Hepatitis C virus reveals a novel early control in acute immune response.
Noëlla Arnaud, Stéphanie Dabo, Daisuke Akazawa, Masayoshi Fukasawa, Fumiko Shinkai-Ouchi, Jacques Hugon, Takaji Wakita, Eliane F Meurs

To cite this version:
Noëlla Arnaud, Stéphanie Dabo, Daisuke Akazawa, Masayoshi Fukasawa, Fumiko Shinkai-Ouchi, et al.. Hepatitis C virus reveals a novel early control in acute immune response.. PLoS Pathogens, 2011, 7 (10), pp.e1002289. 10.1371/journal.ppat.1002289. pasteur-00635743

HAL Id: pasteur-00635743
https://pasteur.hal.science/pasteur-00635743
Submitted on 25 Oct 2011

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers. L’archive ouverte pluridisciplinaire HAL, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d’enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.
Hepatitis C Virus Reveals a Novel Early Control in Acute Immune Response

Noëlla Arnaud\textsuperscript{1}, Stéphanie Dabo\textsuperscript{1}, Daisuke Akazawa\textsuperscript{2}, Masayoshi Fukasawa\textsuperscript{3}, Fumiko Shinkai-Ouchi\textsuperscript{3}, Jacques Hugon\textsuperscript{4}, Takaji Wakita\textsuperscript{2}, Eliane F. Meurs\textsuperscript{4*}

\textsuperscript{1} Institut Pasteur, Hepacivirus and Innate Immunity, Paris, France, \textsuperscript{2} National Institute of Infectious Diseases, Department of Virology II, Tokyo, Japan, \textsuperscript{3} National Institute of Infectious Diseases, Department of Biochemistry and Cell Biology, Tokyo, Japan, \textsuperscript{4} Institut du Fer à Moulin, INSERM UMR 839, Paris, France

Abstract

Recognition of viral RNA structures by the intracytosolic RNA helicase RIG-I triggers induction of innate immunity. Efficient induction requires RIG-I ubiquitination by the E3 ligase TRIM25, its interaction with the mitochondria-bound MAVS protein, recruitment of TRAF3, IRF3- and NF-κB-kinases and transcription of Interferon (IFN). In addition, IRF3 alone induces some of the Interferon-Stimulated Genes (ISGs), referred to as early ISGs. Infection of hepatocytes with Hepatitis C virus (HCV) results in poor production of IFN despite recognition of the viral RNA by RIG-I but can lead to induction of early ISGs. HCV was shown to inhibit IFN production by cleaving MAVS through its NS3/4A protease and by controlling cellular translation through activation of PKR, an eIF2α-kinase containing dsRNA-binding domains (DRBD). Here, we have identified a third mode of control of IFN induction by HCV. Using HCVcc and the Huh7.25.CD81 cells, we found that HCV controls RIG-I ubiquitination through the di-ubiquitine-like protein ISG15, one of the early ISGs. A transcriptome analysis performed on Huh7.25.CD81 cells silenced or for PKR and infected with JFH1 revealed that HCV infection leads to induction of 49 PKR-dependent genes, including ISG15 and several early ISGs. Silencing experiments revealed that this novel PKR-dependent pathway involves MAVS, TRAF3 and IRF3 but not RIG-I, and that it does not induce IFN. Use of PKR inhibitors showed that this pathway requires the DRBD but not the kinase activity of PKR. We then demonstrated that PKR interacts with HCV RNA and MAVS prior to RIG-I. In conclusion, HCV recruits PKR early in infection as a sensor to trigger induction of several IRF3-dependent and MAVS prior to RIG-I. In conclusion, HCV recruits PKR early in infection as a sensor to trigger induction of several IRF3-dependent genes. Among those, ISG15 acts to negatively control the RIG-I/MAVS pathway, at the level of RIG-I ubiquitination. These data give novel insights in the machinery involved in the early events of innate immune response.

HCV infection can trigger important intrahepatic synthesis of several IFN-induced genes (ISGs) in patients [10,11] and in animal models of infection in chimpanzees [12]. Expression of ISGs can be explained at least in part by the ability of HCV to activate the IFN-producing pDCs in the liver through cell-to-cell contact with HCV-infected cells [13]. Intriguingly, despite the recognized antiviral activity of a number of these ISGs, their high expression paradoxically represents a negative predictive marker for the response of these patients to standard combination IFN/ribavirin therapy [14,15,16]. The ubiquitine-like protein ISG15 is among the ISGs which are the most highly induced by HCV [16] and was recently shown to act as a pro-HCV agent [17]. Interestingly, ISG15 was also shown to control RIG-I activity through ISGylation [18].

