Review

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Interferon-stimulated genes and their role in controlling hepatitis C virus

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Summary

Infections with the hepatitis C virus (HCV) are a major cause of chronic liver disease. While the acute phase of infection is mostly asymptomatic, this virus has the high propensity to establish persistence and in the course of one to several decades liver disease can develop. HCV is a paradigm for the complex interplay between the interferon (IFN) system and viral countermeasures. The virus induces an IFN response within the infected cell and is rather sensitive against the antiviral state triggered by IFNs, yet in most cases HCV persists. Numerous IFN-stimulated genes (ISGs) have been reported to suppress HCV replication, but in only a few cases we begin to understand the molecular mechanisms underlying antiviral activity. It is becoming increasingly clear that blockage of viral replication is mediated by the concerted action of multiple ISGs that target different steps of the HCV replication cycle. This review briefly summarizes the activation of the IFN system by HCV and then focuses on ISGs targeting the HCV replication cycle and their possible mode of action.

Introduction

The hepatitis C virus (HCV) is a member of the *Flaviviridae* family where it forms the genus *hepacivirus*. These members have in common a single stranded RNA genome of positive polarity and an enveloped virus particle. Seven HCV genotypes (1–7), in most cases with numerous subtypes (a, b etc.), are distinguished, differing in their nucleotide sequence by ~33% and 25%, respectively [1]. HCV infects only humans and chimpanzees. This narrow host range is due to species-specific host cell factors promoting or restricting HCV replication [2].

Around 2% of the world population is chronically infected with HCV [3] and these people are at high risk to develop liver diseases. Treatment of hepatitis C to the most part depends on pegylated interferon-alpha (pegIFN-α) and ribavirin. A successful therapy is determined by multiple parameters such as age and sex of the patient, duration and degree of liver damage, co-infection with other viruses (e.g., HIV) and alcohol consumption. However, the most important predictors of treatment outcome are distinct genetic polymorphisms in the interleukin (*IL)28B* gene locus [4,5] and the genotype of the infecting virus. More than 80% of the individuals infected with genotype 2 or 3 viruses, but only ~45% of genotype 1-infected individuals, achieve sustained viral response with this treatment regimen. In the latter case, this number has increased to ~75% with the recent implementation of a triple combination therapy, composed of pegIFN-α, ribavirin, and a direct-acting antiviral (DAAs) targeting the HCV protease that resides in non-structural protein 3 (NS3) [6,7]. However, this therapy has serious side effects, is very costly and still depends on the individual patient response to pegIFN-α and ribavirin as deduced from the fact that in treatment-experienced patients, response rates are higher among previous relapsers as compared to patients with a previous breakthrough or non-response [8].

The possibility to propagate HCV in cultured hepatoma cells and the recent availability of cell systems based on immortalized and primary human hepatocytes (PHHs) provided important insights into induction of innate antiviral defense by HCV and viral countermeasures [9]. Moreover, complementing studies with human liver samples have become available [10–12]. The common denominator is that HCV induces an innate antiviral response that suppresses virus replication. However, the mechanisms by which HCV replication is suppressed are poorly understood. In this review, we will briefly summarize how HCV is sensed in the infected cell and how this leads to the activation of the IFN system. Further, we will focus on ISGs and their role in controlling HCV replication, emphasizing those ISGs for which we begin to understand the molecular mechanisms responsible for replication suppression. We will not cover the clinical implications of the IFN response with respect to antiviral therapy or pathogenesis of liver disease as these topics have been covered in excellent recent reviews [5,13,14].

Detection of invading hepatitis C virus

Hepatitis C virus detection by RIG-I-like receptors

The innate immune system comprises several pathogen recognition receptors (PRRs) (Fig. 1). In case of viral infections, major key

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players are the Toll-like receptor (TLR) family and the RIG-I-like receptors (RLRs). Three proteins belong to the latter: retinoic acid inducible gene-I (RIG-I), melanoma differentiation associated gene 5 (MDA5, IFIH1) and laboratory of genetics and physiology 2 (LGP2, DHX58). Upon stimulation, RIG-I as well as MDA5 activate mitochondrial antiviral-signaling protein (MAVS, IPS-1, Cardif, VISA) located at mitochondria, peroxisomes and mitochondria-associated membranes (MAMs) [15,16] (Fig. 1). Activation of MAVS leads to phosphorylation of IFN regulatory factor 3 (IRF3) and IRF7. They are translocated into the nucleus to stimulate transcription of IFN-β and several ISGs.

RIG-I preferentially senses short dsRNA molecules with 5′-triphosphorylated RNA [17–20] whereas MDA5 appears to sense long dsRNA independent of a 5′-triphosphorylated end [17]. In agreement with this specificity, RIG-I seems to be the key sensor of HCV RNA, with the poly(U/UC) tract in the 3′ non-translated region of the HCV genome playing an important role [21,22]. In fact, mouse embryonic fibroblasts (MEFs) from *RIG-I*$^{-/-}$ mice stimulated with HCV RNA do not produce IFN [21] and analogous results were obtained with HuH7-derived human hepatoma cell lines as well as PHHs with attenuated RIG-I expression [23,24].

Whether MDA5 can detect invading HCV remains to be clarified. One study reported suppression of HCV *in vitro* upon overexpression of MDA5 [25]. Moreover, HCV replication is enhanced in cells expressing the V protein of paramyxovirus, which is a known inhibitor of MDA5 [26,27]. However, inhibition of STAT1 (signal transducer and activator of transcription1), rather than MDA5, by the V protein might account for increased HCV replication [28,29]. While other members of the *Flaviviridae* family are detected by both RIG-I and MDA5 [30], the observation that *MDA5*$^{-/-}$ MEFs still produce IFN-β upon stimulation with HCV RNA [21] suggests that RIG-I is the key player in detecting HCV.

