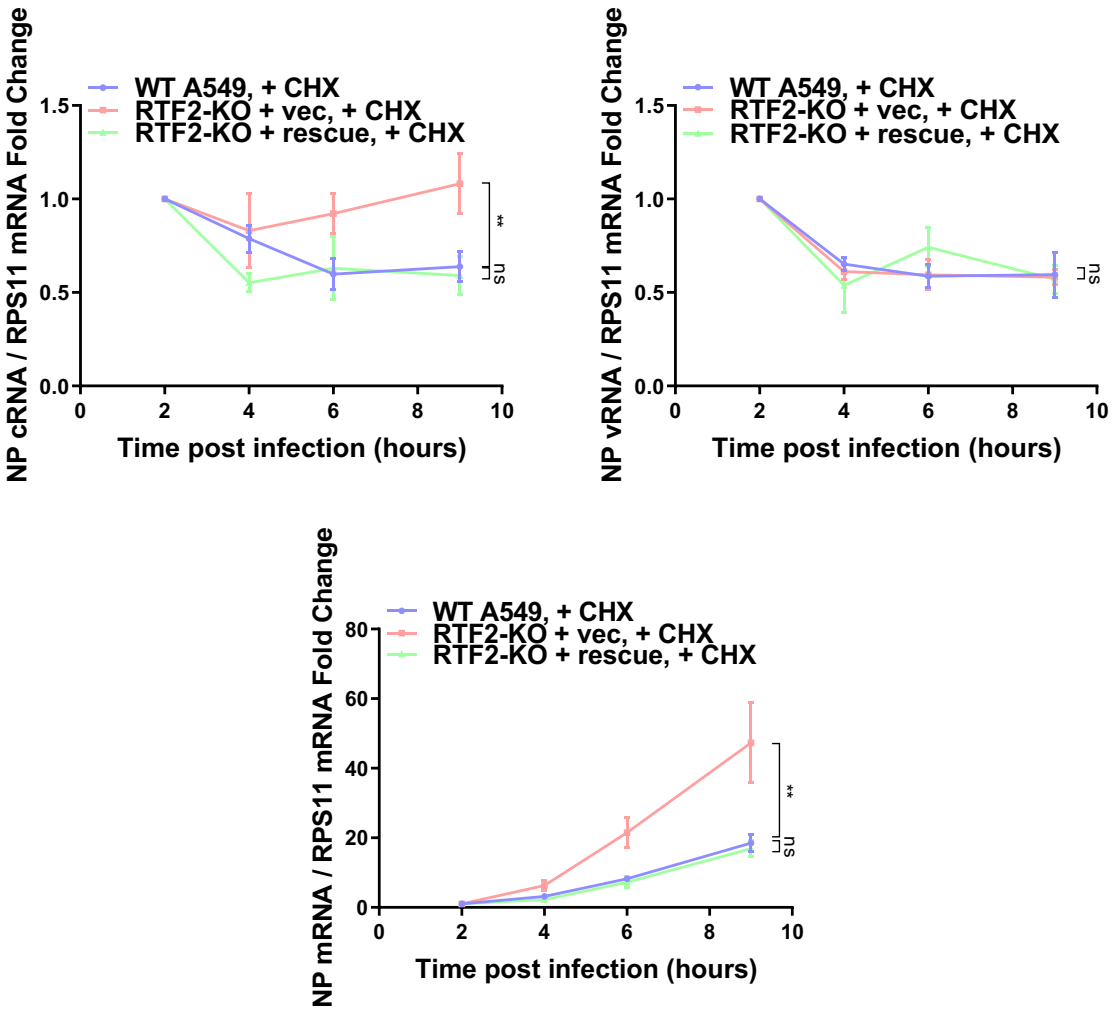

Figure 2N: IAV primary transcription is higher in CHX-treated RTF2-KO cells. IAV NP cRNA (top left) and vRNA (top right), and mRNA (bottom) levels in IFNβ-pretreated and CHX-treated WT A549, RTF2-KO and RTF2-rescued cells are monitored over time.

However, it was unclear whether RTF2 also affects genome replication and/or RNA stability in general. To test the latter, we tracked NP mRNA over time in the presence of baloxavir, a PA endonuclease inhibitor that blocks cap-snatching and hence synthesis of mRNAs. We first infected IFN-pretreated cells with IAV for 6 hours before administering baloxavir to prevent additional synthesis of mRNAs, and tracking the RNA levels over time. We

found no evidence that flu mRNA decays at a slower rate in the RTF2-KO cells than in RTF2-rescued cells (**Figure 2O**), suggesting that the higher mRNA level in RTF2-KO cells might be due to an increased synthesis rate.

In conclusion, RTF2's localization to the nucleus appears to be important for its function in controlling IAV. While we cannot rule out that IAV secondary transcription and genome replication are also affected by RTF2, our experiments do show that RTF2-KO cells lead to elevated primary transcripts at a minimum, likely by affecting mRNA synthesis rather than degradation.

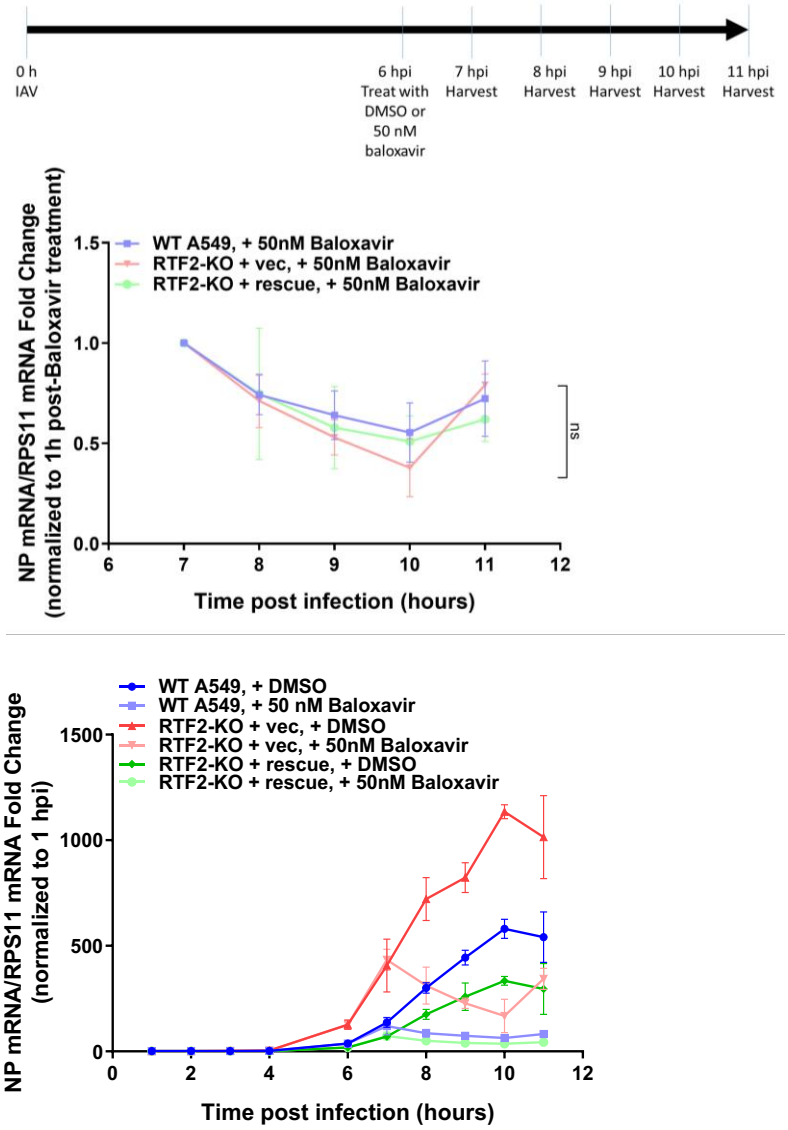

Figure 2O: IAV NP mRNA stability was monitored in infected cells that were treated with baloxavir at 6 hpi. (Top) Schematic showing experiment set-up. IFN-pretreated, IAV-infected cells were treated with baloxavir at 6 hpi to prevent further viral replication. Total RNA was harvested from infected cells at 1 hour intervals from 7 to 11 hpi. (Middle) IAV NP mRNA / RPS11 mRNA fold change, normalized to 1 h post-baloxavir treatment, extracted from baloxavir-treated cells from 7 hpi to 11 hpi. Pairwise comparisons (with Tukey correction) were made within between group at 10 and 11 hpi, and none of the comparisons reached statistical significance. (Bottom) Time course showing changes in NP mRNA levels in DMSO- or baloxavir-treated cells (normalized to 1hpi). The rate of decay of NP mRNA in RTF2-KO cells was not slower compared to WT or RTF2-rescued cells.

