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Interferon-stimulated genes and their antiviral effector functions

John W Schoggins and Charles M Rice

Many viruses trigger the type I interferon (IFN) system, leading to the transcription of hundreds of interferon-stimulated genes (ISGs). The products of these ISGs exert numerous antiviral effector functions, many of which are still not fully described. Recent efforts have been aimed at identifying which ISGs are antiviral and further characterizing their mechanisms of action. IFN effectors vary widely in their magnitude of inhibitory activity and display combinatorial antiviral properties. Collectively, ISGs can target almost any step in a virus life cycle. Some of the most potent antiviral effectors reinforce the system by further inducing IFN or ISGs. Other genes enhance or facilitate viral replication, suggesting that some viruses may have evolved to co-opt IFN effectors for a survival advantage.

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Introduction

The innate immune response provides the first line of defense against viral pathogens. Innate immune activation occurs in an antigen-independent fashion and relies on the ability of the host to recognize pathogens through specific pattern recognition receptors (e.g. Toll-like receptors, RIG-I-like receptors, NOD-like receptors) ([1–3], see other articles in this issue). Engagement of these molecules activates signaling pathways that lead to the production of cytokines, chemokines, and interferons (IFNs), the latter of which bind their cognate receptors, signal through the JAK-STAT pathway, and transcriptionally induce hundreds of interferon-stimulated genes (ISGs) (Figure 1). Type I (IFN α/β) and type III (IL28A, IL28B, IL29) IFNs are often considered the antiviral classes, although type II IFN (IFN γ) also has well-described antiviral properties [4]. Each of these IFNs

induces a unique and partially overlapping set of ISGs [5]. In addition, some ISGs are directly induced by viral infection in the absence of IFN production [6]. This leads to ISG induction through multiple mechanisms that are intersecting and often self-reinforcing.

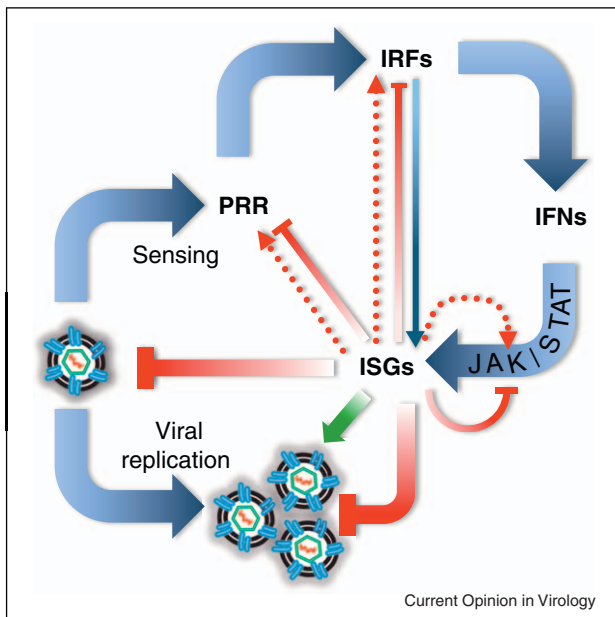
The first ISGs were discovered more than 25 years ago [7,8], and the past decade has seen genome-scale cataloging of these molecules [5]. Depending on cell type, IFN dose, and time of treatment, microarray studies identify 50–1000 ISGs, with 200–500 genes typical of many cell types [9–11]. Insight into the effector functions of ISG-encoded proteins, however, has been limited primarily to a handful of molecules, including the ‘classical ISGs’ PKR (also known as EIF2AK2), MX1, OAS1 and more recently effectors such as APOBEC3G, TRIM5, ZAP, ISG15, ADAR, IFITM1/2/3, tetherin (also known as BST2), and viperin (also known as RSAD2) (please consult www.genenames.org for HUGO Gene Nomenclature Committee (HGNC)-approved designation of ISGs and their encoded proteins). Many of these better-characterized ISGs, particularly the IFN-induced HIV restriction factors, are subjects of recent reviews and will not be discussed in detail [12–14]. Here, we summarize recent efforts to identify new antiviral ISGs and review emerging themes in effector functions and mechanisms.