Hepatitis C virus detection by Toll-like receptors

TLRs are membrane-bound receptors that detect invading pathogens either on the plasma membrane or in endosomes (Fig. 1). In case of HCV, TLR3 and TLR7 that recognize dsRNA and ssRNA,

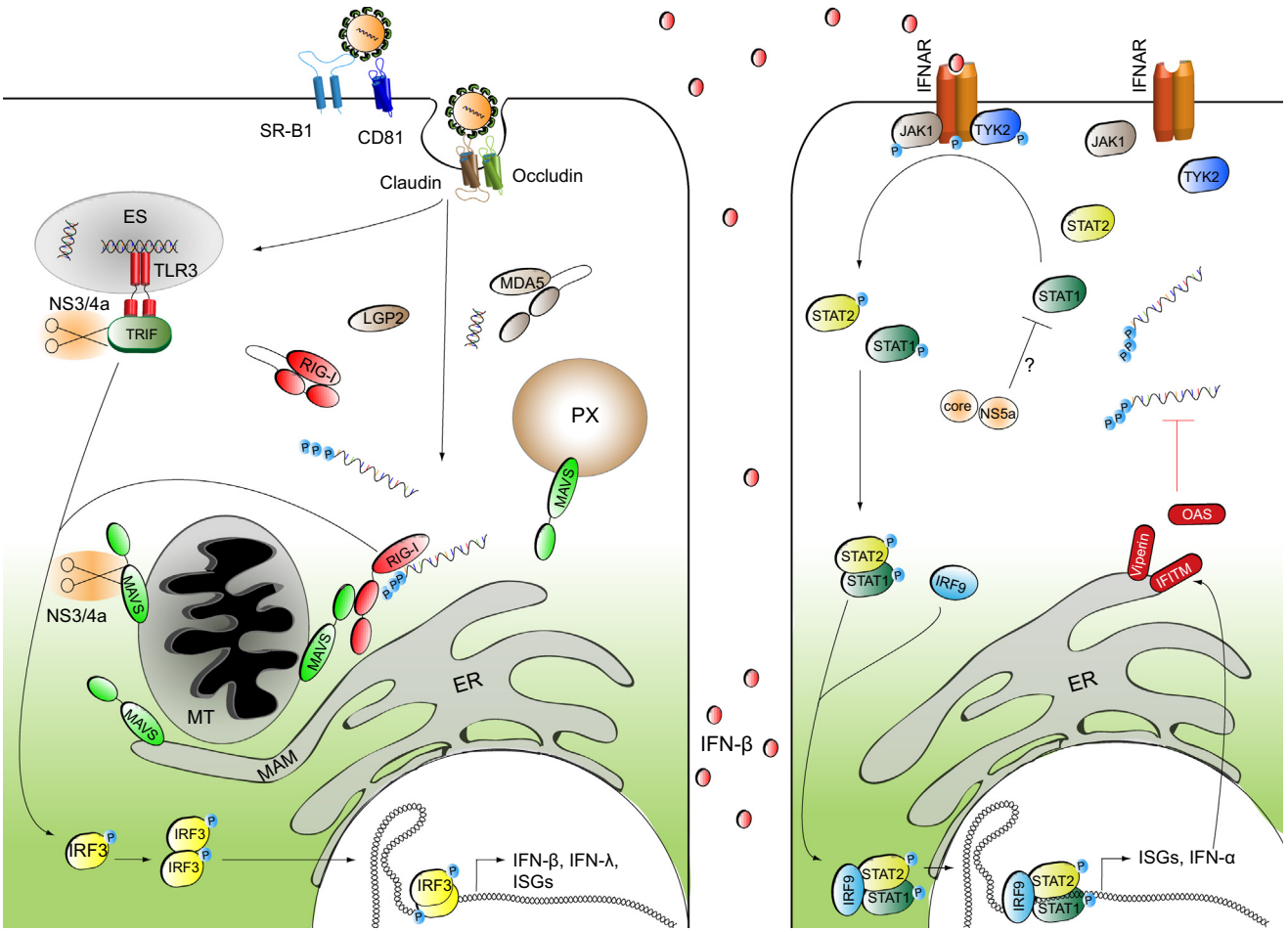

Fig. 1. IFN signaling pathways. Simplified schematic representation of major sensor pathways leading to the expression of antiviral effector genes upon virus infection. Viral RNA is detected by PRRs, such as TLR3 residing in the endosome (ES), or by the cytoplasmic sensors RIG-I or MDA5. Activation of these PRRs leads to the phosphorylation and dimerization of IRF3 that translocates into the nucleus to stimulate transcription of IFN genes as well as genes encoding ISGs and proinflammatory cytokines. Secreted IFNs bind to their cognate receptor, thus activating the JAK/STAT pathway, which leads to the formation of Stat1/2/IRF9 heterotrimers that translocate into the nucleus to induce expression of further ISGs and IFN genes. A subset of these ISGs suppresses HCV replication. Several HCV proteins counteract signaling molecules, most notably MAVS and TRIF that are cleaved by the NS3/4A protease. Additionally, core and NS5A may interfere with the JAK/STAT signaling pathway. ER, endoplasmatic reticulum; MAM, mitochondrion-associated membrane.