RTF2 may affect IFN pathway signaling and antiviral state of cells

Since most of our experiments were conducted in the presence of IFN pre-treatment, we wondered if IFN is required to observe RTF2's role in flu restriction. We found no changes in infection when RTF2 was overexpressed (**Figure 2P**), indicating that RTF2 alone is not sufficient to restrict viral replication.

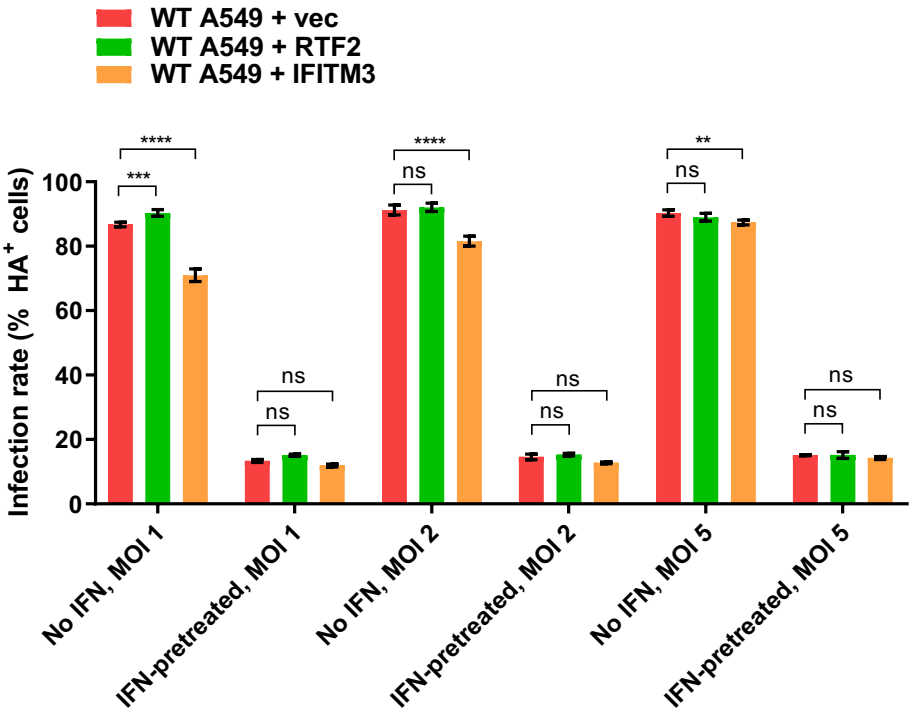

Figure 2P: Overexpressing RTF2 does not confer additional protection against IAV infection in WT A549 cells. WT A549 cells were transduced with either empty vector or RTF2. After 8 days of blasticidin-selection post-transduction, cells were treated with 200U/ml human IFNβ for 24 hours before IAV infection at MOI 5 for 16 hours. At 16 hours post-infection, cells were harvested, stained for HA, fixed, and FACS-sorted to determine infection rate.

To investigate the dependence on interferon, we infected wildtype A549, RTF2-KO and RTF2-rescued cells in the presence or absence of IFN and then measured infection rates based on surface HA levels. We found that IFN-pretreatment (which reduces overall infection rates) enhances the fold increase in infection between RTF2-KO cells and RTF2-rescued cells (**Figure 2Q**). We adopted two other complementary approaches – by using recombinant B18R, a decoy IFNAR protein produced by vaccinia virus to soak up IFN in the culture media, and by using ruxolitinib, a JAK inhibitor, to block IFN signaling. All three approaches suggest that an intact IFN signaling pathway is required to observe the effect of RTF2-KO on infection. A potential reason why the RTF2-KO phenotype is only observed in the presence of IFN may be that IFN activates RTF2, or upregulates an unknown co-factor of RTF2. While we cannot exclude this model, since RTF2 protein and mRNA levels were not upregulated in the presence of IFN (**Figure 2R**), we hypothesized alternatively that RTF2 restricts influenza by positively regulating the response to interferon. Although IFN does not seem to affect RTF2 expression, it is interesting that RTF2 mRNA and protein expression is hugely downregulated upon IAV infection.

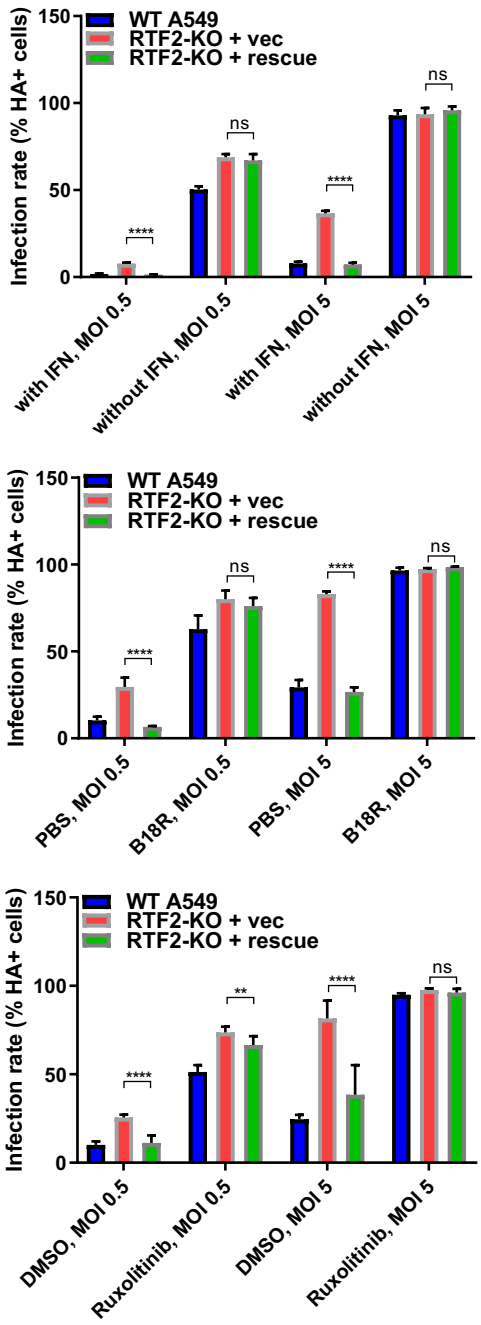

Figure 2Q: IFN-pretreatment enhances the differences in infection rates, as measured by HA staining, between RTF2-KO cells and WT A549/RTF2-rescued cells. (Top) Cells pre-treated with IFNβ or mock-treated. (Middle) Cells pre-treated with IFNβ with or without 0.5 μg/ml IFNAR receptor decoy B18R. (Bottom) Cells pre-treated with IFNβ with or without 5 μM JAK inhibitor ruxolitinib. Shown are combined data from two independent experiments with three technical replicates each. **** $p \leq 0.0001$, by one-way ANOVA followed by unpaired t-test with Bonferroni correction between RTF2-KO and RTF2-rescued cells.