Here, we show that HCV controls IFN induction at the level of RIG-I ubiquitination through the ubiquitine-like protein ISG15, one of the early ISGs. Use of small interfering RNA (siRNA) targeting to compare the effect of ISG15 to that of PKR on IFN induction and HCV replication led to the unexpected finding that HCV infection triggers induction of ISG15 and other ISGs by using PKR as an adapter through its N terminal dsRNA binding domain. This recruits a signaling pathway which involves MAVS, TRAF3.
**Author Summary**

Hepatitis C Virus (HCV) is a poor interferon (IFN) inducer, despite recognition of its RNA by the cytosolic RNA helicase RIG-I. This is due in part through cleavage of MAVS, a downstream adapter of RIG-I, by the HCV NS3/4A protease and through activation of the eIF2α-kinase PKR to control IFN translation. Here, we show that HCV also inhibits RIG-I activation through the ubiquitin-like protein ISG15 and that HCV triggers rapid induction of 49 genes, including ISG15, through a novel signaling pathway that precedes RIG-I and involves PKR as an adapter to recruit MAVS. Hence, we propose to divide the acute response to HCV infection into one early (PKR) and one late (RIG-I) phase, with the former controlling the latter. Furthermore, these data emphasize the need to check compounds designed as immune adjuvants for activation of the early acute phase before using them to sustain innate immunity.

**Results**

**HCV infection negatively controls RIG-I ubiquitination**

We recently reported that the HCV permissive Huh7.25.CD81 cells [19] that we used to identify the pro-HCV action of PKR, did not induce IFN in response to HCV infection, unless after ectopic expression of TRIM25 [8]. We started this study by investigating at which level this defect could occur. A P358L substitution in the endogenous TRIM25 of these cells, revealed by sequence analysis, proved to have no incidence of the ability of TRIM25 to participate in the IFN induction process. Indeed, ectopic expression of a TRIM25 P358L construct was as efficient as a TRIM25wt construct to increase IFN induction in the Huh7.25.CD81 cells, after infection with Sendai virus (SeV) (**Figure 1A**). Like some other members of the TRIM family, TRIM25 is localized in both the cytosol and nucleus and is induced upon IFN treatment [20]. No specific difference between the cellular localization of TRIM25 was observed in the Huh7.25.CD81 cells when compared to Huh7 cells or Huh7.5 cells, which rules out a role for a cellular mislocalization in its inability to participate in IFN induction (**Figure 1B**). TRIM25 was also efficiently induced by IFN (**Figure 1B and Figure S1**). We assayed whether increasing TRIM25 upon IFN treatment could mimic the effect of its ectopic expression and restore IFN induction in response to HCV infection. However, this resulted only in a poor stimulation of an IFNβ promoter (3 to 5-fold), in contrast to its effect on SeV infection (230-fold) (**Figure 1C**). Similarly, HCV infection at higher m.o.i, as an attempt to favour expression of TRIM25, resulted in clear inhibition of HCV RNA expression with however not enough to detect some IFN induction in response to JFH1 infection in those cells (see **Figure 1D**). We found that IFN induction increased when cells were transfected with siRNAs targeting ISG15 while it decreased in cells overexpressing ISG15 (**Figure 2B**). Expression of ISG15 in the presence of the E1, E2 and E3 ligases involved in ISGylation (respectively Ube1L, UbcH8 and HERC5) [22] further inhibits IFNβ induction (**Figure 2B**). Similar results were observed upon infection with Sendai virus (**Figure 3C**). The ISGylation process is strictly dependent on the presence of the E1 ligase Ube1L [23]. Indeed, enhanced IFN promoter activity has been observed in Ube1L−/− cells in response to NDV [18]. In accord with this, depletion of endogenous Ube1L from the Huh7.25.CD81 cells (**Figure S4**), as such or after ectopic expression of ISG15, UbcH8 and HERC5, resulted in an increase in IFNβ induction after infection with HCV (**Figure 2B**). We then analysed the effect of siISG15 on IFNβ induction after infecting the cells with HCV up to 72 hours, in order to pass through the 24 hr time-point where the signaling pathway leading to the transcription of this gene is expected to stop because of the NS3/4A-mediated cleavage of MAVS [8]. The results show that, whereas IFNβ transcription was indeed strongly inhibited after 24 hr in the control cells, it still occurred significantly in the cells expressing siRNA ISG15 (**Figure 2C**). Previous data have shown a positive role for ISG15 on HCV production [24,25]. In accord with this, silencing of ISG15 resulted in clear inhibition of HCV RNA expression with however no significant consequence on the ability of the virions produced to re-infect fresh cells (**Figure 2D**). Analysis of expression of MAVS and NS3, as well as the expression of the core protein as another example of viral protein, then showed that the depletion of ISG15 both decreased and delayed the expression of the viral proteins as compared to the siRNA control cells and that this was correlated by a delay in the NS3/4A-mediated cleavage of MAVS (**Figure 2D**). These results show that ISG15 controls the process of IFN induction during HCV infection by interfering with RIG-I ubiquitination through an ISGylation process and by boosting efficient accumulation of NS3, among other viral proteins, thus favouring its negative control on IFN induction by cleavage of MAVS.

