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# Interferon-stimulated genes: roles in viral pathogenesis

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Interferon-stimulated genes (ISGs) are critical for controlling virus infections. As new antiviral ISGs continue to be identified and characterized, their roles in viral pathogenesis are also being explored in more detail. Our current understanding of how ISGs impact viral pathogenesis comes largely from studies in knockout mice, with isolated examples from human clinical data. This review outlines recent developments on the contributions of various ISGs to viral disease outcomes *in vivo*.

## Addresses

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## Introduction

Interferons (IFN) are secreted cytokines that impact numerous host processes and are well known for their antiviral and antiproliferative properties. Among the three main IFN families, Type I (IFN $\alpha/\beta$ ) and III (IFN $\lambda$ ) IFNs are considered the primary antiviral IFNs, although type II (IFN $\gamma$ ) has well-characterized antiviral effects [1]. IFNs are transcriptionally activated by a highly orchestrated sequence of signaling events composed of viral sensors, adaptor proteins, kinases, and transcription factors (Figure 1) [2,3]. Newly synthesized IFNs are secreted from the infected cell, bind their cognate receptors, and initiate signaling through the JAK/STAT pathway. The result is the transcriptional induction of interferon-stimulated genes (ISGs), which encode direct antiviral effectors or molecules with the potential to positively and negatively regulate IFN signaling and other host responses.

Genome-wide transcriptional profiling has identified hundreds of ISGs [4]. Recent functional screening efforts, both ectopic overexpression and gene silencing, have begun to catalog antiviral ISGs and identify targets for mechanistic characterization [5,6,7\*,8\*,9\*,10\*,11–16]. For the ISGs that have been characterized, their mechanisms generally target conserved aspects of viral infection. Examples include ISGs that modulate nucleic acid

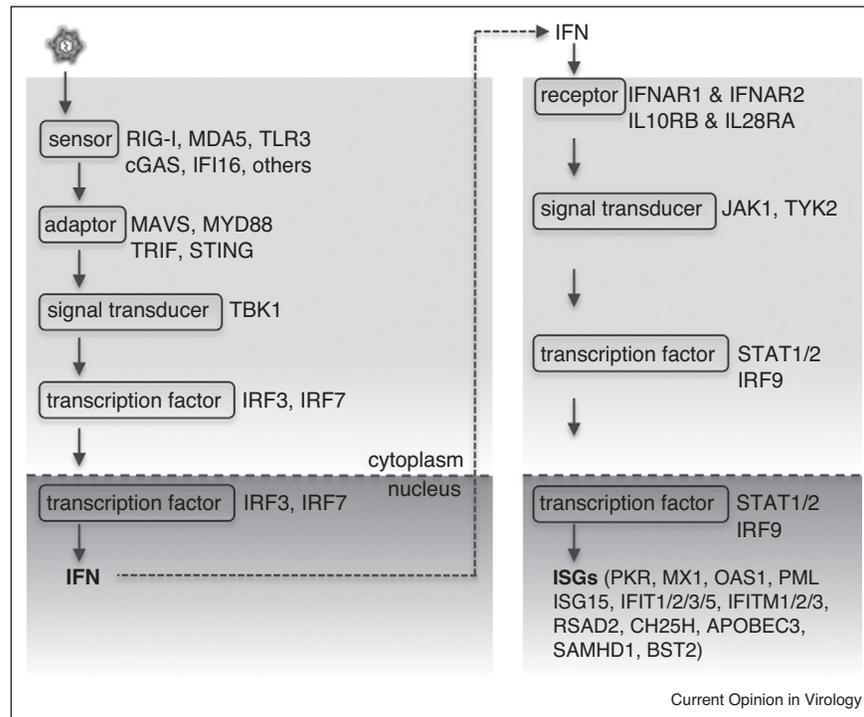
integrity (OAS/RNase L, ADAR1, and APOBEC family members), virus entry (IFITM3), protein translation (PKR, IFIT family members), and virus egress (BST2/tetherin) [17]. The functions of the majority of antiviral ISGs, however, remain unknown. Indeed, determining the mechanisms of antiviral ISGs is a major goal of future research in innate immunity. Moreover, while these recent screening studies provide targets for mechanistic characterization, an additional question that remains is: do *in vitro* ISG studies predict physiologically relevant functions of antiviral molecules *in vivo*?