respectively, appear to be the main sensors. They both reside in endosomes and signal either via TRIF (TIR-domain-containing adapter-inducing interferon-β) or MyD88 to activate IRF3/7 and NFκB (Fig. 1). This finally leads to the production of proinflammatory cytokines and chemokines (Fig. 1). Several lines of evidence suggest that HCV is sensed by these TLRs. First, reconstitution of TLR3 expression in HuH7 human hepatoma cells that normally do not express TLR3 induces an antiviral state upon HCV infection [31,32], which is likely enhanced by the TLR3-dependent transcriptional upregulation of RIG-I (reviewed in [33,34]). Second, a recent study reported dsRNA replication intermediates inducing TLR3 signaling [32]. Third, by using the macrophage cell line THP1, it was found that HCV RNA is sensed by TLR7 leading to the expression of interleukin-1β (IL-1β), which is proposed to be a central factor of liver inflammation [35]. Forth, it was shown that HCV-infected cells activate IFN production by plasmacytoid dendritic cells (pDCs) via TLR7 [36]. In summary, HCV is recognized both by TLR3 and by TLR7, at least in cell cultures.

Interferons and their role in controlling hepatitis C virus

Activation of the interferon response by hepatitis C virus

Based on receptor usage, IFNs are divided into three groups. Type I IFNs comprise a multitude of cytokines with IFN-α and IFN-β being the most important ones with respect to viral infections. Type III IFNs comprise IFN-λ1, IFN-λ2, and IFN-λ3 in humans (also called IL-29, IL-28A and IL-28B, respectively). Moreover, a recent study reported an additional open reading frame, which encodes for IFN-λ4 [4,37]. Type I and type III IFNs signal via the JAK-STAT pathway (Fig. 1). However, they bind to different receptors that in case of type III IFN is expressed mainly in epithelial cells and hepatocytes (reviewed in [37]) whereas the type I IFN receptor is expressed ubiquitously. Type II IFN comprises only IFN-γ, which is mainly produced by T cells and NK cells and acts on multiple cell types including immune cells.

Varying experimental systems such as cell culture models [38–44], transgenic mice with human liver xenografts [45,46], experimentally inoculated chimpanzees [11,47–52] and human liver samples from HCV-infected patients [10–12,53] have been used to characterize the IFN response induced by HCV. The common denominator is that HCV induces an upregulation of ISGs, but the source of IFNs in the infected liver remains to be elucidated. One likely source might be infected hepatocytes themselves, as inferred from the IFN production by PHHs infected with HCV *in vitro* [11,54]. In addition, IFNs and other proinflammatory cytokines can be produced by resident or infiltrating immune cells such as macrophages, Kupffer cells or DCs that can be stimulated, e.g., by HCV RNA-containing exosomes released from infected cells [55,56].

The pattern of ISGs detected in patients with chronic hepatitis C clearly corresponds to a type I or III IFN signature [10,48,57]. Studies based on PHHs infected with cell culture-produced HCV, or liver biopsies from infected patients and chimpanzees show that the ISG expression pattern predominantly reflects a type I and/or type III IFN response [48,51]. Moreover, several groups reported upregulation of type III IFN in PHHs and in liver samples from patients as well as experimentally infected chimpanzees [11,38,49]. However, whether type I or type III IFN is the main driver of HCV-induced ISG expression remains to be determined.

Several single nucleotide polymorphisms (SNPs) have been found in the *IL28B* gene locus, which seem to influence the response to HCV [58–60]. The best studied SNPs do not reside in the coding region of the *IL28A* and *IL28B* genes, but rather upstream (rs12979860, rs8099917) or downstream (rs12980275) of *IL28B* (reviewed in [5]). Of note, the major genotype of SNP rs12979860 (called C/C because of a homozygous cytosine at this position) correlates with better treatment response and viral clearance [61–63]. However, the underlying mechanism is unknown and appears to be independent of a global upregulation of ISGs [64]. Moreover, non-responders have elevated ISG levels irrespective of the SNP genotype [65] arguing that IFN-λ3, encoded by the *IL28B* gene, does not directly control ISG induction.

More recently, a fourth SNP (ss469415590 (TT or ΔG)) was found upstream of *IL28B* [4]. Interestingly, the ΔG variant is a frameshift mutation creating a novel open reading frame, which encodes for a protein that shares 40.8% amino acid sequence similarity to IFN-λ3 and hence has been designated IFN-λ4 [4,37]. Overexpression of an engineered IFN-λ4 fusion protein in a hepatoma cell line induced phosphorylation of STAT1 and STAT2, expression of several ISGs, and suppression of HCV replication. However, in spite of this antiviral activity, the presence of the ΔG variant is in high disequilibrium with rs12979860 and correlates with reduced response to IFN-α treatment as well as low HCV clearance. Since overexpression of the engineered IFN-λ4 protein was found to weakly pre-activate the IFN signaling pathway, which prevented further activation by IFN-α or -λ, it is tempting to speculate that this refractoriness impairs HCV clearance. Alternatively, methylation of a cysteine residue adjacent to the ΔG variant might be important for HCV clearance [66].

Interferon-stimulated genes and their impact on controlling hepatitis C virus

More than 300 ISGs can be upregulated by IFNs. For obvious reasons, most transcriptome profiles have been established by using cell culture systems and only a limited number of profiles are available from *in vivo* studies. In line with the induction of ISGs by HCV infection itself enhanced transcription of ISGs in acutely and chronically HCV-infected chimpanzees as well as chronically infected patients has been observed. Importantly, the subsets of upregulated ISGs detected *in vivo* overlap to a large degree with those detected in IFN-treated cell cultures in *in vitro* [10,11,47,48,51,67,68]. Of note, the spectrum of ISGs induced in cultured cells in the absence or presence of HCV does not differ, supporting the notion that HCV has little effect on the signaling cascade leading to ISG activation, at least *in vitro* (M. Binder and R.B., unpublished).