Identification of antiviral ISGs

Microarray data and knockout studies have suggested that IFN-induced effectors form a diverse and overlapping landscape. Mice defective in one or more of the classical antiviral pathways (MX1, OAS, and PKR) still mount antiviral responses [15], indicating that several factors contribute to protection. This redundancy probably reflects the central importance of ISGs in antiviral defense. A clinically relevant example is seen in patients infected with hepatitis C virus (HCV) who are treated with IFN α -based drug regimens. In those who successfully respond to treatment, ISG levels in the liver are low before treatment and increase significantly after treatment [9]. By contrast, patients who fail to respond to IFN typically have elevated pre-treatment ISG levels, which do not increase dramatically during treatment. Interestingly, higher expression of 36 unique ISGs has been correlated with a reduction in HCV viral load [16]. Until recently, however, direct evidence for the antiviral effects of the majority of ISGs has been lacking.

Several discovery-based screens to identify antiviral ISGs have been performed. Depending on the study, these screens are typically designed to determine ISG effects on virus replication using infectious viruses that can

Figure 1



Diverse roles for ISGs in the IFN antiviral pathway. Incoming viruses are sensed by pattern recognition receptors (PRR), leading to activation of interferon regulatory factors (IRFs) and transcriptional induction of IFNs. Antiviral IFNs signal through the JAK/STAT pathway to induce ISG production. ISGs can also be directly induced by some IRFs in an IFN-independent pathway (thin blue arrow). Some ISGs function to block virus replication (thick red bars), while others have the ability to promote or enhance replication of certain viruses (green arrow). A subset of ISGs are themselves components of the IFN pathway or promote its signaling (red dotted arrows). IFN also induces several negative regulators that can target PRR, IRFs, or JAK/STAT to dampen the response (thin red bars).

undergo a full life cycle (e.g. entry, uncoating, genome replication, particle assembly, egress). Alternatively, replication can be assayed in the absence of virus production with replicons, which are viral genomes that are genetically designed to be competent for replication but unable to produce infectious virus. In two small-scale screens, 29 or 18 ISGs were tested by overexpression for activity against HCV subgenomic replicons. Several ISGs, including, GBP1, IFI6, IFI27, IRF1, IRF9, ISG20, MX1, OAS1, PKR, and viperin, significantly reduced replicon activity [17,18]. A screen of 36 ISGs against dengue and West Nile replicon-containing virus-like particles yielded 5 hits [19]. Viperin, ISG20, and PKR inhibited both replicons, and their enzymatic activities were required for antiviral activity. Viperin, ISG20, IFITM2 and IFITM3 were antiviral against fully infectious DENV, and the IFITM proteins were proposed to target binding, entry, or nucleocapsid uncoating [19]. These results are consistent with the original report of IFITM family members as antiviral factors that target early life cycle steps of several viruses [20^{••}]. In a study with fully infectious Sindbis virus, 7 mouse ISGs (IFIT1, IFIT2,

IFIT3, ISG15, ISG20, viperin, ZAP) were tested for antiviral activity *in vitro* and *in vivo*. The authors confirmed previously reported antiviral activities for ZAP and ISG15 and identified ISG20, IFIT1, and viperin as anti-alphavirus effectors [21].

Building on the need to identify a more complete spectrum of antiviral ISGs, we recently reported a comprehensive overexpression screen in which more than 380 genes were tested for antiviral activity against six viruses: HCV, HIV-1, yellow fever virus (YFV), WNV, Venezuelan equine encephalitis virus (VEEV), and chikungunya virus (CHIKV) [22^{••}]. Statistically significant reductions in virus replication were demonstrated for at least 25 genes, half of which had never been characterized as antiviral (Table 1). Some ISGs had broad activity against many viruses, while others were more restricted in specificity (Figure 2). The collection of genes preferentially inhibiting a given virus, the 'ISG profile', was unique and partially overlapping with the profiles of other viruses, particularly those in the same family. With respect to HCV, there was little overlap in the group of effectors that inhibited fully infectious virus when compared to genes that had previously been identified to target subgenomic replicons [17,18,22^{••}]. These differences suggest that antiviral ISG effects on replicons may not correlate with activity against infectious virus. Interestingly, a small subset of ISGs was shown to enhance replication in a virus-specific manner, highlighting the inherent complexity of the IFN system (Figure 1). This growing body of knowledge on ISG effector functions is beginning to illuminate several interesting features of the global IFN system.