## IFN responses and ISGs in viral pathogenesis

The role of IFNs in viral pathogenesis is well established in mice and humans. Both type I IFN receptor knockout (*Ifnar*<sup>-/-</sup>) and STAT1 knockout (*Stat1*<sup>-/-</sup>) mice are highly susceptible to numerous viruses [18–21]. Mice with defects in IFN or ISG production, due to genetic ablation of *Rig-I*, *Mda5*, *cGas*, *Thr3*, *Mavs*, *Irf1*, *Irf3*, or *Irf7* also exhibit enhanced viral pathogenesis [7\*,22–26]. Similarly, humans with inborn deficiencies in *STAT1*, *TYK2*, *NEMO*, *TBK1*, *TLR3*, *UNC93B1*, *TRIF*, or *TRAF3* have defective IFN responses and are often highly susceptible to one or more viral diseases [27].

The disease outcomes in mice and humans with these various genetic deficiencies strongly implicate IFNs, and by extension, ISGs as primary genes controlling the replication and spread of viruses *in vivo*. However, of the hundreds of known ISGs, relatively few have been characterized *in vivo* for their contributions to antiviral immune responses. This is likely due to several reasons. First, the breadth of the ISG response was not uncovered until use of genome-wide transcriptional profiling over a decade ago [4]. Second, until the publication of recent ISG screening efforts, relatively few of the hundreds of ISGs had been characterized for antiviral phenotypes *in vitro*. Since the decision to carry out genetic studies in mice typically requires strong rationale from validated *in vitro* studies, *in vivo* targeting has been limited to a few genes. Third, genes that control IFN production (e.g., *Rig-I/Ddx58*) or signaling (e.g., *Stat1*) are more attractive targets for genetic ablation in mice since their absence would *a priori* be predicted to confer strong viral phenotypes. By contrast, deleting a single ISG from a large pool of IFN-induced genes carries an inherent risk of not detecting a viral phenotype due to redundancy in the ISG effector system. Nonetheless, a subset of these downstream ISGs has been targeted for genetic disruption in mice, with promising results (Table 1). The data from these studies are providing new insight into ISG specificity against distinct classes of virus and in unique cell or tissue types.

Figure 1



Overview of the general innate immune response to viral infection. Incoming or replicating viral genomes are detected by viral sensors. Adaptor proteins initiate a signaling cascade that results in activation of transcription factors. Type I/III IFNs are produced and secreted from the infected cell. They act on neighboring cells through the JAK/STAT pathway, leading to widespread ISG induction. The majority of downstream ISGs that have been targeted for deletion in mice are listed in this figure.

### PKR, MX1, OAS-RNase L

Three of the so-called 'classical ISGs' include: interferon-induced, double-stranded RNA-activated protein kinase (PKR, encoded by *Eif2ak2*), the myxovirus (influenza

virus) resistance 1, interferon-inducible protein p78 (MX1 or MXA, encoded by *Mx1*) and the 2',5'-oligoadenylate synthetase/RNase L system (OAS1, OAS2, OAS3, encoded by *Oas1*, *Oas2*, *Oas3*). The mechanisms of these

Table 1

#### Viral pathogenic outcomes in ISG-deficient mice

ISG	Virus	Pathogenic outcome (lethality, titers, or disease)
<i>Eif2ak2/Pkr</i> , <i>Oas1b</i> , <i>Rnase1</i> (single or double mutants)	EMCV, VSV, HSV-1, HSV-2, CVB4, MHV, VV, flavivirus	Increased
<i>Trim19/Pml</i>	SINV, Friend retrovirus	No difference, subclinical
<i>Isg15</i>	LCMV, EMCV, SFV	Increased
<i>Zbtb16/Plzf</i>	CHIKV, MHV68, HSV-1, FLUAV, FLUBV, SINV, VV	Increased
<i>Ifit1</i>	LCMV, VSV	No difference
<i>Ifit2</i>	EMCV, SFV	Increased
<i>Ifitm3/Ifitm</i> locus	WNV, HCoV (lacking 2'-O-methyltransferase activity)	Increased
<i>Rsad2/viperin</i>	VSV, WNV, MHV-A59	Increased
<i>Ch25h</i>	FLUAV, RSV	Increased
<i>Apobec3</i>	WNV, CHIKV	Increased
<i>Samhd1</i>	FLUAV	No difference
<i>Bst2/tetherin</i>	MHV68	Not determined, increased virus replication
	MMLV, Friend retrovirus	Increased
	HIV-1-based vectors	Not determined, increased transduction
	MMLV, MCMV, VSV	Mixed, subclinical