**ISG15 strengthens the pro-HCV activity of PKR**

ISG15 [24,25] and PKR [8,9] emerge as two ISGs with pro-HCV activities, instead of playing an antiviral role. We then assayed the effect of a combined depletion of PKR and ISG15 on HCV replication and IFN expression in the Huh7.25.CD81 cells. As shown in **Figure 2D** and **B**, siRNAs targeting ISG15 were sufficient both to inhibit HCV replication (**Figure 3A**) and to increase IFNβ expression, either measured by RT-qPCR (**Figure 3B**) or by using an IFNβ-luc reporter

and IRF3 but not RIG-I. Altogether, our results present a novel mechanism by which HCV uses PKR and ISG15 to attenuate the innate immune response.
Figure 1. HCV infection negatively controls RIG-I ubiquitination. (A) Huh7.25.CD81 cells were transfected for 24 hrs with 150 ng of the pGL2-IFNβ-FLUC; 40 ng of the pRL-TK-RLUC reporter plasmids alone or in presence of 150 ng of a plasmid expressing HA-TRIM25, either as such (TRIM25wt) or containing the P358L substitution (a SNP rs75467764 with no reported pathology). Cells were infected or not with SeV (40 HAU/ml) for 24 hrs. IFN expression was expressed as fold induction of luciferase activity. Error bars represent the mean ± S.D. for triplicates. (B) Huh7, Huh7.5 and Huh7.25.CD81 cells were either untreated or treated with 500 U/ml IFNα for 24 hrs. TRIM25 was detected by immunoblot after preparation of nuclear and cytosolic fractions from 25 μg of cell extracts. Detection of Actin, RIG-I and phosphorylated Histone 3 (HH3-P) served as controls. (C–D) Huh7.25.CD81 cells were transfected with the reporter plasmids as in A, a few hours before being treated with Sendai virus (40 HAU/ml) or with JFH1 at an m.o.i of 0.2 (C) or increasing from 0.2 to 10 (D). At the times indicated, IFN expression was expressed as fold induction of luciferase activity. Error bars represent the mean ± S.D. for triplicates. Induction of
cells silenced for ISG15 are able to induce IFN in response to HCV infection, as shown in Figure 3B, they are unable to do so when PKR is overexpressed. This suggests that PKR may also interfere with the process of IFN induction, independently of ISG15, a possibility that remains to be explored.

A role for PKR in gene induction in response to HCV infection has not been described before. Additional information was therefore obtained through a transcriptome analysis of 2165 genes in the Huh7.25.CD81 cells treated with control siRNAs or siRNAs targeting PKR and infected with HCV for 12 hrs. Out of the most significant 422 genes that were identified, 99 were unmodified or barely modified and 33 were down-regulated, while 290 genes were found to be up-regulated by HCV infection (data not shown). Among those, HCV infection triggered up-regulation of 49 genes which are directly dependent on PKR expression (Table S1). Forty percent of these genes (20) belong to the family of the ISGs, with ISG15 among the most induced genes (Table S1). In the reciprocal situation, only 17 genes depended on PKR for their down-regulation by HCV infection, with no link to a particular family of genes and limited variation both in number and intensity (Table S1). Thus, induction of ISGs upon HCV infection may occur through a novel signaling pathway that involves PKR.

Induction of ISG15 by HCV is independent of RIG-I, involves MAVS/TRAF3 association with PKR and involves the DRBD region but not the catalytic activity of PKR