In the absence of immuno-competent small animal models that are fully permissive for HCV, no mechanistic *in vivo* evaluation of specific ISGs is possible. Thus, our knowledge about the mechanisms by which ISGs control HCV replication is based on *in vitro* models. Below, we will first summarize screens conducted to identify ISGs that are involved in the control of HCV and then focus on those ISGs for which insights into the underlying mechanism are available.

Identification of ISGs targeting hepatitis C virus

To identify genes responsible for the suppression of HCV replication in IFN-treated cells, several biased and unbiased screens have been conducted in hepatoma cells. Identified ISGs restrict-

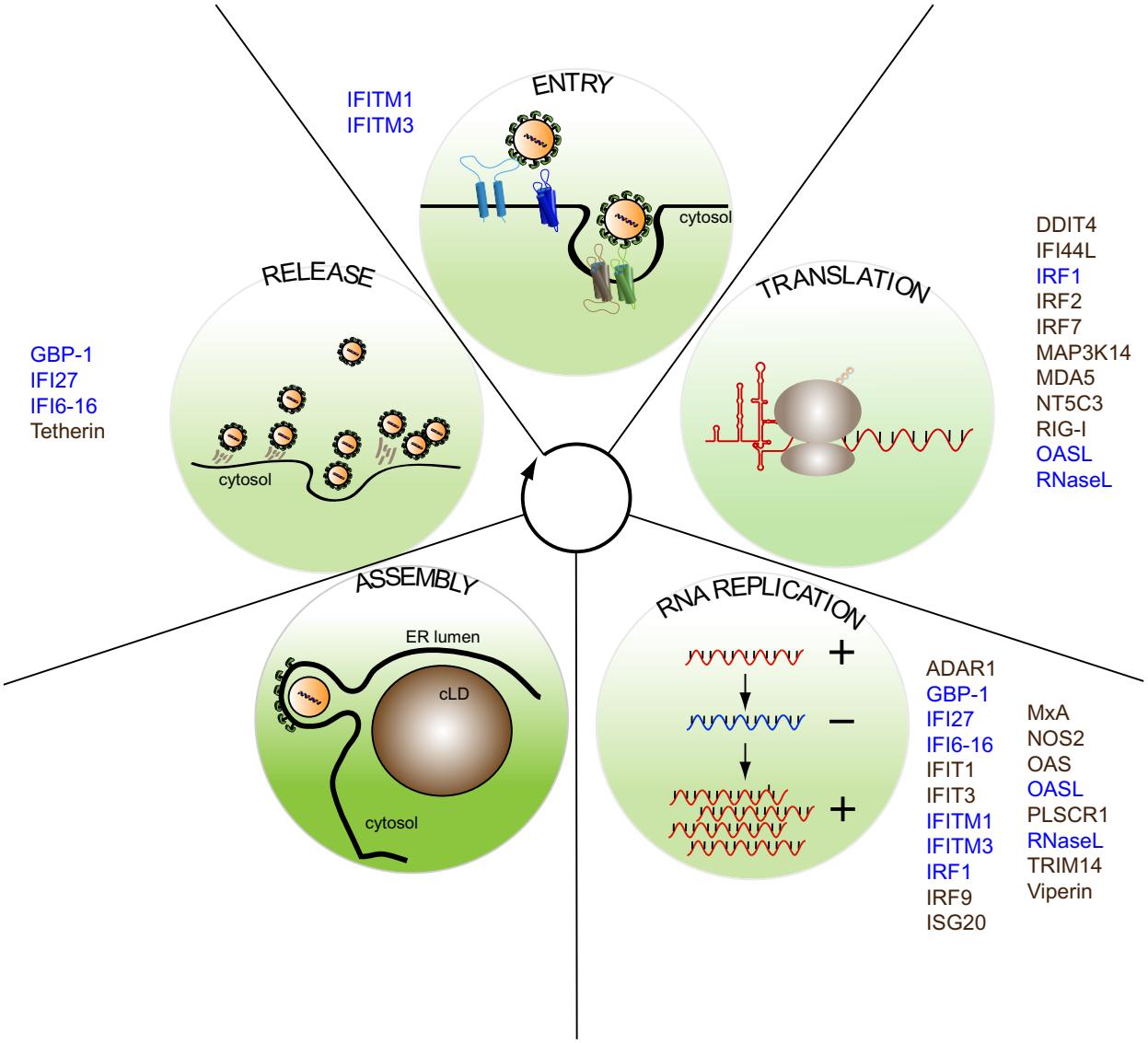

Fig. 2. Phases of the HCV replication cycle and ISGs targeting these phases. Individual steps of the HCV replication cycle are given in the filled circles. These are virus entry, RNA translation, replication of the plus-strand (+) RNA genome via a minus-strand copy (−), assembly of infectious HCV particles that seems to require cytosolic lipid droplets (cLDs), and release of infectious HCV particles. ISGs targeting these individual steps are given in the respective sector. ISGs written in dark brown letters target only one step; ISGs marked in blue target multiple steps.

ing or promoting HCV replication are summarized in Fig. 2 as well as Tables 1 and 2, respectively, that also specify the used ISG abbreviations.