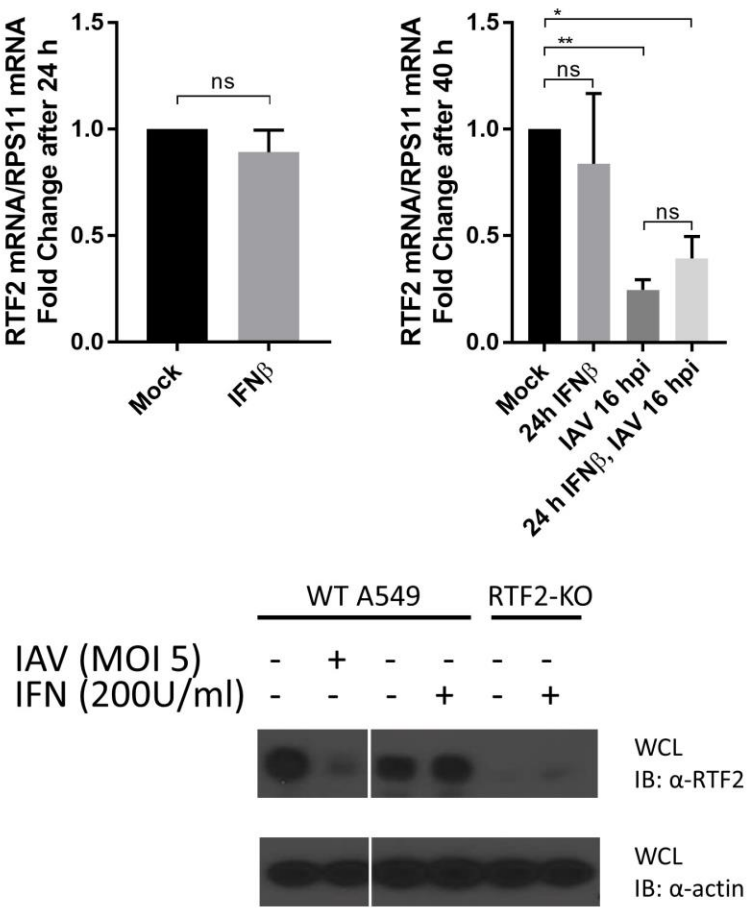

Figure 2R: RTF2 expression is not upregulated by IFN, but is downregulated upon IAV infection. (Top Left) RTF2 mRNA level (normalized to RPS11 mRNA) appears unchanged after 24 hours of 200U/ml IFNβ exposure. (Top Right) Cells were treated with IFNβ (at 200U/ml for 24 hours), infected with IAV (MOI 5 for 16 hours), or pre-treated with IFNβ for 24 hours before IAV infection. At 40 hours, cells were lysed to extract RNA before qPCR was performed to quantify RTF2 mRNA and RPS11 mRNA levels. RTF2 mRNA level appears to be unaffected by IFNβ treatment alone, but is reduced upon IAV infection. (Bottom) Western blot showing that a mild reduction of RTF2 protein level in IAV-infected cells, but no such decrease in cells exposed to just IFNβ alone.

To investigate the latter hypothesis, we performed RNA-sequencing to compare the transcriptomes of RTF2-KO and RTF2-rescued cells in the presence of IFN. Principal component analysis (PCA) shows that one dimension is associated with IFN exposure while the other with IAV infection (**Figure 2S**). While the transcriptomes of RTF2-KO and RTF2-rescued cells were quite similar in the absence of IFN exposure (and IAV infection), RTF2-KO cells showed lower induction of ISGs relative to wildtype or RTF2-rescued cells (**Figure 2T**). Among the reduced ISGs, we found the known influenza restriction factors, IFITM1 and IFITM3 (**Figure 2U**), which validated at the protein level (**Figure 2V**). Consistent with this observation, we found that phosphorylation of STAT1, a major transcription factor responsible for upregulating ISGs during the IFN signaling, was reduced in RTF2-KO cells (**Figure 2V**), further supporting RTF2's role in modulating the IFNAR pathway.

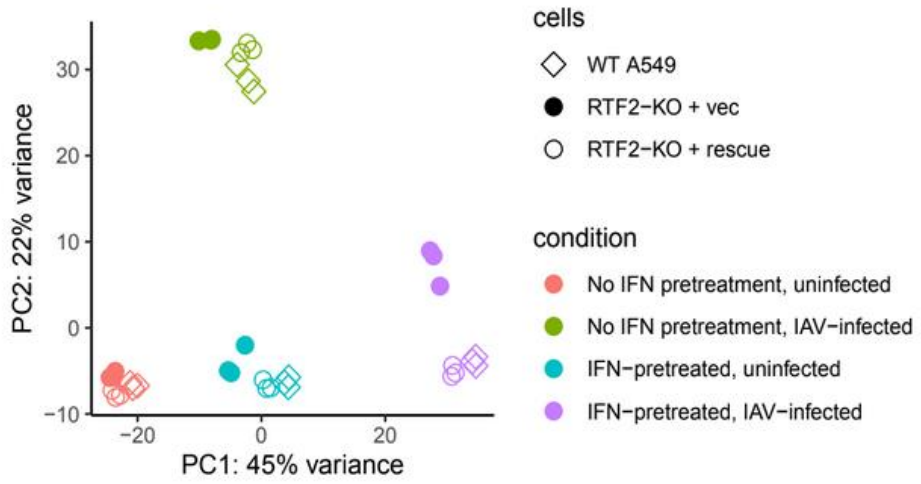

Figure 2S: Principal Component Analysis of gene expression profiles of WT A549, RTF2-KO and RTF2-rescued cells under different conditions. Top positively weighted genes in PC1 are IFITM1, OAS2 and MX1, and top positively weighted genes in PC2 are NGFR, FOS and TNFRSF10D.

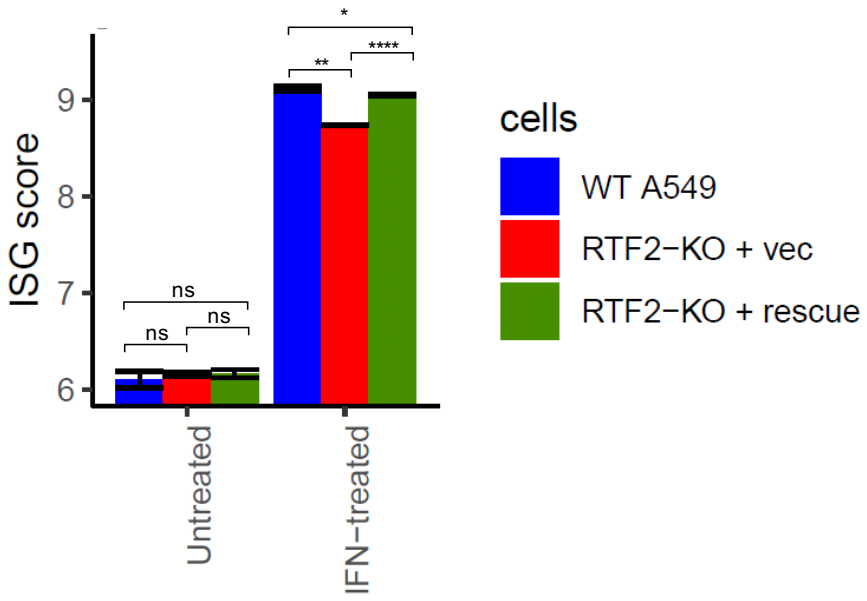

Figure 2T: IFN response score based on RNA-seq of WT A549, RTF2-KO and RTF2-rescued cells with or without IFN (200U/ml IFNβ for 24 hours).