Abbreviations: CHIKV, chikungunya virus, CVB, coxsackie virus, EMCV encephalomyocarditis virus, FLUAV, influenza A virus, FLUBV, influenza B virus, HCoV, human coronavirus, HIV, human immunodeficiency virus, HSV, herpes simplex virus, LCMV, lymphocytic choriomeningitis virus, MCMV, murine cytomegalovirus, MMLV, Moloney murine leukemia virus, RSV, respiratory syncytial virus, SFV, Semliki Forest virus, SINV, Sindbis virus, VSV, vesicular stomatitis virus, VV, vaccinia virus, WNV, West Nile virus.

effectors have been studied for years and are the subjects of recent reviews [28–30]. Briefly, PKR is a known inhibitor of cellular and viral mRNA translation, and is involved in a variety of cellular processes including inflammation and apoptosis. Several members of the OAS enzyme family are activated by double-stranded RNA to catalyze the formation of 2',5'-oligoadenylates, which activate cellular RNase L to degrade viral genomes. MX1 is a dynamin-like GTPase that appears to target viral nucleocapsids, resulting in viral inhibition prior to the establishment of replication. Most inbred strains of laboratory mice are deficient in functional MX1 protein expression and are more susceptible to influenza A virus infection than non-laboratory, wild type strains [28]. Thus, any ISG knockout made on a standard mouse background must minimally be considered a functional 'double knockout' of *Mx1* and the ISG of interest.

Studies have shown that mice deficient in RNase L, PKR, or both have a range of pathogenic phenotypes in response to numerous viruses. The viruses tested in these mice include encephalomyocarditis virus [31,32] vesicular stomatitis virus [33–35], herpes simplex virus types 1 and 2 [36–38], coxsackievirus B4 [39], West Nile virus [40], murine coronavirus [41], and vaccinia virus [42]. Depending on the study, pathogenic outcomes for many of these viruses typically include increased mortality, higher viremia and/or viral burden in various tissues, and accelerated onset of clinical disease. However, the effects are not universal since *Rnase1*<sup>-/-</sup> mice are not more susceptible than wild type mice to retroviral infection [43], and *Rnase1*<sup>-/-</sup> x *Pkr*<sup>-/-</sup> knockouts exhibit only subclinical phenotypes when infected with Sindbis virus [44]. Mice with a natural mutation in *Oas1b*, the so-called *Fkv* or flavivirus resistance gene [45], are also well characterized for their susceptibility to a range of flaviviruses [46,47]. Taken together, these *in vivo* studies establish roles for PKR and the antiviral OAS system in controlling pathogenic viruses, and highlight the virus-specific inhibitory nature of these ISGs.

#### PML and PLZF

The promyelocytic leukemia (PML) protein was originally discovered as part of an oncogenic fusion protein with the retinoic acid receptor alpha in patients with acute promyelocytic leukemia [48]. Encoded by *Trim19*, PML is an IFN-induced protein that organizes the formation of structurally distinct nuclear bodies composed of permanent proteins Daxx and SP100, as well as a number of transient proteins such as P53 and ATM. PML bodies regulate a wide range of cellular processes, including responses to DNA damage, stress, apoptotic stimuli, and viral infection. Regarding the latter, the antiviral mechanisms of PML in cell culture appear to be varied, depending on the virus. Examples of PML-mediated antiviral function include inhibition of viral transcription, sequestration of viral proteins, and reduction of IFN

antiviral efficacy. When *Trim19*<sup>-/-</sup> (formerly *Pml*<sup>-/-</sup>) mice were challenged with lymphocytic choriomeningitis virus (LCMV), increased viral titers were observed in spleen when compared to wild type mice [49]. *Trim19*<sup>-/-</sup> mice also exhibited more severe footpad swelling reactions after subcutaneous intrafootpad inoculation. When the knockout mice were infected with a hepatotropic strain of LCMV, a pronounced terminal immunopathological hepatitis that correlated with elevated liver transaminase levels was observed. When *Trim19*<sup>-/-</sup> mice were infected with a low dose of LCMV by intracerebral inoculation, they exhibited a more severe cytotoxic T cell-mediated choriomeningitis compared to wild type mice. A more recent study has implicated a PML binding protein, the transcription factor PLZF (encoded by *Zbtb16*), as critical for regulating the expression of a subset of ISGs during the IFN response [50]. *Zbtb16*<sup>-/-</sup> mice are more susceptible to EMCV infection than wild type mice, and are not protected by IFN treatment prior to Semliki Forest virus (SFV) infection. Accordingly, SFV titers were 3 logs higher in numerous organs of *Zbtb16*<sup>-/-</sup> mice when compared to wild type. These *in vivo* studies provide direct evidence that PML and related pathways contribute to controlling viral spread and immunopathological outcomes of certain viral infections.