Efficacy of antiviral ISGs

One interesting property of ISG-mediated antiviral activity is the magnitude with which a single IFN effector can inhibit virus replication. Not surprisingly, the first antiviral ISGs discovered were very potent (e.g. MX1, PKR, OAS1), with newer additions to this highly active group reported in the last decade (TRIM5, ZAP, APO-BEC3G, IFITM3). As more effectors are uncovered, however, it is becoming clear that a gradient of antiviral activity exists, with many acting as only modest inhibitors [19,21,22^{••}]. From the host perspective, inducing a diverse range of weak effectors may be preferable to upregulating a group of extremely potent genes, as the latter could result in a toxic cellular environment. The experimental observation of these intermediate phenotypes supports the hypothesis that IFN effectors work in combination to achieve a fully functional antiviral state [23]. Indeed, when combinations of two ISGs are expressed together, the magnitude of antiviral activity is usually greater than either gene alone [22^{••}]. This effect was observed for HCV in hepatoma cells, HIV in MT4 T cells, and for YFV in fibroblasts, suggesting a paradigm that crosses cell types and viral species. Conversely, for Sindbis virus, simultaneous knockdown of

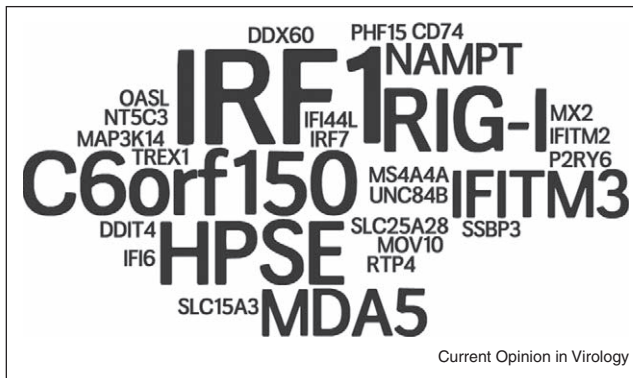
Table 1

Antiviral interferon-stimulated genes

Gene symbol	Targeted viruses	Viral life cycle	Mechanism related to antiviral activity	Ref.
ADAR	HCV(r), HDV [enhances CHIKV, HIV-1, MV, VEEV, VSV, WNV, YFV]	replication	viral RNA editing, suppress PKR	[22**,46]
APOBEC3	HIV-1, other retroviruses	replication	cytidine deamination of viral genome	[14]
BST2 (tetherin)	filovirus, FLUAV, HIV-1, LASV, VSV	egress/budding	block release of nascent virions	[47–49]
C6orf150 (MB21D1)	CHIKV, VEEV, WNV, YFV	translation	unknown	[22**]
CD74	HIV-1	replication	unknown	[22**]
DDIT4	HCV	translation	unknown	[22**]
DDX58 (RIG-I)	numerous RNA and DNA viruses	translation, replication	viral sensing, activation of IRFs	[50,51]
DDX60	HCV, PV, VSV	translation (HCV)	promote RIG-I-like receptor signaling	[22**,42*]
EIF2AK2 (PKR)	numerous RNA and DNA viruses	translation	targets EIF2A	[52]
GBP1, GBP2	EMCV, HCV(r), VSV	replication	unknown	[53]
HPSE	CHIKV, VEEV, WNV, YFV	unknown	unknown	[22**]
IFI44L	HCV	translation	unknown	[22**]
IFI6/G1P3	HCV(r), YFV	unknown	unknown, possibly antiapoptotic	[22**,54]
IFIH1 (MDA5)	numerous RNA and DNA viruses	translation, replication	viral sensing, activation of IRFs	[50,51]
IFIT1/2/3/5	FLUAV, HPV, MHV*, RVFV, SINV, VSV, WNV*	translation, replication	target EIF3 subunits, target HPV helicase, bind 5'-triphosphate RNA	[25,26**,27**,28*]
IFITM1/2/3	DENV, filovirus, FLUAV, HIV-1, SARS-CoV, VSV, WNV, YFV	entry	unknown, possibly target endocytic pathway	[32,48,55]
IRF1	numerous RNA and DNA viruses	similar to IFN	IFN induction, direct ISG induction	[22**,35,56]
IRF7	numerous RNA and DNA viruses	similar to IFN	IFN induction, direct ISG induction	[22**,57,58]
ISG15	FLUAV, HIV-1, HSV-1, JEV, MHV-68, SINV, VΔE3L	various	modulate protein function by ISGylation	[59,60]
ISG20	BVDV, DENV, EMCV, FLUAV, HCV, SINV, VSV, WNV(v), YFV	viral RNA synthesis	exonuclease activity	[19,61,62]
MAP3K14 (NIK)	HCV	translation	unknown, possibly NF-κB activation	[22**]
MOV10	HIV-1, HCV	post-entry (HIV-1)	unknown	[22**,63]
MS4A4A	HCV	translation	unknown	[22**]
MX1 (MxA)	CVB, FLUAV, HCV(r), HPIV3, LACV, MV, SFV, THOV, VSV, others	primary transcription, nucleocapsid shuttling	formation of highly ordered oligomers	[64]
MX2 (MxB)	HIV-1, HNTV, LACV, RVFV, VSV	unknown	unknown	[22**,64]
NAMPT (PBEF1)	VEEV, WNV	unknown	unknown	[22**]
NT5C3	HCV	translation	unknown	[22**]
OAS1/2/3	CHIKV, DENV, EMCV, HCV(r), SFV, SINV, WNV	replication	activate RNaseL to degrade viral genome	[65]
OASL	HCV, HCV(r)	translation	unknown	[22**,66]
P2RY6	CHIKV	unknown	unknown	[22**]
PHF15	WNV	unknown	unknown	[22**]
PML (TRIM19)	numerous RNA and DNA viruses	various	organize multiprotein nuclear bodies	[67]
RSAD2 (viperin)	DENV, DENV(v), FLUAV, HCMV, HCV(r), SINV, WNV(v)	egress (FLUAV)	perturb lipid rafts (FLUAV), promote TLR7/9 signaling	[29,30,31**,42*]
RTP4	YFV	unknown	unknown	[22**]
SLC15A3	CHIKV	unknown	unknown	[22**]
SLC25A28	CHIKV	unknown	unknown	[22**]
SSBP3	HCV	translation	unknown	[22**]
TREX1 (AGS1)	YFV	unknown	unknown	[22**]
TRIM5	HIV-1, other retroviruses	before reverse transcription	target incoming capsids, promote innate signaling	[14]
TRIM25	FLUAV, VSV	similar to IFN	activate RIG-I via ubiquitination	[68]
SUN2 (UNC84B)	HIV-1	unknown	unknown	[22**]
ZC3HAV1 (ZAP)	EBOV, FLUAV, MBGV, NDV, retrovirus, SINV	post-entry, translation	target viral RNA, promote RIG-I signaling	[38–40,41*]