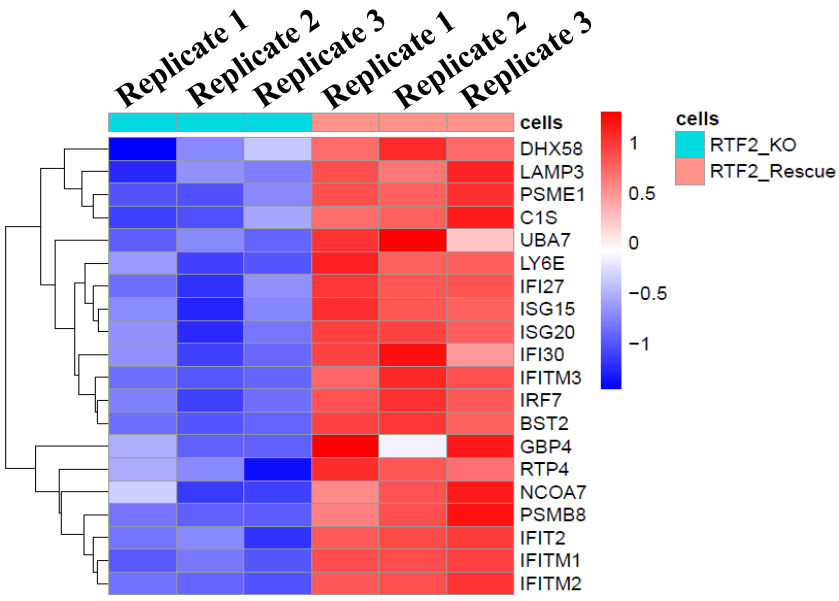

Figure 2U: Heatmap showing ISGs that are differentially expressed in IFN-pretreated RTF2-KO and RTF2-rescued cells. Shown are three technical replicates, with the row-normalized values for each gene.

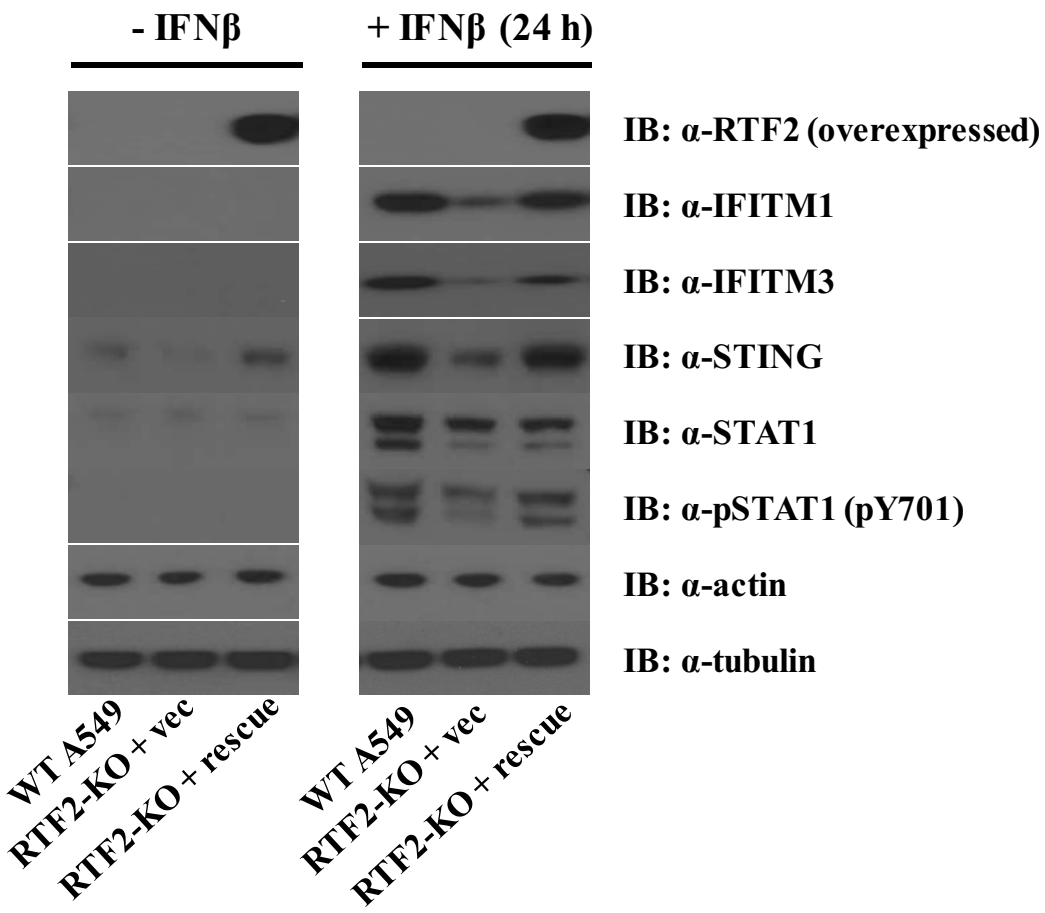

Figure 2V: IFNβ-treated RTF2-KO cells have lower ISG induction. Western blot showing total STAT1, phosphorylated STAT1 (pY701), IFITM1, IFITM3, and STING levels in IFNβ-treated WT A549, RTF2-KO and RTF2-rescued cells. Cells were mock-treated or exposed to 200U/ml IFNβ for 24 hours.

Given that RTF2 might play a role in positively regulating the IFN response, we hypothesized that RTF2-KO should result in higher infection rates by other viruses, especially viruses that are susceptible to the effects of IFN signaling. To test this, we infected RTF2-KO cells with clinical isolates of IAV such as the 1999 New Caledonia strain and the 2009 California H1N1 pandemic strain, as well as vesicular stomatitis virus (VSV). Consistent with our hypothesis, RTF2-KO cells showed higher NP mRNA levels for both the A/New Caledonia/20/1999 and A/California/04/2009 viruses (**Figure 2W**), and higher VSV N mRNA levels (**Figure 2X**).

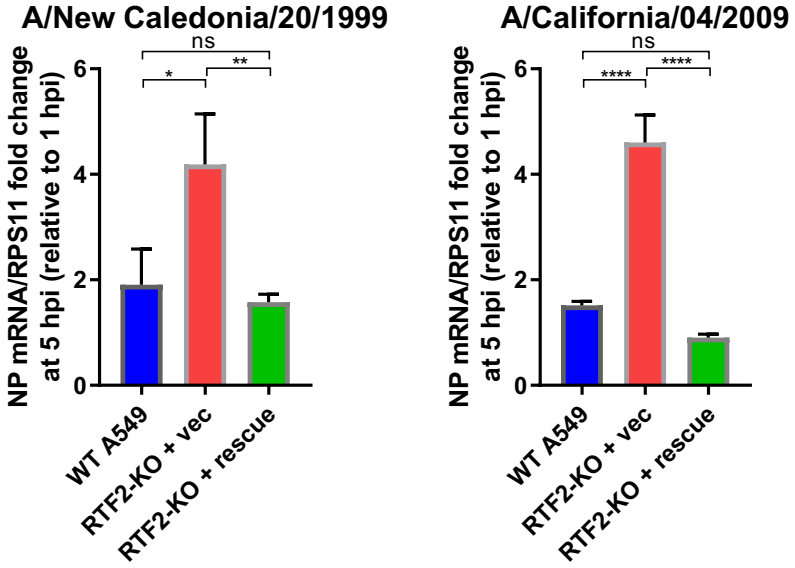

Figure 2W: RTF2-KO cells display increased NP mRNA levels when infected by other IAV strains. WT A549, RTF2-KO, and RTF2-rescued cells were pre-treated with 200U/ml IFNβ before infection with A/New Caledonia/20/1999 (Left) or A/California/04/2009 (Right) at MOI 5 for 5 hours. RNA was harvested at 5 hpi for qPCR to measure NP mRNA levels. **** $p \leq 0.0001$, ** $p \leq 0.01$, * $p \leq 0.05$, by one-way ANOVA followed by Tukey's multiple comparison test.

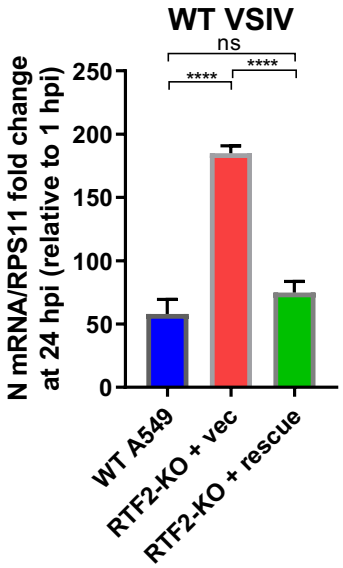

Figure 2X: RTF2 restricts VSV replication. WT A549, RTF2-KO, and RTF2-rescued cells were pre-treated with 200U/ml IFNβ before infection with WT VSV virus at MOI 5 for 24 hours before RNA was harvested for qPCR to measure N mRNA levels. **** $p \leq 0.0001$, by one-way ANOVA followed by Tukey's multiple comparison test.