Infection with RNA viruses or transient transfection with dsRNA can directly and rapidly induce early ISGs, such as ISG15, through IRF3, after activation of the RIG-I/MAVS pathway and recruitment of TRAF3, an essential adapter which recruits the downstream IRF3 kinases Tbk1/IKKε. We have shown that the RIG-I pathway was not operative during HCV infection in the Huh7.25.CD81 cells, precisely due to the presence of ISG15. To determine how ISG15 induction through PKR relates to or differs from the RIG-I/MAVS pathway, the Huh7.25.CD81 cells were treated with siRNAs aimed at targeting separately PKR, RIG-I, MAVS, TRAF3 and IRF3 (Figure S7) and infected with HCV. The results clearly showed that induction of ISG15 in response to HCV infection depends on PKR, MAVS, TRAF3 and IRF3 but not on RIG-I (Figure 5A). The participation of IRF3 was further confirmed by immunofluorescence studies which showed its nuclear translocation at 6 hours post-infection (Figure 8B). ISG15, as well as ISG56, was also clearly induced in response to HCV infection in two other HCV permissive cell lines, such as Huh7 and Huh7.5 cells, and this induction was abrogated in absence of siRNAs targeting PKR (Figure 8D and Figure S6). Importantly, since Huh7.5 cells express a non-functional RIG-I/MAVS pathway due to a mutation in RIG-I, result with these cells supports the notion that the ability of HCV to trigger induction of ISGs through PKR is independent of RIG-I. To have more insights on this novel PKR signaling pathway, PKR was immunoprecipitated at early times points following infection of Huh7.25.CD81 cells with HCV and the immunocomplexes were analysed for the presence of MAVS, TRAF3 and RIG-I. Both MAVS and TRAF3, but not RIG-I, associate with PKR in a time dependent manner, beginning at 2 hrs post-infection (Figure 5C). Strikingly, these associations were abrogated by the cell-permeable peptide PRI which is analogous to the first dsRNA binding
Figure 2. HCV controls RIG-I ubiquitination through ISG15. (A) Huh7.25.CD81 cells were transfected for 24 hrs with 25 nM of siRNA (Control or ISG15) and for another 24 hr with 5 μg of a His-Myc-Ubiquitin plasmid in absence or presence of 5 μg of a plasmid expressing HA-TRIM25. The cells were infected with JFH1 (m.o.i = 0.2). At the times indicated, cell extracts were processed for analysis of RIG-I ubiquitination and the expression of the different proteins in the total cell extracts. (B) Huh7.25.CD81 cells were first transfected with siRNA Control (25 nM), siRNA ISG15 (25 nM), siRNA Ube1L (50 nM) or left untreated. After 24 hrs, the untreated cells were transfected with a plasmid expressing HA-ISG15 (500 ng) alone or in presence of plasmids expressing E1, E2 and E3 (1 μg each) while a set of cells transfected with siRNA Ube1L received plasmids expressing HA-ISG15, E2 and E3. After 24 hrs, the cells were infected with JFH1 (m.o.i = 6) for the times indicated. Stimulation of endogenous IFNβ RNA expression was determined by RTqPCR and expressed as fold induction. The degree of statistical significance is indicated by stars after calculation of the p-values (from left to right: 0.0005, 0.0076, 0.0003, 0.047 and 0.0023). (C–D) Huh7.25.CD81 cells, transfected with 25 nM of siRNA (Control or ISG15) for 48 hrs, were infected with JFH1 (m.o.i = 6) for the times indicated. Expression of IFNβ or HCV RNA, determined by RTqPCR, was expressed as fold induction (C; IFNβ) or as copies (D; HCV). Error bars represent the mean ± S.D for triplicates. Expression levels of IFNβ RNA at the start of infection were 2.1 × 10^4 (siControl) and 4 × 10^4.
domain (DRBD) of PKR [8], while unaffected by C16, a chemical compound which inhibits the catalytic activity of PKR (Figure 5D). In line with this, PRI but not C16, abrogated the ability of HCV to induce ISG15 (Figure 5E). The same result was obtained for induction of ISG56 (Figure S10). We then used human primary hepatocytes (HHP) to determine whether HCV was also able to induce ISGs through PKR in a more physiological cellular model. A follow-up of the infection over a period of 96 hours showed that JFH1 was replicating correctly in those cells as well as leading to induction of ISG15 (10-fold) and to some induction of IFN-β (2.5-fold). These cells were infected with JFH1 for 8 hours in the absence or presence of PRI, making convenient

Figure 3. ISG15 strengthens the pro-HCV activity of PKR. (A–B). The Huh7.25.CDH1 cells were transfected with 25 nM of the different siRNA (Control, ISG15, PKR), separately or together. After 48 hrs, cells were infected with JFH1 (m.o.i = 0.2). At the times indicated, expression of HCV or IFN-β RNA was determined by RTqPCR and expressed as copies of JFH1 RNA (A) or as fold induction (IFN-β; B). The expression levels of IFN-β RNA at the start of infection was 6.96×10^5 copies. (C) Two sets of Huh7.25.CDH1 cells were first transfected with siRNA ISG15, siRNA PKR separately and together for 24 hrs, then transfected with the reporter plasmids IFN-β-firefly luciferase (pGL2-IFN-β), pRL-TK Renilla-luciferase for another 24 hrs and infected with JFH1 (m.o.i = 0.2) for the times indicated. In each case, IFN expression was expressed as fold-induction over control cells that were simply transfected with pGL2-IFN-β-FLUC/pRL-TK-RLUC. The graph represents the level of firefly luciferase activity normalized to the ratio R-luc RNA/GAPDH RNA. Such normalization is required because of the negative control of general translation through PKR after 12 hrs post-infection [8]. Error bars represent the mean ± S.D for triplicates. (D) Huh7.25.CDH1 cells, in 100 cm² plates, were transfected with siRNA Control or siRNA ISG15 or transfected with a plasmid expressing HA-ISG15 for 48 hrs and infected with JFH1 (m.o.i = 6). At the indicated times post-infection, cell extracts (2.2 mg) were processed for immunoprecipitation of PKR or for incubation with mouse IgG as a control of specificity (asterisk). The immunoprecipitated complexes were run on two different NuPAGE gels and blotted using Mab 71/10 or anti-phosphorylated PKR antibodies (PKR-P). The presence of PKR and PKR-P was revealed using the Odyssey procedure. The ratio PKR-P/PKR in the absence or in the presence of ISG15, either endogenous or endogenous and ectopic, is shown in Figure S5.