#### ISG15

Interferon stimulated gene 15, (encoded by *Isg15*) is a small, ubiquitin-like molecule that has well characterized antiviral properties. A recent, comprehensive review discusses ISG15 function, activity, and contributions to innate immune responses *in vitro* and *in vivo* [51]. In brief, ISG15 has numerous antiviral functions including inhibition of virus release, ISGylation of both viral and host proteins, and immunomodulatory cytokine-like properties in its unconjugated form. *Isg15*<sup>-/-</sup> mice have been challenged with at least ten viruses, and many of these infected mice showed increased mortality when compared to wild type mice [51,52]. Studies in *Ube1*<sup>-/-</sup> mice, which lack the ISG15 conjugating enzyme, indicate that while ISG15 conjugation is required for antiviral activity against some viruses (e.g., influenza virus), it is dispensable for viruses such as CHIKV [51]. The complexity of ISG15 function is highlighted further by the recent identification of humans with inherited ISG15 deficiency. These patients were found to have increased susceptibility to mycobacterial, but not viral disease, and the phenotypes were correlated with impaired IFN $\gamma$  immunity mediated by secreted ISG15 [53••]. ISG15 may be unique in that its role in the immune response is species-specific.

#### IFIT and IFITM families

The interferon-induced protein with tetratricopeptide repeats (IFIT) family and the IFN-induced transmembrane protein family (IFITM) family have been aggressively studied in recent years. These proteins are the

subjects of several recent reviews [54–57]; thus only highlights of the latest developments with respect to the roles of these effectors in viral pathogenesis will be addressed here. The human and mouse IFIT family consists of IFIT1, IFIT2, and IFIT3, and humans have an additional member not found in mouse, IFIT5. The IFIT family members have a variety of antiviral mechanisms, including translational inhibition, recognition of viral RNAs that lack 2'-O methylation, and recognition of viral RNAs that contain 5'-triphosphates. *Ifit1*<sup>-/-</sup> mice have been well characterized with respect to enhanced susceptibility to mutant viruses (WNV, poxvirus, and coronavirus) lacking 2'-O-methyltransferase activity [54]. *Ifit2*<sup>-/-</sup> mice were recently generated and were shown to be highly susceptible to VSV-mediated neuro-pathogenesis and lethality [58<sup>•</sup>]. When challenged with a neurotropic coronavirus or WNV, *Ifit2*<sup>-/-</sup> mice showed greater signs of clinical disease, accompanied by increased infection in the central nervous system [59<sup>•</sup>,60<sup>•</sup>]. These studies support a role for *Ifit2* in controlling replication and pathogenic outcomes of neurotropic viruses and highlight differential *in vivo* functions for related IFIT family members.

The IFITM family consists of four (human) or six (mouse) proteins, all of which are highly homologous and presumably structurally related. However, they do exhibit preferential antiviral specificities that appear to be linked to their mechanisms of action. In general, IFITMs have been shown to inhibit an early viral entry step, with numerous studies implicating virion fusion [54,61–64]. Their specificity is thought to be dictated in part by the cellular location of virion fusion, for example, late endosomes or lysosomes. IFITM proteins were originally shown to potently inhibit influenza A virus and several flaviviruses [64], and the list of targeted viruses continues to grow [54]. *Ifitm3*<sup>-/-</sup> mice were recently generated and showed fulminant viral pneumonia and a striking mortality phenotype when challenged with an influenza A virus that exhibits otherwise low pathogenicity in wild type mice [65<sup>••</sup>]. Similar results were obtained with mice that lack the entire *Ifitm* locus [66<sup>•</sup>]. *Ifitm3*<sup>-/-</sup> mice have also been shown to be more susceptible to respiratory syncytial virus [67]. It should be noted, again, that these laboratory *Ifitm*<sup>-/-</sup> strains are also functional *Mx1*-null mice. The striking pathogenic phenotypes with influenza A virus must therefore be considered in the context of this double *Ifitm/Mx1*-null genotype. Of the hundreds of human ISGs that have been tested *in vitro* for antiviral activity against influenza A virus, *IFITM3* and *MX1* appear to be two of the most potent [7<sup>•</sup>]. Thus, the *Ifitm/Mx1*-null mouse is likely an extreme example of innate ISG-mediated immunodeficiency, at least in the context of influenza A virus infection. Notably, *IFITM3* is one of the few ISG effectors that have been linked to pathogenic outcomes of viral infection in humans. A genetic survey of patients hospitalized with seasonal influenza showed