Collectively, our data suggest that RTF2 contributes positively to the cellular response to IFN via the IFNAR pathway, as loss of RTF2 results in lower levels of pSTAT1 and ISGs, and impairment of the IFN-induced antiviral response.

Discussion

Based on our data, two models of how RTF2 may act as an antiviral factor emerge. First, RTF2 may be a direct antiviral effector of IAV polymerase and inhibits transcription. Alternatively, RTF2 may be a positive regulator of the IFN signaling pathway, and globally regulates the antiviral state of the cell in response to IFN exposure. Based on our available data, we cannot definitively distinguish between either model.

While we do not know if RTF2-KO also affects early steps of infection, we found that RTF2-KO cells exhibited a higher rate of primary transcription (rather than lower degradation). In addition, the elevated luciferase activity in the minigenome reporter assay, which is based on transfected plasmids instead of infection with live viruses, thus bypassing all the earlier steps in the viral life cycle also suggests that, at a minimum, RTF2 affects influenza polymerase complex activity. In addition, mislocalization of RTF2 outside the nucleus abolishes its ability to restrict IAV replication.

As the minigenome reporter assay was not performed in IFN-pretreated cells, one could hypothesize that RTF2 directly inhibits the activity of the IAV polymerase complex, perhaps by interacting with the viral polymerase complex and/or viral RNAs, without other IFN-induced proteins or activation. RTF2 and its homologs have been reported to interact with DNA[244,247] and possibly RNA[248]. In a recent published abstract, Zhai *et al.* made the following claims: (1) the monomeric crystal structure RTF2 protein has two RING-like domains, (2) proximity-dependent labeling revealed RTF2's interaction with spliceosomal components, and (3) RNA sequencing of

RTF2-knockdown cells revealed that intron retention was altered[251]. Based on these, they proposed that RTF2 is a novel E3 ligase that regulates pre-mRNA splicing, consistent with the report by Sasaki *et al.*[248]. If RTF2 could indeed interact with RNAs, then it is possible that RTF2 could also recognize viral RNAs and inhibit polymerase activity, similar to how nuclear TRIM25 can bind to RNA and block chain elongation[203]. Biochemical approaches such as an *in vitro* replication assay with purified components may reveal whether RTF2 is a direct inhibitor of IAV polymerase. Alternatively, if RTF2 is an E3 ligase, it could also regulate the levels of viral proteins by targeting them for proteasomal degradation, similar to how TRIM22 targets NP for proteasomal degradation[200]. Overexpressing RTF2 by itself does not confer additional protection in wildtype A549 cells, suggesting that RTF2 might require additional co-factors that are IFN-induced. Investigating if RTF2 interacts with viral RNAs and/or viral proteins, and monitoring viral protein levels in the presence and absence of proteasome inhibitors could help evaluate if these different models are true.

On the other hand, since transfection of DNA plasmids can induce IFN production in a STING and TBK1-dependent manner[252,253], we cannot rule out the possibility that the luciferase readout has been confounded by the antiviral response to transfected plasmids. A549 cells express both TLR9[254] and cGAS[255,256], and can upregulate IFN in response to foreign DNA. Hence, it is possible that the deficiency of RTF2 results in a globally lower IFN-induced response, which in turn permits higher IAV polymerase activity. In order to test this hypothesis, either DNA sensing and/or IFN signaling has to be blocked. If RTF2-KO cells still produce higher luciferase signal in the absence of DNA sensing and/or IFN signaling, then it would suggest that RTF2 has a more direct effector function on the viral polymerase.

Even if RTF2 does have a direct effector function on IAV polymerase activity, it does not necessarily mean that this is the main mechanism through which RTF2 exerts antiviral effect. It is increasingly appreciated that restriction factors could have dual functions[257]. Examples include IFITM3 acting as a feedback inhibitor of type I IFN induction via autophagosomal degradation of IRF3[258], and SAMHD1 inhibiting NFκB and IRF7 activation by inhibiting phosphorylation of IκBα and IRF7, independent of its dNTPase activity[259]. Although these reports reflect negative regulation of type I IFN signaling, ISGs could also positively regulate IFN signaling. For example, viperin, which is thought to inhibit budding[221], can also enhance TLR signaling and type I IFN production in plasmacytoid dendritic cells, by recruiting TRAF6 and IRAK1 to lipid bodies to enhance K63-linked ubiquitination of IRAK1 to activate IRF7[260]. Another example is ZAPS associating with RIG-I to promote its oligomerization and ATPase activity to facilitate IRF3 and NFκB signaling pathways, which in turn lead to robust type I IFNs production[261]. It is possible that RTF2 could be both a direct antiviral effector, and a positive regulator of type I IFN signaling concurrently.

Given the importance of type I IFN in antiviral innate immunity[262], it is not surprising that the lower pSTAT1 level and lower ISG induction in RTF2-KO cells would result in higher IAV infection. Although we only investigated the primary transcription step of the IAV life cycle here, it is possible that multiple steps in the viral life cycle are affected too, owing to the reduced induction of ISGs. In addition, the higher VSV mRNA levels in RTF2-KO cells also suggests that the restriction imposed by RTF2 likely extends beyond inhibiting primary transcription in the nucleus. This is because VSV is a cytosolic negative-sense RNA virus that does not enter the

nucleus for transcription and/or replication. Future work could investigate if RTF2 affects additional viruses from different families. If a broad panel of unrelated viruses all replicate better in RTF2-KO cells, then it would suggest that RTF2 exerts antiviral effects by positively regulating the IFN pathway (or another unknown broad-spectrum restriction factor that shuttles between the nucleus and cytosol). If this were true, then the reason why mislocalizing RTF2 outside of the nucleus results in enhanced IAV replication is not only because RTF2 can no longer inhibit IAV polymerase activity, but rather, RTF2 can no longer access the signal transduction and gene regulation machineries in the nucleus necessary for an efficient IFN-induced antiviral response.

A major unanswered question is how RTF2 regulates the IFN response. Without additional supporting data, one could only speculate how RTF2 may regulate IFN response. The first and simplest model would be that RTF2 directly interacts with proteins in the IFN signaling pathway. Perhaps RTF2 directly binds to STAT1 and prevents its dephosphorylation or degradation, or perhaps RTF2 facilitates loading of interferon-stimulated gene factor 3 (ISGF3) onto interferon-sensitive response elements in ISG promoters.

A second model could involve ssDNA generation at stalled replication forks. One clue comes from that fact that persistent RTF2 at stalled replication forks results in increased ssDNA[244]. It was recently proposed that SAMHD1 could limit IFN signaling by stimulating the activity of exonuclease MRE11 to promote degradation of nascent DNA at stalled replication forks[263]. This enhanced MRE11 activity results in lower levels of ssDNA, and correspondingly lower cGAS-STING activation and ISG induction[263]. A possible scenario is that RTF2 generates

ssDNA at replication forks, and knocking out RTF2 results in lower amount of ssDNA and cGAS-STING activation. This might have resulted in a lower basal antiviral tone of the cell, and hence a slower and/or lower upregulation of ISGs in response to IFN treatment by RTF2-KO cells.