A FACS-based phenotypic screen was used to determine the antiviral activity of 389 ISGs upon overexpression in HuH7 and HuH7.5 cells [25]. Lentivirus-based transduction was used to introduce each ISG individually and transduced cells were infected with an HCV reporter virus. A reduction of HCV-specific signal in ISG-expressing cells indicated inhibition of viral replication. In this way, strongest antiviral effects were observed for IRF1, RIG-I, MDA5, IRF2, and IRF7. While this result underscores the important role of these RNA-sensing and key signaling molecules in mounting an antiviral response against HCV, additional ISGs were detected suppressing HCV replication to a much lesser extent. These ISGs included DDIT4, NT5C3, IFI44L, MAP3K14, and OASL (Table 1). It is likely that these factors exert a more direct effect against HCV, e.g., by enhancing turnover of RNA or slowing down RNA translation. The fact that none of these ISGs was sufficient to suppress HCV replication to an extent observed by IFN treatment suggests that virus inhibition is brought about by the concerted action of several ISGs.

In contrast to this overexpression approach, a whole-genome RNA interference-based screen was performed by Zhao and co-workers [69]. The authors used HuH7 cells containing a stably replicating subgenomic HCV replicon and determined rescue of viral replication by knockdown of a given gene with cells that had been treated with IFN-α. With this approach, 93 genes were identified contributing to suppression of HCV replication. Identi-

Table 1. Interferon-stimulated genes described to restrict the HCV replication cycle.

ISG	Abbreviation	Cell system[1]	Infection or replicon-based test[2]	Test by over expression or knock-down of ISG[3]	[Ref.]
Adenosine deaminase, RNA-specific	*ADAR1*	HuH.BB7	rep	kd	[123]
DNA-damage-inducible transcript 4	*DDIT4*	HuH7	inf/rep	oe	[25]
Guanylate binding protein 1	*GBP-1*	HuH7	rep	oe/kd	[44]
IFN alpha-inducible protein 27	*IFI27*	HuH7	rep	oe/kd	[44]
IFN induced protein 44-like	*IFI44L*	HuH7	inf/rep	oe	[25]
IFN alpha-inducible protein-16	*IFI6-16*	HuH7	rep	oe/kd	[44]
IFN induced protein with tetratricopeptide repeats 1	*IFIT1*	IHH	inf	oe/kd	[41]
IFN induced protein with tetratricopeptide repeats 3	*IFIT3*	HuH7-Lunet	rep	kd	[54]
IFN induced transmembrane protein 1	*IFITM1*	HuH7-Lunet	rep	kd	[54]
		IHH	inf	oe/kd	[41]
		HuH7	rep	oe/kd	[94]
IFN induced transmembrane protein 3	*IFITM3*	HuH7-Lunet	rep	oe/kd	[54]
		HuH7.5	rep	oe	[93]
		HuH7	rep	oe/kd	[94]
IFN regulatory factor 1	*IRF1*	HuH7	inf/rep	oe	[25]
IFN regulatory factor 1	*IRF1*	HuH7	rep	oe	[44]
IFN regulatory factor 2	*IRF2*	HuH7	inf/rep	oe	[25]
IFN regulatory factor 7	*IRF7*	HuH7	inf/rep	oe	[25]
IFN regulatory factor 9	*IRF9*	HuH7	rep	oe	[44]
IFN stimulated gene 15	*ISG15*	HuH7	rep	oe	[122]
IFN stimulated gene 20	*ISG20*	HEK293	rep	oe	[101]
Mitogen-activated protein kinase kinase kinase 14	*MAP3K14*	HuH7	inf/rep	oe	[25]
Melanoma differentiation associated protein-5	*MDA5*	HuH7	inf/rep	oe	[25]
Myxovirus resistance protein A	*MxA*	HuH7	rep	oe	[44]
Nitric oxide synthase 2	*NOS2*	HuH7-Lunet	rep	oe/kd	[54]
5'-Nucleotidase, cytosolic III	*NT5C3*	HuH7	inf/rep	oe	[25]
2'-5'-Oligoadenylate synthetase	*OAS*	HuH7	rep	oe	[44]
2'-5'-Oligoadenylate synthetase 1	*OAS1*	HuH7	genome transf.	oe	[113]
2'-5'-Oligoadenylate synthetase 3	*OAS3*	HuH7	genome transf.	oe	[113]
2'-5'-Oligoadenylate synthetase-like	*OASL*	HuH7	inf	oe	[25]
		HuH7	rep	oe	[110]
Protein kinase R	*PKR*	HuH7	rep	oe/kd	[44]
		HEK293	rep	oe	[101]
		HuH7	rep	kd	[124]
		HuH7	rep	oe/kd	[94]
Phospholipid scramblase 1	*PLSCR1*	HuH7-Lunet	rep	oe/kd	[54]
Retinoic acid inducible gene-I	*RIG-I*	HuH7	inf/rep	oe	[25]
Ribonuclease L	*RNASEL*	HuH7-Lunet	rep	oe/kd	[54]
		HuH7	inf	oe	[113]
Tetherin		HuH7.5	inf	oe	[125]
Tripartite motif containing 14	*TRIM14*	HuH7-Lunet	rep	oe/kd	[54]
Virus inhibitory protein, ER-Associated	*Viperin*	HEK293	rep	oe	[101]
		HuH7	rep	oe	[68]
		HuH7.5	inf/rep	oe	[104]
		HuH7	inf	kd	[105]

[1] Cell line used for the study.
[2] 2ISGs were validated by using either infection of cells with HCV (inf) or subgenomic replicons (rep) or transfection of genomic *in vitro* transcripts (genome transf.).
[3] Antiviral effects were determined by using overexpression of a given ISG (oe) or by knockdown (kd) of the respective ISG in cells treated with IFN and measuring the impact on HCV replication.

fied hits were enriched for genes involved in IFN signaling, RNA translation, and mRNA processing [69]. One of these genes, *SART1*, was characterized in more detail. This factor was not induced by IFN and reported to play a more general role in regulation of ISG expression, which would explain its antiviral effect [69].