enrichment for a minor *IFITM3* allele (SNP rs12252-C) that generates a splice variant of *IFITM3* with reduced antiviral efficacy [65<sup>••</sup>]. This human clinical data strongly support the mouse studies and helps solidify *IFITM3* as an antiviral effector that is critical for controlling pathogenic outcomes of certain viral infections.

### RSAD2/viperin

Viperin (encoded by *Rsad2*) is a radical SAM domain-containing molecule with diverse antiviral activities, most of which appear to tie into lipid biosynthetic or fatty acid metabolic pathways [68,69]. *In vitro*, viperin has been implicated in controlling a number of viruses, including human cytomegalovirus, influenza A virus, Sindbis virus (an alphavirus), and flaviviruses such as WNV and dengue virus. *In vivo*, *Rsad2*<sup>-/-</sup> mice have been challenged with influenza A virus, WNV, and chikungunya virus (an alphavirus). Lethal challenge with influenza A virus showed that viperin knockout and wild type mice were similar with respect to mortality rates, viral titers in lungs, and lung pathology [70]. By contrast, *Rsad2*<sup>-/-</sup> mice were more susceptible to WNV and CHIKV infection when compared to wild type mice. In a footpad model of CHIKV infection, *Rsad2*<sup>-/-</sup> mice did not exhibit enhanced mortality. However, these mice showed higher virus replication in footpad, increased viremia in blood, and more pronounced joint swelling and subcutaneous edema [71]. After subcutaneous or intracranial challenge with WNV, *Rsad2*<sup>-/-</sup> mice had higher rates of mortality and increased viral replication in brain and spinal cord [72]. Thus, with respect to positive-stranded RNA viruses (flavivirus and alphavirus), these *in vivo* studies largely corroborate some *in vitro* findings. The lack of a distinct phenotype in *Rsad2*<sup>-/-</sup> mice after influenza A virus infection stands in contrast to *in vitro* studies. This may be due to numerous reasons, including experimental parameters of the *in vivo* studies, compensatory action of additional ISGs *in vivo*, or cell type specificity of viperin action in mice.

### 25-Hydroxycholesterol and CH25H

The sterol biosynthetic pathway has recently been implicated in antiviral immunity. 25-Hydroxycholesterol (25HC) is a naturally occurring oxysterol synthesized from cholesterol by the enzyme cholesterol 25-hydroxylase (*Ch25h* in mice; *CH25H* in humans). Metabolomic and genetic screens independently uncovered 25HC as a potent antiviral lipid mediator [73,74<sup>•</sup>]. 25HC modulates lipid composition and likely inhibits virus infection early in the life cycle, at either fusion or at a post-entry step. Indeed, several enveloped viruses are susceptible to 25HC-mediated inhibition, but a non-enveloped adenovirus is not. When challenged with murine gammaherpesvirus MHV68, *Ch25h*<sup>-/-</sup> mice were more susceptible to infection [74<sup>•</sup>]. 25HC also had direct antiviral effects against HIV-1 replication in a humanized mouse model of infection. These studies establish a role for 25HC in suppressing

virus replication *in vivo*. However, additional studies in these mice are needed to evaluate the contribution of CH25H and 25HC in controlling viral pathogenesis.