doi:10.1371/journal.ppat.1002289.g003
Figure 4. HCV triggers a PKR-dependent pathway early in infection to induce ISG15 and other genes. (A–C) The cDNAs reversed transcribed from the RNAs extracted from the Huh7.25.CD81 cells for the experiment described under Figure 3A were analysed by qPCR for the expression of ISG15 (A) and ISG56 (B). Expression levels of ISG15 and ISG56 RNA at the start of infection were respectively $1 \times 10^5$ and $1.18 \times 10^5$ copies. A novel set of Huh7.25.CD81 cells were transfected with siRNA Control, siRNA ISG15, siRNA PKR separately and together for 48 hrs. They were then transfected with the reporter plasmids ISG56-FLUC and pRL-TK-RLUC and infected with JFH1 (m.o.i = 0.2). At the times indicated, the effect of the different conditions of silencing on the reporter expression was analyzed after normalization performed as described under Figure 3C (C). Results are expressed as fold induction. Error bars represent the mean ± S.D for triplicates. (D) Huh7.25.CD81 cells were either transfected with 25 nM of siRNA Control or siPKR for 24 hrs and infected with SeV for the times indicated. Expression of endogenous ISG15 was determined by RTqPCR and expressed as fold induction. Error bars represent the mean ± S.D for triplicates. The expression levels of ISG15 RNA at the start of infection were respectively $4.91 \times 10^4$ copies (siControl) and $5.44 \times 10^4$ copies (siPKR). (E) Huh7.25.CD81 cells were either transfected with 25 nM of siRNA Control or
use of the cell-penetrating ability of this peptide. Longer period of treatment with PRI were not investigated for practical reasons (see Materials and Methods). The results showed that PRI was significantly inhibiting the induction of ISG15 while it had no effect on that of IFNβ (Figure 5F). Altogether, these data demonstrate that HCV triggers induction of early ISGs through MAVS and TRAF3 by using PKR as an adapter protein.

PKR interacts both with MAVS and TRAF3 and binds HCV RNA ahead of RIG-I.

The ability of HCV to control activation of the RIG-I/MAVS pathway after induction of ISG15 through a novel PKR/MAVS pathway suggests that PKR has the possibility to bind MAVS prior to RIG-I. To determine this, we established the kinetics of these interactions, after treating the Huh7.25.CD81 cells with siRNAs targeting ISG15 prior to HCV infection. This was necessary in view of the negative control of ISG15 on RIG-I. MAVS was immunoprecipitated from the cell extracts at different times post-infection and the presence of PKR and RIG-I was examined in the immunocomplexes, as well as that of TRAF3, used as marker of the MAVS signaling pathway. As expected, only PKR was able to associate with MAVS and TRAF3 in the control cells (Figure 6A) whereas both PKR, RIG-I and TRAF3 were found in the immunocomplexes in the absence of ISG15 (Figure 6B). The PKR/MAVS association took place at 4 hrs post-infection in the control cells but was observed 2 hrs earlier in the ISG15-depleted cells. Whether ISG15 plays a role in the regulation of the PKR/MAVS association remains to be determined. However, the presence of TRAF3 in association with MAVS at 2 hrs post-infection in the control cells (Figure 6A) correlates with its association with PKR (Figure 5C) which indicates that the MAVS pathway can be activated through PKR as soon as 2 hrs post infection. In ISG15 knock-down cells, the RIG-I/MAVS association occurred later at 6 hrs post-infection with an increase in TRAF3 association at 9–12 hrs post infection. Altogether, these data revealed that HCV infection triggers an earlier interaction of MAVS with PKR than with RIG-I.

Finally, we asked whether PKR was able to associate with HCV RNA and how this association can be compared to that of RIG-I. PKR and RIG-I were immunoprecipitated at 2, 4 and 6 hrs post-infection and the presence of HCV RNA was analysed in the complexes. The results showed that PKR associates with HCV RNA with best efficiency at 2 hrs post-infection. Importantly, this association was strongly inhibited in presence of PRI, thus confirming the importance of PKR DRBD in the process. In contrast, the association of HCV RNA with RIG-I was detected only at 6 hrs post-infection. Interestingly, the association between RIG-I and HCV RNA was not affected by PRI, which rules out the possibility that the initial formation of a complex between PKR and HCV RNA was a pre-requisite for the subsequent binding of RIG-I to HCV RNA. Immunoprecipitation of PKR at 1, 2, 4 and 6 hrs post-infection, in presence of an inhibitor of ribonucleases also did not lead to detection of RIG-I in the complexes (Figure S11). Association of HCV RNA with eIF2α, used as negative control, was not significant, thus showing the specificity of the assay (Figure 6C). Whether a direct interaction of PKR with HCV RNA represents the initial event leading to the MAVS-dependent induction of early ISGs remains now to be characterized. Altogether, these data reveal an earlier mobilization of PKR than RIG-I in response to HCV infection which leads to activation of a MAVS-dependent signaling pathway.