A third speculative model concerns RTF2's potential interaction with spliceosomal components. Perhaps RTF2, because of its role in regulating pre-mRNA splicing, is necessary to generate specific isoforms that are critical in IFN signaling and/or ISG expression. Alternatively, because of RTF2's interaction with spliceosomal components, RTF2 might impair IAV's antagonism of the antiviral response. IAV endonuclease PA-X has been reported to interact with the host splicing machinery to gain access to host transcripts for host shutoff[264]. Although PA-X does not degrade ISG transcripts specifically, host shutoff can dampen the cell-intrinsic response to infection, as evidenced by higher ISG expression in the absence of PA-X[264]. Perhaps RTF2 interferes with PA-X from degrading ISG mRNAs. All these remain speculative, and understanding which proteins RTF2 interact with will be a crucial first step in unraveling the mechanism.

In addition to the experiments described above that might help distinguish between different models of RTF2's mechanism, it would also be interesting to investigate the downregulation of RTF2 mRNA and protein levels relative to housekeeping genes in IAV-infected cells (**Figure 2Q**). One could hypothesize that IAV has evolved to reduce RTF2 levels in infected cells to counteract RTF2's antiviral activity, suggesting, but definitely not proving, that RTF2 is a bona fide antiviral factor. Future experiments investigating how IAV induces such

downregulation, and whether other viruses induce such downregulation, might be informative to RTF2's mechanism of action too.

Knocking out RTF2 also affects the transcription of additional gene beyond the ISGs. For instance, genes that are less upregulated in IFNβ-treated RTF2-KO cells compared to RTF2-rescued cells include those involved in DNA synthesis, metabolism, interleukin signaling, interferon signaling, and protein glycosylation (**Figure 2Y**). This is in line with our observation that there is slower growth of RTF2-KO cells (data not shown), and data from Broad Institute Cancer Dependency Map, which reports the dependencies of particular genes in the various cancer cell models. In addition, besides indicating that RTF2 might be essential in other cell lines, the Dependency Map portal also reveals that there is a co-dependency of RTF2 on a ribosomal protein gene RPS21 (Pearson correlation of 0.34). This suggests that RTF2 may have other functions in the cell, such as regulating transcription or translation, and raises the possibility that the higher luciferase reporter signal observed in RTF2-KO cells in the reporter assay might be due to RTF2's effect on cellular machinery even beyond the context of interferon-stimulated antiviral restriction factors. Additional experiments should be performed to compare rates of transcription of different constructs used in the reporter assay in RTF2-KO and RTF2-rescued cells to clarify this possibility.

In addition, when we specifically examined the Gene Ontology (GO) biological processes (BP) categories of differentially expressed genes in RTF2-KO and RTF2-rescued cells, we obtained far fewer statistically significant categories in the absence of IFNβ than in the presence of IFNβ. In the absence of IFNβ exposure, genes involved in extracellular matrix organization,

translational initiation, and cell adhesion seem to be upregulated in RTF2-KO cells compared to RTF2-rescued cells, while genes involved in oxidation-reduction process, daunorubicin metabolic process and doxorubicin metabolic process are downregulated in RTF2-KO cells relative to RTF2-rescued cells. On the other hand, when comparing IFNβ-treated RTF2-KO and RTF2-rescued cells, the only statistically significant GO BP category of genes that is differentially upregulated in RTF2-KO cells compared to RTF2-rescued cells is positive regulation of translation. Downregulated genes in RTF2-KO cells relative to RTF2-rescued cells fall into the following GO BP categories: type I interferon signaling pathway; oxidation-reduction process; negative regulation of viral genome replication; antigen processing and presentation of exogenous peptide antigen via MHC class I, TAP-dependent; daunorubicin metabolic process; doxorubicin metabolic process; response to virus; interferon-gamma-mediated signaling pathway; positive regulation of smooth muscle cell proliferation; antigen processing and presentation of peptide antigen via MHC class I; and immune response. This is in line with our earlier observation that IFNβ pretreatment is needed to observe the effect of knocking out RTF2 on IAV infection rate. While we did not pursue this direction further, gene expression data of RTF2-KO and RTF2-rescued cells, with and without IFNβ treatment, could be accessed through NCBI GEO database.

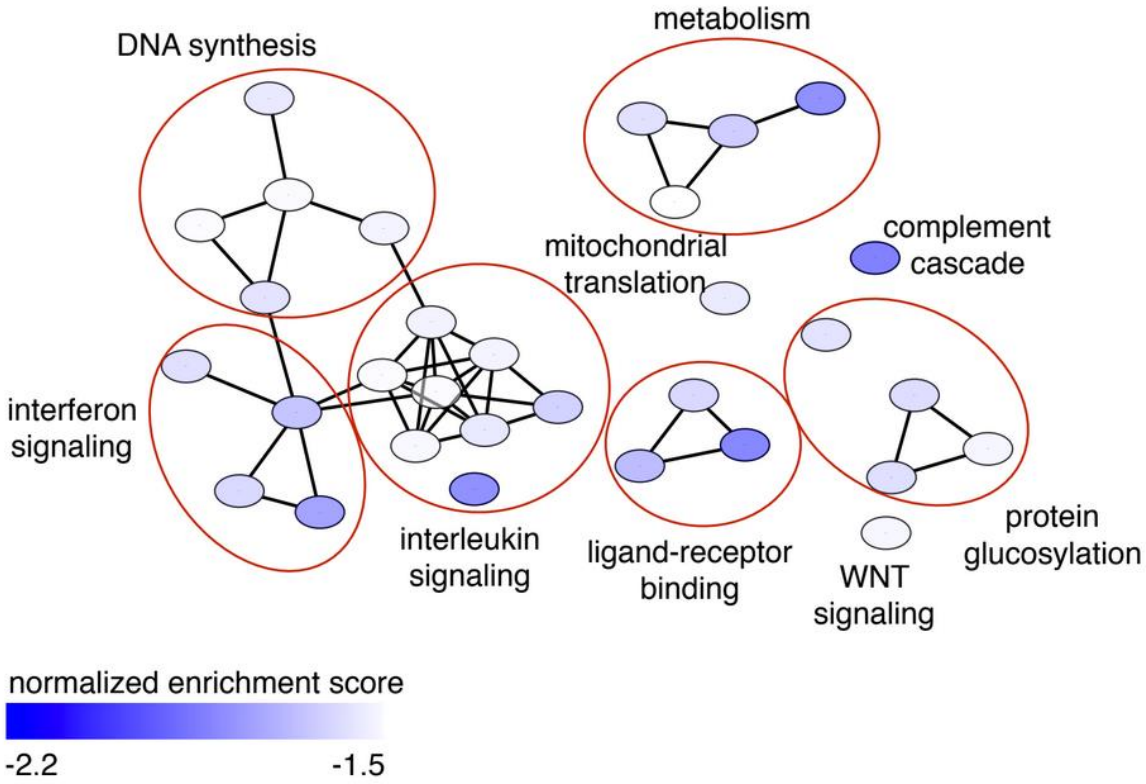

Figure 2Y: Network analysis showing different sets of genes that are differentially expressed between IFNβ-treated RTF2-KO cells and RTF2-rescued cells.

In summary, our work has shown that RTF2 is an antiviral factor that has to be localized to the nucleus to restrict IAV replication. RTF2 inhibits polymerase activity, as shown via a minigenome luciferase reporter assay, and impairs IAV primary transcription, as shown in CHX-pretreated cells. This is likely due to impaired mRNA synthesis, as we did not find evidence of slower IAV mRNA decay in RTF2-KO cells. While it remains untested if RTF2 inhibits other steps in the flu life cycle, RTF2 is important for positively regulating the IFN pathway and upregulating other antiviral ISGs such as IFITM1 and IFITM3. Hence, RTF2 likely blocks the other steps in IAV life cycle too.

Methods

Cell culture, Reagents, Virus Strains

For IFN signaling inhibition, we used recombinant viral B18R protein (R&D systems; 8185-BR-025), and ruxolitinib (Invivogen; tlrl-rux). Baloxavir was obtained from MedChemExpress (HY-109025A). Cycloheximide was obtained from CST (2112). For microscopy, we used rabbit serum for blocking (Sigma; R9133) and Vectrashield antifade mounting medium with DAPI (Vector labs; H-1200).