By using an analogous 'gain-of-function' RNA interference-based screen, Metz and co-workers identified 7 ISGs that rescue HCV replication in IFN-α or IFN-γ treated cells [54]. All of these genes are induced by either IFN, showing a substantial overlap of the ISG spectrum triggered by these cytokines. Nevertheless, some

Table 2. Interferon-stimulated genes described to promote the HCV replication cycle.

ISG	Abbreviation	Cell system[1]	Infection or replicon-based test[2]	Test by over expression or knock down of ISG[3]	[Ref.]
IFN stimulated gene 15	*ISG15*	MH1	rep	oe/kd	[118]
		HuH7.5	inf	oe	[119]
		HuH7.25.CD81	inf	kd	[84]
Protein kinase R	*PKR*	HuH7.25.CD81	inf	kd	[84]
		HuH7	inf	kd	[83]
Ubiquitin specific peptidase 18	*USP-18*	HuH7.5	inf	kd	[121]

[1] Cell line used for the study.
[2] ISGs were validated by using either infection of cells with HCV (inf) or subgenomic replicons (rep).
[3] Antiviral effects were determined by using overexpression of a given ISG (oe) or by knockdown (kd) of the respective ISG in cells treated with IFN and measuring the impact on HCV replication.

differences exist. For instance, phospholipid scramblase 1 and inducible nitric oxide synthase 2 were identified as main effectors of IFN-γ. Similar to the results by Schoggins and co-workers [25], it was found that the antiviral state blocking HCV replication requires the combined activity of multiple ISGs [54].

Very recently, an unbiased genome-wide siRNA screen was performed to identify ISGs as well as non-transcriptionally induced genes required for the antiviral effect of IFN-α [70]. HuH7-derived cells were transfected with siRNAs and after stimulation with IFN-α infected with HCV. By using an image-based analysis, 9 genes were identified to be responsible for IFN-α-mediated suppression of HCV replication (Table 1). Furthermore, the individual contribution of each of these candidates to inhibit distinct steps in the HCV replication cycle was determined. Thereby, effector groups were identified targeting viral entry, replication, RNA production or virus particle production/release. Three of the candidates, MYST1, ALG10B, and PDIP1, were found to target nearly all steps of the HCV life cycle. These findings underscore that both ISGs and non-ISGs are required for efficient suppression of HCV.

In addition to the high-content screens described above, several more targeted screens have been conducted. Although a direct comparison of obtained results is flawed by differences of used experimental approaches, several ISGs have been identified consistently. These include members of the IFITM family, PKR, and viperin (Table 1).

Key Points

- Upon HCV infection of liver cells, the virus is recognized by cellular sensors leading to induction of type I and III IFN. This induction is counteracted by the NS3-4A protease. Whether other viral proteins also impair IFN induction is contradictory
- Type I and III IFN production results in the upregulation of a broad range of ISGs. Several of them suppress HCV replication in a concerted action
- By and large, HCV is sensitive to the IFN-induced antiviral state. This is exploited by currently used IFN-based antiviral therapy
- It remains to be determined how HCV can persist in presence of an IFN-induced antiviral state

Possible anti-hepatitis C virus mechanisms of selected ISGs

Protein kinase R

PKR is a central component that links pathogen sensing to stress [71]. The kinase is a cytosolic sensor of viral dsRNA [72] and PKR activation results in a global suppression of RNA translation and thus, protein synthesis. Binding to dsRNA promotes PKR homodimerization and activates the kinase domain that phosphorylates the alpha subunit of eukaryotic initiation factor 2 (eIF2α). This factor is essential for initiation of cap-dependent mRNA translation and its phosphorylation blocks this process [73]. In addition, translation arrest leads to the formation of complexes that are composed of non-translated mRNAs and RNA-binding proteins in so-called stress granules [74,75]. Apart from that, PKR links pathogen sensing to metabolic homeostasis, virus-induced autophagy [76], NF-κB signaling [77], and activation of the inflammasome [78].

In vitro binding studies suggest that after HCV infection, PKR is activated as a result of binding to distinct RNA structures in the IRES residing in the 5′ NTR of the genome [79]. However, the benefits and/or disadvantages of this activation for HCV replication are controversial. PKR was initially recognized as an effector ISG that is able to inhibit HCV replication [43,80]. Two viral proteins, NS5A and E2, were reported as PKR inhibitors by direct binding to the kinase. Comparison of genotype 1b full-length virus sequences of IFN-α-responsive and non-responsive patients suggested that HCV variants, unable to escape IFN antiviral effects, accumulated mutations in the C-terminal region of NS5A. This region was designated the IFN sensitivity determining region (ISDR) [81]. Cell culture assays and *in vitro* studies suggest that the ISDR overlaps with the PKR binding region in NS5A, arguing that ISDR mutations might cause a loss of PKR binding and thereby reduce susceptibility to IFN therapy [82]. This is an attractive hypothesis, however, recent studies propose that PKR acts as an HCV proviral rather than antiviral factor [54,83,84]. It has been suggested that by activation of PKR, translation of ISG mRNAs is attenuated without affecting translation of the HCV genome that is mediated by the IRES and thus, eIF2α independent [83,85]. While these results appear to be conflicting, it is possible that activation and inhibition of PKR occur in a sequential manner, with activation predominating at the early stage of infection and inhibition of PKR at a later stage when high amounts of NS5A have accumulated. However, how the reported enhancement of

PKR autophosphorylation induced by interaction with the HCV core protein fits into this scenario remains to be determined [86].