Discussion

Hepatitis C virus can attenuate IFN induction at multiple levels in infected hepatocytes, such as through the NS3/4A-mediated MAVS cleavage [7,27] and by using the eIF2α kinase PKR to control IFN and ISG expression at the translational level [8,9]. Here, we have identified another process by which HCV controls IFN induction at the level of RIG-I ubiquitination through ISG15 and an ISGylation process. Importantly, we have shown that ISG15 is rapidly induced, among other ISGs, in response to HCV infection, through a novel signaling pathway that involves PKR, MAVS, TRAF3 and IRF3 but not RIG-I. In this pathway, PKR is not used for its kinase function but rather as an adapter protein with its dsRNA binding domain (DRBD) playing an essential role in this mechanism (Figure 7). By transcriptome analysis, we showed that HCV induces a number of ISGs in the HCV-permissive Huh7.25.CD81 cells and we confirmed the induction of two of these, ISG15 and ISG56, in other HCV-permissive cells, such as Huh.7.5 and Huh7 cells. In addition, induction of ISG15 by HCV in a PKR-dependent manner was confirmed in human primary hepatocytes. The ability of HCV to trigger high expression levels of ISG15 and ISG56, as well as other ISGs, has previously been reported in models of HCV-infected chimpanzees [10,12,28] and in HCV-infected patients [14,15,16]. Induction of ISGs thus represents a general propriety of the response of the cells to HCV. In addition to this, natural variations in intra-hepatic levels of ISG15 in vivo may increase the susceptibility of some patients to HCV infection. The ability of HCV to control RIG-I activity through ISG15 is important to note in view of several reports which highlight the importance of a role for ISG15 in the maintenance of HCV in livers [15,16] or in the control of HCV replication in cell cultures [17,25]. Our data provide an explanation for the presence of ISGs at high expression levels in HCV-infected patients [14,15,16] and in models of HCV-infected chimpanzees [10,12,28] in the absence of, or with poor IFN expression.

The 15 Kda ISG15, or Interferon Stimulated Gene 15 [29], also known as ubiquitin cross reactive protein (UCRP) [30], can be conjugated (ISGylation) to more than 150 cellular protein targets [31] through the coordinated action of three E1, E2 and E3-conjugating enzymes, in a process similar but not identical to ubiquitination. While both ubiquitin and ISG15 can use the same E2 enzyme UbcH8, Ube1L functions as a specific E1 enzyme for ISG15, in spite of its 45% identity with Ube1, the E1 enzyme for ubiquitin [32]. The major E3 ligase for human ISG15 is HEC5 [33].

Interestingly, RIG-I was identified as a target for ISG15, among other IFN-induced proteins or proteins involved in IFN action [31]. However, its activity appears to be negatively controlled by ISG15 and the ISGylation process, either as shown previously after cotransfection with the ISG15 and the ISG15-conjugating enzymes [18] or as shown here, in a model of infection with HCV. Indeed, ISG15 is now emerging as playing a proviral role in case of HCV infection. Several reports now highlight the importance of a role for ISG15 in the control of HCV replication in cell cultures [17,25] as well as in the maintenance of HCV in livers and...
Table 1. PKR-dependent up-regulated genes upon HCV infection.