CHIKV, chikungunya virus; CVB, coxsackie B virus; DENV, dengue virus; EBOV, Ebolavirus; EMCV, encephalomyocarditis virus; FLUAV, influenza A virus; HBV, hepatitis B virus; HCV, hepatitis C virus [(r), replicon]; HDV, hepatitis delta virus; HIV, human immunodeficiency virus; HPIV3, human parainfluenza virus type 3; HPV, human papillomavirus; HSV, herpes simplex virus; JEV, Japanese encephalitis virus; LACV, La Crosse virus; LASV, Lassa virus; MBGV, Marburg virus; MHV, mouse hepatitis virus; MHV-68, murine gammaherpesvirus-68; MV, measles; NDV, Newcastle disease virus; PV, poliovirus; RVFV, Rift Valley fever virus; SARS-CoV, SARS coronavirus; SFV, Semliki Forest virus; SINV, Sindbis virus; THOV, Thogoto virus; TMEV, Theiler's encephalomyelitis virus; VEEV, Venezuelan equine encephalitis virus; VSV, vesicular stomatitis virus; VV, vaccinia virus; WNV, West Nile virus [(v), virus-like particles]; YFV, yellow fever virus; *, WNV and MHV lacking 2'-O-methyltransferase activity.

Figure 2



Frequency of antiviral ISG activity against six viruses. Data from large-scale ISG screens presented in ref [22**] were analyzed for the frequency with which a given ISG was shown to have antiviral activity against six viruses (HCV, HIV-1, YFV, WNV, VEEV, CHIKV). A list of genes and the number of viruses each gene targeted was submitted to wordle.net to generate a word cloud of ISGs. The font size is directly proportional to the prevalence of antiviral activity, with IRF1 demonstrating antiviral activity against all six viruses.

ISG20 and ZAP can reverse IFN-mediated inhibition of virus replication better than individual knockdown of ZAP or ISG20 [21].