#### Retroviral restriction factors: APOBEC3, SAMHD1, BST2/tetherin

Of the known IFN-inducible retrovirus restriction factors, several have been targeted for gene deletion in mice, including *Apobec3*, *Samhd1*, and *Bst2/tetherin*. Humans have seven *APOBEC3* genes, with *APOBEC3G* being the best characterized with respect to antiviral function. *APOBEC3G* encodes a cytosine deaminase that restricts HIV-1 by deleterious modification of reverse transcribed viral DNA [75]. Since mice only have one *Apobec3* gene, it is presumed that this single gene would confer anti-retroviral activity in mice similar to the human homologs. Indeed, when *Apobec3*<sup>-/-</sup> mice were infected with Moloney murine leukemia virus (MMLV), they showed higher levels of infection in bone marrow and spleen [76]. Moreover loss of one or both copies of *Apobec3* resulted in a more rapid development of T cell leukemia. In a separate study, *Apobec3*<sup>-/-</sup> mice were infected with Friend retrovirus, followed by systemic IFN $\alpha$  treatment [77]. In wild type mice, IFN treatment significantly reduced viral loads in plasma and proviral loads in spleen and bone marrow, but this effect was compromised in *Apobec3*<sup>-/-</sup> mice. Together, these studies suggest that *Apobec3* contributes significantly to IFN-induced antiviral effects against retroviruses in mice.

SAMHD1 is a dTNP hydrolase that reduces the concentration of intracellular dNTP pools, and this activity is implicated in the ability of SAMHD1 to restrict retroviruses in non-cycling cells [78]. Two variants of *Samhd1* knockout mice have been generated to assess the role of this effector in controlling retroviral infection *in vivo* [79,80]. Notably, *Samhd1*<sup>-/-</sup> mice do not exhibit the severe autoimmune disease that is characteristic of human Aicardi-Goutieres syndrome patients with *SAMHD1* mutations. Using different experimental systems and HIV-1-based vectors, both studies show some level of *Samhd1*-mediated control of HIV-1 vector transduction. However, pathogenesis in this relatively new mouse model has not been addressed.

Tetherin (encoded by human *BST2*) is a membrane bound protein that inhibits viral particle release from the cell surface [75]. Originally identified to block HIV-1 release, tetherin has now been shown to have similar activities against other enveloped viruses [81]. *Bst2*<sup>-/-</sup> mice were recently generated, and challenged with several viruses [82]. Systemic infection of tetherin knockout mice with MMLV resulted in a slightly higher, but not statistically significant, viral burden in spleen compared to wild type mice. Both strains also had similar tissue viral burdens after systemic infection with murine cytomegalovirus or VSV. Surprisingly, local intranasal infection of VSV or influenza

B virus resulted in lower viral titers in lungs of knockout mice compared to wild type, but only at early time points post-infection. Another unexpected result indicated that *Bst2*<sup>-/-</sup> mice secreted less IFN than wild type mice in response to viral challenge, suggesting that tetherin may play a role in IFN induction. While additional pathogenesis studies need to be performed in *Bst2*<sup>-/-</sup> mice, this single study highlights a potentially complex phenotype for the antiviral function of tetherin in mice.

#### Perspectives

In recent years, the pace of discovery of ISG-mediated antiviral mechanisms has rapidly increased. As new antiviral ISGs are identified and characterized, their contributions to immune responses *in vivo* will need to be addressed. While the major genes that modulate IFN production or signaling have been targeted for deletion in mice, relatively few downstream ISG effector knockouts exist. Of those ISGs that have been targeted, most have shown observable phenotypes with respect to viral pathogenesis, although the phenotypes are typically more modest when compared to mice lacking major IFN signaling components such as IFNAR1 or STAT1. This is not unexpected given the presumed redundancy in the ISG effector system. Additional insight into ISG mechanisms *in vivo* may be obtained by crossing ISG knockout mice to a *Stat1*<sup>-/-</sup> background, thereby precluding interference from endogenous IFN signaling. Alternatively, ISG transgenics, or 'knock-ins', onto a *Stat1*<sup>-/-</sup> background may help determine which ISGs are sufficient to inhibit viruses *in vivo*. Such an approach would complement current knockout strategies, which mostly assess the necessity of an ISG in host antiviral responses. Moreover, most *in vitro* and *in vivo* ISG studies to date have focused on single genes in isolation, either by ectopic expression or gene silencing/deletion. Moving forward, new approaches to understand the combinatorial nature of ISG interaction networks will be needed. This may be accomplished by proteomic studies in cell culture, or by multiple gene deletions in mice, perhaps via recent CRISPR technologies [83]. Nonetheless, the fact that single ISG deletions have observable pathogenic phenotypes *in vivo* suggests that these genes do provide critical functions in protecting the host from viral disease. They may, therefore, be valid targets for the development of novel antiviral therapeutics.

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