| SiPKR mock/siCt | Name                | Access. N.    | siCtMock | siCt HCV | siCtMock' | siPKRHCV | LOG2* |
|-----------------|---------------------|---------------|----------|----------|-----------|----------|-------|
| 0.6             | ISG56               | NM_001548     | 15.0     | 885.8    | 10.2      | 7.6      | -6.3  |
| 0.7             | ISG15               | NM_005101     | 593.6    | 26061.9  | 410.6     | 283.5    | -6.0  |
| 0.7             | IFI 9-27/IFITM1     | NM_003641     | 27.8     | 817.7    | 15.1      | 10.1     | -5.5  |
| 1.2             | IFI1-8U             | NM_006435     | 24.0     | 597.7    | 10.9      | 7.2      | -5.3  |
| 1.1             | Olfactory Receptor 91I | NM_001005211 | 26.6     | 473.1    | 10.1      | 4.8      | -5.2  |
| 1.6             | IFI1-8U             | XM_084845     | 17.7     | 365.4    | 9.3       | 6.5      | -4.9  |
| 0.8             | OASp100             | NM_006187     | 46.4     | 909.9    | 40.0      | 33.5     | -4.5  |
| 0.8             | IFI6-16             | NM_002038     | 83.4     | 10040.6  | 45.9      | 24.1     | -4.5  |
| 0.6             | Ub2L6               | NM_004223     | 392.7    | 4078.9   | 281.2     | 128.7    | -4.5  |
| 0.9             | OAS 1               | NM_016816     | 49.9     | 704.0    | 31.8      | 21.6     | -4.4  |
| 0.9             | ISG12               | NM_005532     | 46.3     | 592.54   | 38.3      | 29.2     | -4.1  |
| 0.8             | IFP 35              | NM_005533     | 36.3     | 369.7    | 26.6      | 16.9     | -4.0  |
| 0.6             | PARP-9              | NM_031458     | 29.5     | 318.5    | 37.8      | 25.6     | -4.0  |
| 0.5             | GABA-B receptor 1   | NM_006398     | 29.5     | 500.8    | 26.0      | 28.5     | -4.0  |
| 0.7             | Lysp100B            | NM_003113     | 8.7      | 93.0     | 8.5       | 6.1      | -3.9  |
| 0.8             | PDIP1               | NM_033405     | 27.4     | 146.6    | 28.0      | 12.8     | -3.6  |
| 0.9             | PKR                 | NM_002759     | 48.2     | 306.8    | 47.0      | 26.0     | -3.5  |
| 1.1             | RIG-I               | NM_014314     | 23.5     | 223.2    | 18.9      | 21.9     | -3.0  |
| 1.1             | RIG-I               | NM_004585     | 7.4      | 42.5     | 6.3       | 6.0      | -2.6  |
| 0.7             | Phospholipid scramblase | PM21105     | 170      | 1137.2   | 189.9     | 153      | -3.1  |
| 1.1             | STAT1 beta          | NM_012420     | 24.9     | 95.3     | 35.0      | 21.3     | -2.7  |
| 1.3             | RIG-I               | NM_004585     | 7.4      | 42.5     | 6.3       | 6.0      | -2.6  |
| 0.7             | ZNF532              | NM_018181     | 32.8     | 146.5    | 25.5      | 23.8     | -2.3  |
| 0.6             | NNMT                | NM_006169     | 52.8     | 143.8    | 50.26     | 28.9     | -2.2  |
| 1.1             | ISG1-8U             | XM_084845     | 32.0     | 146.8    | 23.1      | 22.7     | -2.2  |
| 1.1             | HIF00               | NM_153833     | 45.3     | 199.9    | 34.7      | 32.9     | -2.2  |
| 0.8             | ISG20               | NM_002201     | 129.3    | 338.3    | 107.5     | 61.4     | -2.2  |
| 1.1             | PSMB10              | NM_002801     | 16.2     | 75.1     | 14.5      | 15.0     | -2.2  |
| 1.3             | ZC3HAV1             | NM_024625     | 8.3      | 26.0     | 6.9       | 4.9      | -2.1  |
| 0.9             | SOD2                | NM_006363     | 348.5    | 1612.2   | 311.0     | 334.3    | -2.1  |
| 0.7             | PARP12              | NM_022750     | 269.9    | 875.2    | 296.9     | 224.2    | -2.1  |
| 0.7             | NMI                 | NM_000486     | 32.1     | 136      | 37.4      | 37.0     | -2.1  |
| 0.8             | NEDD9               | NM_006403     | 5.7      | 19.0     | 5.7       | 4.7      | -2.0  |
| 1.1             | SAMHD1              | NM_015474     | 17.1     | 49.5     | 13.6      | 9.7      | -2.0  |
| 0.7             | AKT2                | NM_001626     | 13.4     | 20.0     | 14.2      | 5.4      | -2.0  |
| 0.5             | ARG1                | NM_000045     | 245.9    | 282.6    | 231.4     | 67.0     | -2.0  |
| 0.8             | BHLHB2              | NM_003670     | 76.4     | 128.0    | 71.5      | 30.5     | -2.0  |
| 0.8             | LGALS3BP            | NM_005567     | 22.0     | 72.0     | 16.1      | 13.6     | -2.0  |
| 1.3             | ZNF292              | XM_048070     | 22.3     | 31.4     | 20.2      | 7.3      | -2.0  |
| 1.1             | STAT1               | NM_007315     | 53.3     | 275.3    | 48.2      | 64.9     | -1.9  |
| 0.7             | TBA3_HUMAN          | NM_006009     | 28.2     | 43.6     | 33.4      | 13.7     | -1.9  |
| 0.5             | TM4SF20             | NM_024795     | 45.2     | 51.0     | 36.9      | 11.1     | -1.9  |
| 1.4             | ERAP2               | NM_022350     | 9.8      | 19.2     | 8.8       | 4.6      | -1.9  |
| 0.8             | USP18               | XM_001126794.1| 215.1    | 979.2    | 201.8     | 245.9    | -1.9  |
| 1               | USP18               | XM_001126794.1| 129.6    | 617.0    | 127.3     | 165.5    | -1.9  |
pinpoint ISG15 as among the predictor genes of non-response to IFN therapy [14,15,16].