Gene combination studies have the potential to define pathways of IFN effector functions, much like epistasis analysis in classical genetics. For example, IFI6 and the recently identified antiviral effector C6orf150 both inhibit YFV and, when combined, the effect is stronger [22**]. This result suggests that their mechanisms of action are based on two independent, parallel processes, or that one gene potentiates the other. Similar results were observed for ISGs that enhance YFV replication, namely LY6E and MCOLN2 [22**]. By contrast, HPSE, which encodes the heparan sulfate cleaving enzyme heparanase, has very potent activity against YFV. When combined with the antiviral effector RTP4, the level of replication inhibition is unchanged, suggesting that HPSE functions ‘upstream’ of RTP4 in the cascade of antiviral functions [22**]. Although the antiviral mechanism of heparanase action is unknown, its enzymatic activity suggests that it may inhibit binding of viruses that utilize heparan sulfate for entry. This strong block to the first step in the viral life cycle would mask any detectable inhibition at a later stage. Additional combinatorial studies may be useful for further defining ISG interactions and assigning effector functions to distinct stages in the viral life cycle.

ISG induction levels versus antiviral activity

ISGs are induced to vastly different levels during viral infection or IFN treatment, and expression levels are often dependent on time, dose, and cell type. A rational hypothesis is that the ISGs most highly induced during

infection or IFN treatment are those that control viral replication the most effectively. Indeed, a goal of infected cell microarray analysis is often to identify highly induced ISGs as good candidates for follow-up studies [24]. In principle, this strategy is sound, but accumulating evidence suggests that the magnitude of ISG induction does not always correlate with the strength of antiviral effector function. A key example is the family of IFIT proteins (IFIT1,2,3,5). IFIT family members are among the most highly induced ISGs in response to IFN treatment or viral infection [25]. Given this high level induction, it is surprising that overexpression of IFITs confers only modest or no inhibitory antiviral activity [18,19,21,22**,26**]. Recent insight into the role of IFIT family members during viral infection provides clues to this apparent discrepancy. Using a proteomics approach, Pichlmair *et al.* showed that several IFIT family members form a multiprotein complex in which IFIT1 sequesters viral RNAs containing 5' triphosphates [26**]. Loss of IFIT expression, particularly IFIT1, conferred a growth advantage to viruses that generate 5'-triphosphate RNAs, supporting the authors' model that IFITs form an effector complex to execute IFN antiviral activity. Using a genetics approach, two groups have recently demonstrated that certain viruses use a virally encoded 2'-O-methyltransferase to specifically evade inhibition by IFIT proteins [27**,28*]. For example, a mutant WNV lacking 2'-O-methyltransferase activity (WNV-E218A) was inhibited by murine IFIT1/2, while wild type WNV was not. Moreover, WNV-E218A was avirulent in wild type mice but virulence was completely restored in *Ifit1*^{-/-} knockout mice [27**]. This observation suggests that viral evasion mechanisms may counteract the high levels of IFIT expression during infection. Further studies will be needed to determine if other highly expressed ISGs assemble multiprotein effector complexes and/or serve as targets for viral immune evasion.

Another IFN effector whose expression level is discordant with antiviral activity is viperin. When expressed in cells before infection, viperin is able to inhibit several viruses, including HCMV, Sindbis virus, influenza A virus, and dengue virus [18,19,21,29]. However, viperin was originally identified as an antiviral effector that is highly induced in fibroblasts infected with human cytomegalovirus (HCMV) or treated with IFN [30]. Thus, with respect to HCMV, it seems paradoxical that this virus would induce high levels of an antiviral protein. Seo *et al.* have now reported that the HCMV-encoded vMIA protein co-opts viperin, forcing its relocalization from the endoplasmic reticulum to mitochondria, where viperin interferes with cellular ATP generation [31**]. Modulation of ATP levels results in disruption of the actin cytoskeleton, which ultimately enhances HCMV infection. This remarkable example of ISG hijacking helps to explain the discrepancy between viperin levels and the apparent lack of antiviral activity during normal infection.