NS5A is not the only viral protein assumed to interfere with PKR. One study reported an inhibition by the viral envelope glycoprotein E2 via direct interaction with PKR [87]. Interestingly, the interaction site in E2 was mapped to a highly conserved amino acid sequence with high sequence homology to PKR autophosphorylation and eIF2α phosphorylation sites. In addition, the extent of sequence homology correlated with the IFN-resistance phenotypes of different HCV genotypes [87]. Although this is an elegant way to explain how HCV might block PKR, the physiological relevance is not clear. In the study by Taylor and colleagues, only E2 was used for expression-based interaction assays, even though proper folding and membrane association of E2 require co-expression with E1 [88]. Thus, these observations need to be revisited by using more authentic viral proteins and adequate cell culture systems.

To conclude, studies on inhibition or activation of PKR by HCV are conflicting. This controversy is further emphasized by the discrepant reports related to the effects that PKR silencing, inhibition or overexpression have on HCV replication that range from inhibitory [84] to non-existent [25,54]. One obvious explanation is the differences of used experimental conditions that are very critical when analyzing rather subtle effects. One other source of discrepancy is the multiple roles that PKR has in controlling cell homeostasis, making experimental outcomes very dependent on cell status. Thus, the role PKR plays in IFN-mediated suppression of HCV replication remains to be determined. We note that in our hands neither silencing of PKR expression in IFN-α-treated cells, nor PKR overexpression has an effect on HCV replication in the commonly used cell line HuH7 [54].

Interferon-inducible transmembrane proteins

The IFITM family consists of 4 members: IFITM1, IFITM2, IFITM3, and IFITM5. With the exception of the latter, all members are ubiquitously expressed in humans and their expression is upregulated by all types of IFNs. IFITMs contain two anti-parallel transmembrane domains, a leucin-zipper motif, and a short cytoplasmic domain. A membrane-proximal cysteine residue can be modified post-translationally by S-palmitoylation controlling IFITM localisation in membrane compartments [89]. IFITM proteins appear to counteract a wide range of viruses, but the mode of action remains unclear [41,90–93]. In case of HCV, IFITM1, and IFITM3 have been reported to interfere with viral entry and RNA replication, respectively [41,54,93,94]. IFITM1 is able to bind to two of the HCV co-receptors: the tetraspanin CD81 and the tight junction protein occludin [94,95]. These co-receptors have to interact with each other to enable virus particle entry into hepatocytes. It is speculated that IFITM1, and to a minor extent IFITM3, disrupt co-receptor interaction, thus inhibiting the entry process [94]. However, two alternative hypotheses have been put forward. First, IFITMs might inhibit the fusion of the viral envelope membrane with endosomes or lysosomes by altering lipid components or blocking acidification of virus-containing endocytic vesicles. Second, IFITMs might alter vesicle trafficking in a way that the invading viruses are redirected to a non-fusogenic pathway [92,96]. Apart from viral entry inhibition, IFITM proteins also suppress HCV replication as deduced from knockdown as well as overexpression studies [41,54,93]. Interestingly, the combined knockdown of IFITM1 and IFITM3 has synergistic effects arguing for a redundant antiviral mode-of-action [54].

The mechanism of replication inhibition by IFITMs remains to be determined. One study describes an interference of IFITM3 with HCV IRES-mediated RNA translation, however, this hypothesis needs further proof as in this study also inhibition of cap-dependent RNA translation was observed and a rather artificial *in vitro* system based on extracts from HeLa cells has been used [93]. Given the 'membrane activity' of IFITMs, it is much more intuitive to speculate that IFITMs have a negative influence on formation of the membranous web, which is the site of HCV RNA replication. A recent study describes an inhibitory effect of IFITM3 on cholesterol homeostasis [97]. Amongst others, cholesterol synthesis is regulated by the vesicle-membrane-protein-associated protein A (VAP-A) and oxysterol-binding protein (OSBP). Interestingly, IFITM1, IFITM2, and IFITM3 can bind to VAP-A and inhibit its interaction with OSBP. This results in cholesterol-enriched multivesicular bodies and late endosomes that inhibit release of several viruses into the cytosol. In the context of HCV infection, the impact of IFITMs on cholesterol homeostasis might have consequences on the formation of the membranous web, which is a highly specialized membrane compartment that contains high amounts of cholesterol [98]. Moreover, IFITM-mediated sequestration of VAP-A, which is an important cofactor for efficient HCV RNA replication, might contribute to this inhibitory effect.

Viperin

Viperin (RSAD2) localizes to the ER and lipid droplets (LDs), which are both important for HCV replication [99]. This protein belongs to the radical S-adenosyl-L-methionine (SAM) superfamily [100] that is characterized by a SAM domain, which is responsible for methylation of nucleic acids and proteins. It was speculated that SAM transferase activity is important for the antiviral activity of viperin [101]. However, mutagenesis studies showed that not the SAM domain, but rather the N-terminal amphipathic helix and the C-terminal region of the protein are necessary for suppression of HCV replication [102]. The N-terminal helix of viperin is responsible for its anchoring to the ER and LDs [102]. Importantly, this helix inhibits protein secretion and induces ER membrane curvature [103]. During HCV infection, viperin resides in small cytoplasmic foci at the ER–LD interface. These foci are thought to correspond to viral replication complexes. It is thought that viperin interacts via its C-terminal region with the core protein and NS5A that also localize to LDs and LD-proximal ER membranes [102,104,105]. In addition, viperin binds to VAP-A, an important host factor of HCV replication [104,105] that also regulates trafficking and biogenesis of lipids and sterols [106]. Therefore, one proposed mechanism of viperin's antiviral activity is the disintegration of the membranous HCV replication compartment as a result of altered NS5A – VAP-A interaction. This might cause an alteration of the lipid composition of the membranous web thereby inhibiting HCV replication. Viperin has also been found to bind to and inhibit farnesyl diphosphate synthetase (FPPS) [107]. Like VAP-A, FPPS is involved in the cholesterol and isoprenoid biosynthesis at the ER membrane [108]. It is assumed that the inhibition of FPPS by viperin may change the type or quantity of lipids in the ER membrane and thus affect the composition of the ER-derived HCV replication complex. Given the recent establishment of a

method to isolate functional membrane-associated HCV replication complexes, these attractive hypotheses can now be addressed experimentally [98].