Additional viruses were obtained from the following sources: Vesicular stomatitis virus was kindly gifted by Dr. Sean Whelan's lab. Influenza A/New Caledonia/20/1999, A/California/04/2009 viruses were kindly gifted by Dr. Daniel Lingwood's lab.

Plasmids

Additional site-directed mutagenesis was performed on the codon-mutated RTF2 rescue plasmid to generate mislocalized forms of RTF2. For ER-retention mutant, a signal peptide (MLLSVPLLLGLLGLAAAD)[265] was added to the N-terminus, and a KDEL motif was added to the C-terminus of the NLS mutant. For the plasma membrane-targeting mutant, a myristoylation motif (GCIKSKRKDNLNDDGVD)[266] was added to the N-terminus of the NLS mutant. Plasmids used in the luciferase reporter assay were previously described[109].

Antibodies

Sources of antibodies besides those listed in the previous Methods section were as follows: From Abcam, Anti-IFITM1 (ab224063) (1:2000). From Cell Signaling Technology, Anti-IFITM3 (59212T) (1:2000), Anti-STING (13647) (1:2000), Anti-p-STAT1 Y701 (9167)

(1:2000), Anti-TATA Binding Protein (8515s) (1:2000). From ThermoFisher, Rabbit anti-Mouse IgG (H+L) Secondary Antibody, Alexa Fluor 488 conjugate (A-11059) (1:500), Mouse anti-alpha tubulin antibody (DM1A) (14-4502-82) (1:2000).

Microscopy

Cells were grown in chamber slides (Nunc™ Lab-Tek™ II Chamber Slide™ System, ThermoFisher) overnight. Media was aspirated, then the cells were rinsed with 1x PBS before they were fixed in fresh 4% formaldehyde for 15 minutes at room temperature. Cells were then rinsed with 1x PBS thrice, for 5 minutes each, before they were permeabilized and blocked in rabbit serum for 1 hour at room temperature, and then stained with anti-RTF2 primary antibody (1:250) at 4^{o}C overnight. Cells were then rinsed with 1x PBS thrice, 5 minutes each, and then stained with rabbit anti-mouse secondary antibody conjugated with Alexa Fluor 488 for 1 hour at room temperature in the dark. Cells were then rinsed with 1x PBS thrice, 5 minutes each, then mounted and allowed to cure overnight before imaging. For live-cell imaging, mCherry-tagged RTF2-expressing cells were grown in regular 6-well plates in DMEM. Cells were imaged on a wide-field epifluorescence microscope (Applied Scientific Instrumentation) with the images acquired using Micro-Manager software. Acquisition settings and exposure times were kept consistent across experiments.

Biochemical fractionation

Cell fractionation protocol was adapted from the Ficoll-Digitonin protocol as previously described[267]. Briefly, 1.5 x 10^6 trypsinized WT A549 cells were washed with PBS twice and resuspended in 600 µl of HEPES-Sucrose-Ficoll solution (HSF, 20 mM HEPES-KOH, 6.25%

Ficoll, 0.27 M sucrose, 3 mM $CaCl_2$, 2 mM $MgCl_2$) with 50 μg/ml digitonin, and EDTA-free Protease inhibitor cocktail (Roche). Cells were kept on ice for 10 minutes before they were spun down at 1000 x g for 3 minutes. The supernatant was collected and then further centrifuged at 15,000 x g for 10 minutes to generate cytosol fraction. The nuclei pellet from the first centrifugation was rinsed with HSF buffer once, and spun down again at 1000 x g for 3 minutes. The supernatant was collected as the washed fraction, and the pellet was then lysed in 50 μl of RIPA with EDTA-free Protease inhibitor cocktail (Roche) on ice for 20 minutes. The lysed nuclei were then centrifuged at 10,000 x g for 10 minutes, before the supernatant was collected as the nuclear fraction for western blot analysis.

Minigenome luciferase reporter assay for influenza A virus replication

To measure viral polymerase activity, we utilized a vRNA-luciferase reporter system. Briefly, A549 cells were transfected with a vRNA reporter plasmid expressing firefly luciferase under a viral UTR. The cells were also transfected with influenza A virus PA, PB1, PB2, NP and Renilla luciferase plasmids. 24 hours post-transfection, cells were lysed and mixed with Dual Glo substrate (Promega) according to Manufacturer's protocol. Luminescence was measured and quantified using a Synergy H1 multi-mode microplate reader (BioTek).

CHX experiments

For experiments involving CHX treatment, cells were exposed to either DMSO or 50 μg/ml CHX for 2 hours towards the end of the 24 hours IFN pre-treatment prior to infection; CHX was retained in the infection and cell culture media until the cells were harvested.

RNA stability experiment

At 6 hpi, baloxavir (or DMSO) was added to the serum-free incubation cell culture media to achieve a final concentration of 50nM baloxavir. Starting from 7 hpi to 11 hpi, media was aspirated from the corresponding wells and cells were lysed with RNeasy Mini Kit (Qiagen) lysis buffer supplemented with β-mercaptoethanol according to manufacturer's instructions. RNA was then extracted and processed for RT-PCR and qPCR as described above.

IFN dependence experiments

Cells were incubated in either complete DMEM containing 200U/ml of IFN β, or media without any IFNβ. For the corroborating experiments, cells were pre-treated with 0.5 μg/ml IFNAR receptor decoy B18R, or 5 μM JAK inhibitor ruxolitinib, for 2 hours prior to IFNβ treatment, and the respective blockers were left in the media throughout the entire duration of the subsequent 24-hour IFN treatment.

RNA sequencing

The Smart-Seq2 protocol[268] was employed to perform transcriptomic analyses of the different cells. Total RNA was extracted using the RNeasy Mini Kit (Qiagen) according to manufacturer's protocol. The cDNA was synthesized from 1ng of total RNA using SuperScript II reverse transcription, followed by PCR amplification and quality check using high-sensitivity DNA Bioanalyzer chip (Agilent). 0.15 ng of cDNA was then used for the tagmentation reaction carried out with the Nextera XT DNA sample preparation kit (Illumina) and further PCR amplification. Paired-end reads of 38-bp of the amplified library were generated on a NextSeq 500 instrument (Illumina) and aligned to the hg19 (GENCODE v21) transcriptome using STAR

v2.6. RSEM v1.3.1 was then used to quantify expression of all genes. RNA-sequencing data has been uploaded to the NCBI Gene Expression Omnibus (GEO) database (accession number: GSE146403). Downstream analysis of the resulting genes x samples expression matrix was performed in R. Specifically, differential expression was calculated with the R package DESeq2. ISG score was calculated by summing up the DESeq2 normalized expression values of ISGs, as obtained from the hallmark interferon alpha response gene set.

RNA-extraction and qPCR

Additional primers used for qPCR were as follows:

Influenza A/New Caledonia/1999 NP

F: 5' – TGAGGGACGACTGATCCAGA – 3'

R: 5' – ATGTGAGTCAAACCAGCCGT – 3'

Influenza A/California/2009 NP

F: 5' – GCTTGTGTGTATGGGCTTGC – 3'

R: 5' – TCTGGACCCCTCTTGTGGAA – 3'

VSV N

F: 5' – ATCGGGAAAGCAGGGGATAC – 3'

R: 5' – TTTGTCATCTGCGCTGGTTC – 3'

Conclusions and Future Directions

Our study has identified a novel restriction factor of IAV replication, RTF2, via a genome-wide CRISPR-KO screen. Loss of RTF2 leads to increased IAV replication, as measured in multiple different assays. Nuclear localization is important for RTF2's antiviral function. RTF2 restricts IAV, at least in part through blocking primary transcription, and in part through positively regulating IFN signaling.