Mechanisms of action: virus life cycle and host processes

With hundreds of genes induced by IFN, it is reasonable to assume that any step of the viral life cycle (entry, uncoating, transcription, translation, assembly, egress) could be targeted for inhibition. Interestingly, however, when a panel of eight antiviral ISGs was tested for effects on the HCV life cycle, each gene impaired primary translation of the incoming genome [22^{••}]. The magnitude of translation inhibition correlated with the overall reduction in virus replication at later time points. These results suggest that, at least for HCV, targeting early, post-entry life cycle events is a common mode of ISG action. IFITM proteins have also been found to inhibit early steps in the life cycles of various viruses, possibly by blocking entry or viral particle trafficking [19,20^{••},32,33]. Studies in other systems suggest that virus assembly and egress are also viable ISG targets. For example, BST2 is an ISG encoding tetherin, which is known to prevent release of HIV-1 virions from the cell surface [14]. Recent work has shown that egress of influenza virus is also targeted by an ISG. Viperin interacts with the cellular enzyme farnesyl diphosphate synthase to perturb lipid rafts, resulting in inhibition of influenza virus release [29]. As the mechanisms of more IFN effectors are uncovered, it is likely that their modes of action will collectively span the majority of virus life cycle stages.

An emerging paradigm for ISG-mediated activity in the context of host processes is feedback into antiviral pathways (Figure 1). Major IFN signaling components such as RIG-I-like receptors, IRFs, and STAT1, are known to be induced by IFN [5,10], as are proteins that activate these pathways, such as TRIM25 [34]. Among these, IRF1 is a prominent antiviral effector against numerous viruses. IRF1 was originally characterized nearly 20 years ago in a study demonstrating that it exerts antiviral functions in the absence of IFN [35]. In support of this observation, the expression of IRF1 in human *STAT1*^{-/-} fibroblasts results in the induction of more than 100 genes, the majority of which are ISGs, but not IFN genes [22^{••}]. IRF1 therefore appears to execute an IFN-like antiviral program in the absence of both STAT1 signaling and IFN gene induction. Mechanistically, this IFN-independent IRF1 antiviral phenotype has been linked to signaling from the RIG-I-like receptor adapter protein IPS1 localized specifically on peroxisomes, but not mitochondria [36[•]]. Similar to IRF1, IRF7 is an ISG that can also activate ISG expression in the absence of IFN signaling [37]. Thus, IRF-mediated transcriptional cascades may be intrinsic antiviral mechanisms that allow rapid ISG expression before IFN itself can be produced.

Recent work implicates other ISGs in promoting antiviral signaling. The potent zinc antiviral protein (ZAP),

encoded by ZC3HAV1, is well known for its ability to inhibit retroviruses, filoviruses, and alphaviruses [38–40]. The short isoform of this protein, ZAPS, was recently shown to be selectively induced by 5'-triphosphate-modified RNA and capable of binding RIG-I [41[•]]. The ZAPS/RIG-I interaction leads to IFN induction, which can be abrogated by siRNAs targeting ZAPS. Similar to ZAPS, DDX60, which has modest antiviral activity [22^{••}], binds RIG-I and MDA5 [42[•]]. Knockdown of DDX60 interferes with IFN and ISG expression in virus-infected cells. Viperin was also recently shown to promote IFN induction [43[•]]. In contrast to ZAPS or DDX60, which abrogate RIG-I-like receptor signaling, viperin interacts with the Toll-like receptor 7 (TLR7) and TLR9 signal mediators IRAK1 and TRAF6. TLR7-dependent and TLR9-dependent IFN production is impaired in plasmacytoid dendritic cells from mice lacking viperin (*Rsad2*^{-/-} mice). Finally, PKR was recently proposed to contribute to antiviral immunity by regulating IFN mRNA integrity [44]. Together, IRF1, ZAPS, DDX60, viperin, and PKR join a growing class of downstream IFN effectors whose antiviral activities, partly, serve to reinforce upstream signaling pathways. A recently recognized small class of IFN-induced negative regulators probably also contribute to the overall picture, providing an inducible mechanism to ward off excessive inflammation and tissue damage [45] (Figure 1).

Conclusions

With genome-wide microarray analyses, the majority of ISG messenger RNAs have now probably been identified, with the possible exception of genes in unique cell types whose IFN profiles have not yet been determined. Next-generation sequencing platforms hold high promise for uncovering potential IFN-induced microRNAs and non-coding RNAs, both of which may significantly impact the antiviral efficacy of IFN. Recent functional data are rapidly accelerating the identification of antiviral ISGs and the characterization of their mechanisms of action with respect to the viral life cycle and host cell processes. Collectively, these studies are revealing novel concepts and emerging themes that are helping to accurately define the 'antiviral state.' Ongoing work will provide a rich platform to understand how a pleiotropic molecule such as IFN orchestrates its antiviral program. Furthermore, taking advantage of these naturally occurring viral inhibitors may be an effective strategy in the development of novel drugs to treat human viral diseases.

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