The oligoadenylate synthetase/ribonuclease L system

RNase L was one of the first identified restriction factors of HCV [109]. Subsequent studies could confirm that the components of this pathway are important for the counteraction of HCV infection [25,54,110]. RNase L contains 9 so-called ankyrin repeats serving as protein-protein interaction platforms, several protein kinase-like motifs, and a ribonuclease domain. This domain has homology to IRE1 (inositol-requiring protein 1), an enzyme important for the unfolded protein response (UPR) [111]. RNase L is constitutively expressed in a wide range of tissues, but requires stimulation of enzymatic activity by a particular oligonucleotide (2′,5′-oligoadenylate) that is generated by the IFN-inducible 2,5-linked oligoadenylate synthetases (OAS1, OAS3, and OASL). Similar to PKR, OAS proteins require activation by dsRNA that is generated during viral replication. Synthesized 2′,5′-oligoadenylate binds to RNase L to induce a conformational change leading to its homodimerization and activation of the endonuclease activity. RNase L cleaves viral as well as cellular RNAs in single-stranded regions, mainly after UU or UA dinucleotides leaving a 5′-OH and a 3′-monophosphate [112]. This unspecific cleavage counteracts several viral pathogens, including HCV. Recent studies showed that all three OAS proteins induce RNase L-dependent antiviral activity against HCV [25,110,113]. Some of the cleavage products of the HCV genome might serve as ligands for RIG-I and MDA5 [114] thereby enhancing the induction phase of the IFN pathway. Interestingly, HCV genomes of less IFN-responsive genotypes (1a and 1b) have a lower frequency of UA and UU dinucleotides as compared to genotypes with higher IFN response (genotypes 2a, b and 3a, b) [109], indicating that the OAS/RNase L system contributes to the control of HCV replication also *in vivo*.

Interferon-stimulated gene 15

ISG15 is one of the most highly induced ISGs. It is a 15 kDa protein with two ubiquitin-like domains in the C- and N-terminal region. Comparable to ubiquitin, ISG15 can be conjugated to lysine residues of target proteins. This so-called ISGylation occurs through the sequential reaction of an E1-activating, an E2-conjugating, and an E3 ligation enzyme. Mass spectrometry-based studies identified more than 160 host proteins that are ISGylated, including the important dsRNA sensors PKR and RIG-I [115]. ISGylation has two effects: first, it alters protein property directly by addition of ISG15; second, it reduces degradation of the target protein by competing with ubiquitin conjugation [116]. In addition, ISG15 can be secreted and acts like a cytokine by modulating immune responses such as activation of T cells and NK cells leading to the production of IFN-γ [117].

Reports on the role of ISG15 in the HCV replication cycle are controversial, but the majority of studies argue for a proviral effect. For instance, ISG15 overexpression has been reported to increase HCV replication while RNAi-mediated silencing of ISG15 expression was found to inhibit HCV replication, albeit to a rather low extent (~2 to 3-fold) [118,119]. At the first glance, the proviral role of ISG15 appears counterintuitive, but it was found that ISG15 overexpression inhibited induction of IFN-β by HCV [120]. In line with this report, silencing of USP18 expression, which is a negative regulator of ISGylation, potentiates IFN-α mediated HCV suppression [121]. These results suggest that ISG15 counteracts the IFN-α response by ISGylation, e.g., of important signaling factors, and this negative regulation is counteracted by USP18 that removes ISG15 from target proteins. Although this is a plausible assumption, one report seems to contradict it. Kim and Yoo reported reduced HCV replication upon overexpression of ISG15 or ISG15-conjugating enzymes [122]. This destabilization was blocked by a particular arginine for lysine substitution in NS5A, arguing for an ISGylation-dependent antiviral mechanism. However, these results have been obtained in highly selected HCV replicon cell clones and thus, need to be revisited in more authentic culture systems and by using replication-competent HCV isolates from different strains and genotypes. We note that high hepatic ISG15 levels were found to correlate with low antiviral IFN-response during the early phase of antiviral therapy, supporting the notion that ISG15 is a negative regulator of the IFN system [118].

Conclusions

With the advent of HCV cell culture models, important insights into the suppression of viral replication by the IFN-induced antiviral state have been gained. Nevertheless, several key questions remain to be clarified.

1. High-content screens have identified numerous ISGs that might contribute to IFN-mediated control of HCV. However, for a few of them we begin to understand the underlying mechanism and for most of them, the mechanism is not known.
2. A concerted action of multiple ISGs is responsible for the suppression of HCV replication, but the relevant ISGs remain to be identified. Determination of the 'real' ISG set will require systems that allow combination of multiple ISGs.
3. Given the frequent discrepancies reported for HCV-targeting ISGs, more authentic cell culture systems as well as standardization of used methods are urgently needed. Since many results were obtained by using rather artificial experimental systems, re-evaluations in more appropriate models are required.
4. Validation of ISGs identified in cell culture or deduced from analyses of patient samples in adequate *in vivo* models is necessary. This will require a fully permissive and immuno-competent mouse model, which is not yet available.

Conflict of interest

The authors declared that they do not have anything to disclose regarding funding or conflict of interest with respect to this manuscript.

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