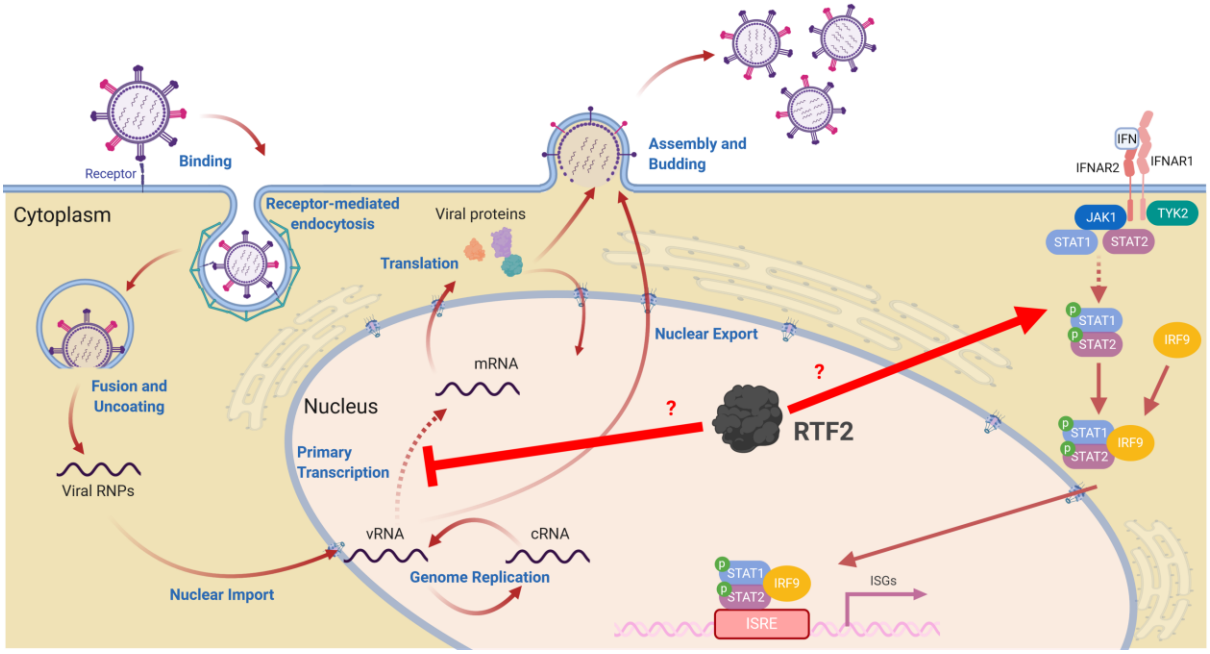

Figure 3: Schematic of how RTF2 may restrict influenza. RTF2 may directly restrict primary transcription and/or have a more general role in regulating the cell's antiviral state in response to IFN stimulation.

Although the detailed molecular mechanism of action for RTF2 has yet to be worked out, our work advances the field by elucidating a novel cellular function of this understudied gene. Previously Inagawa *et al.* reported that the *Schizosaccharomyces pombe* RTF2 homolog could be involved in site-specific replication termination by stabilizing stalled replication forks at the

specific replication barrier *RTS1* element[247], while Sasaki *et al.* reported that the *Arabidopsis thaliana* homolog might be regulating pre-mRNA splicing in an ubiquitin-dependent fashion to affect embryonic development[248]. An abstract published by Zhai *et al.* claimed to have solved the crystal structure of RTF2, and that RTF2 is a E3 ligase that regulates pre-mRNA splicing[251]. The only published study characterizing RTF2 in human cells reported that RTF2 needs to be removed from stalled replication forks during induced DNA damage in order to maintain genome integrity[244]. The common thread across these studies is that RTF2 appears to interact with some form of nucleic acids and regulate their processing. It is increasingly appreciated that there is crosstalk between DNA replication, DNA damage response, and innate immune pathways[269]. Understanding RTF2's roles, even outside the context of antiviral immunity, would thus be insightful in figuring out how it functions as a restriction factor.

Our work also underscores a point that despite the many screens that had been published and identification of many restriction factors, additional novel factors (which may be context-specific) remain to be found. Although our screen recovered genes in the IFN signaling pathway, we did not recover previously reported classical antiviral ISGs. As discussed in the earlier chapter, this might be due to a combination of factors: (1) small effect size of each individual antiviral effector gene, (2) redundancy of the many ISGs upregulated upon IFN exposure, (3) incomplete deletion of genes by CRISPR.

Future improvements to such screens could involve using a lower IFN dose, increasing the size of the screen to detect lower effect size genes, and performing a combinatorial screen where multiple antiviral genes are knocked out in the same cell. Using a lower IFN dose might

reduce redundancy of ISGs if fewer ISGs get induced per cell. Without other restriction factors compensating, the impact of knocking out any particular ISG is potentially larger and easier to detect. However, this is a fine line to tread because a lower IFN dose might also lead to lower induction of said ISG within the cell, further reducing its contribution and effect size. A much larger screen, using more guides per cell, and starting with more cells per guide in the pre-IAV infection population, would have to be conducted for improved statistical power to recover genes that might have small, but reproducible effect, in restricting IAV. Lastly, one could perform combinatorial screens (perhaps by creating two or more guide libraries or expressing multiple sgRNAs per vector) or infect cells with high MOI of sgRNA lentiviruses before using single-cell approaches to detect the combinations of sgRNAs in each cell. This allows for direct investigation of how different restriction factors work together to achieve protection conferred by IFN treatment.

These improvements to screens will help address important knowledge gaps in the field, including, but not limited to, the following: (1) what is the minimum number of restriction factors, each with a modest effect size, required to achieve a level of protection that is comparable to IFN treatment; (2) what individual restriction factors are effective in restricting specific viruses; (3) which host factors work together (either in the same complex or in the same pathway) and which host factors can have additive effects; (4) whether restriction factors synergize or do they only confer additive protection. Addressing such questions would help inform the development of novel therapeutics, either by mimicking the mechanisms of these restriction factors, or coming up with ways to temporarily upregulate the minimum set of genes

effective in restricting a particular virus to avoid potential side-effects. With the advancements seen in gene therapy and synthetic biology, this might be possible in the future.

Another interesting direction to pursue would be to develop scalable assays that investigate late stages of the viral life cycle. As discussed earlier, our screen only allows recovery of factors that affect viral replication up to the point of HA trafficking. Recent work by Dittmann *et al.*, employing an image-based screen of an ISG library comprising 400 cDNAs, identified a novel restriction factor that reduces virus spread[215]. This labor-intensive, arrayed format of screening a limited (non-genome-wide) library identified the first extracellular direct-acting antiviral factor, which would not have been recoverable in screens that flow-sort on infected cells (like ours). The development of optical pooled screens such as the one published by Feldman *et al.*[270] allows demultiplexing genetic perturbations, making the combinatorial knockouts feasible. As this optical screen uses a microscopy approach, it might be possible to adapt an assay to screen for late stages in the viral life cycle. This will complement the more commonly employed FACS-based enrichment and survival-based selection screening approaches. OhAinle *et al.* also recently developed another novel approach, where they engineered HIV-1 viruses to package lentiviral genomes that encode CRISPR sgRNAs as progeny viruses bud from CRISPR/Cas9-mediated knockout cells. Host factors affecting virus replication were identified by amplifying and sequencing sgRNAs encoded by the packaged lentiviral genomes of budded viruses in the supernatant. Simply put, the progeny viruses were "barcoded" to reveal the genetic perturbations present in the cell from which they came from. Such an approach allows identification of host factors involved in the entire life cycle of the virus, and could be a useful tool. Although the pooled optical screen and virus-packageable

screen are currently still limited in their capacity and have yet to be scaled up for genome-wide screens, it would be exciting to see what discoveries could be made when these approaches are adapted and/or scaled up for genome-wide screens.

While great progress has been made in understanding cellular antiviral defenses in the past decades, the contributions of the many host antiviral factors to controlling specific viruses has yet to be determined. As each virus evolves a unique strategy to counteract host restriction factors, one might expect to find few pan-virus effectors[2,233], but instead, many effectors that have directly evolved to cope with different viruses. Elucidation of how RTF2, and other factors, restrict viral replication will continue to provide insights into how our cells contend with the multitude of viruses.

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