At present, we do not know at which level ISGylation regulates IFN induction in response to HCV infection. An HCV-mediated increase of ISG15 would favour preferential binding of ISG15 over that of ubiquitin to the E2 enzyme Ubch8 and hence enhance the spatio-temporal availability of UbcH8-ISG15 for HERC5 over that of Ubch8-ubiquitin for TRIM25. It may also lead to inhibition of TRIM25, through autoISGylation [21,34], which would decrease its ability to ubiquitinate RIG-I. We showed that overexpression of HERC5 together with UbcH1, Ubch8 and ISG15 was increasing the ability of ISG15 to inhibit IFN induction by HCV (Figure 2B). All three enzymes UbcH1, Ubch8 and HERC5 belong to the family of genes induced by IFN and it has been reported that ISGylation is optimum in a context of IFN treatment [18,35]. Therefore, it is tempting to speculate that elevated levels of ISG15 in some HCV-infected patients would bring the most favorable context for the virus when those patients are under IFN therapy. This would be in accord with the clinical data showing that HCV-induced high expression of ISG act as a negative predictive marker for response to IFN therapy.

It is doubtful that viruses with high IFN-inducing efficiency, such as Sendai virus may control RIG-I through ISG15 and PKR. However, viruses that avoid inducing IFN may have use of the PKR pathway. A good example might be that of Hepatitis B Virus (HBV) [36,37,38]. PKR expression was previously reported to be elevated in HCC liver from chronically HBV-infected patients [39] and a relationship between PKR and IFN induction during HBV infection would be important to evaluate.

At present, we have established that HCV RNA interacts with PKR as soon as 2 hours post-infection. This interaction occurs prior the interaction of HCV RNA with RIG-I, which suggests that PKR may rapidly detect structures containing the incoming HCV RNA genome. Indeed, PKR has been reported to bind the dsRNA domains III and IV of HCV IRES [40] in addition to its ability to also bind 5’ triphosphorylated ss or dsRNA structures [41]. Whether PKR behaves as a pathogen recognition receptor for HCV RNA, like RIG-I, remains to be clarified. It is however clear that, in contrast to RIG-I, PKR acts here in favor of the pathogen rather than in favor of the host defense. We have established that the HCV RNA/PKR interaction depends on the first DRBD present at the N terminus of PKR and is independent on its kinase activity. The ability of PKR to serve as adapter in signaling pathways is not a total surprise since it has been previously shown to activate NF-κB through interaction of its C terminus with members of the TRAF family, such as TRAF5 and TRAF6 [42]. PKR contains also TRAF interacting motifs in its N terminus [42] and an association between TRAF3 and PKR has been reported upon cotransfection in 293T cells [43]. Intriguingly, PKR was previously reported to participate in the induction of IFNβ, in association with MAVS, through activation of NF-κB or ATF-2 but not or partially IRF3; however these studies were not performed in the absence of RIG-I [44,45,46]. The mode of interaction between PKR, TRAF3 and MAVS, independently of RIG-I, and how it leads to a preferential induction of ISGs and not of IFNβ in response to HCV infection in contrast with the RIG-I/MAVS pathway remains to be determined. Based on our data, we propose now to divide the innate response to acute HCV infection into two phases: an early acute phase in which PKR is activated and a late acute phase that depends on RIG-I, the early phase controlling activation of the late phase. It is now essential to progress towards the generation of specific pharmaceutical inhibitors targeting PKR in order to abrogate the early acute phase to the benefit of the RIG-I-driven late phase. In a more general view, care should now be taken in the choice of compounds designed to be used as immune adjuvants, such as to be devoid of activation of the early acute PKR phase. This will ensure their efficiency as to activate properly the innate immune response through the late acute RIG-I phase.

### Methods

**Cell cultures and viruses**

The culture of Huh7, Huh7.5, Huh7.25,CD81 cells, the preparation of Sendai virus stocks (≈2000 HAU/ml) and of HCV JFH1 stocks (≈6.10^4 FFU/mL and ≈6.10^5 FFU/mL) was as described [8,47]. Preparation and cultures of human primary hepatocytes was as described [48]. Of note, the ability of the Huh7.25,CD81 cells to induce IFN in response to SeV without prior IFN treatment (40-fold) was not observed in our previous study [8]. The ability of Sendai virus to induce IFN is related to the presence of copyback DI (Defective Interfering) genomes [49]. The higher IFN inducing ability of the novel Sendai virus stock may have come from an important accumulation of these copyback DI genomes, during its growth in chicken eggs.

**PKR inhibitors**

The C16 compound [50] and the cell-permeable PRI peptide [51] were provided by Jacques Hugon. These drugs were applied (200 nM for C16 and 30 mM for PRI) one hour before the end of the 2 hr-incubation time with JFH1 and re-added to the medium after washing the cells with phosphate buffered saline (PBS). Note that PRI loses its effect very rapidly, probably through degradation in the cells, and requires to be added every hour to the cells until the end of treatment.

**Expression vectors**

TRIM25 was cloned from the IFN-treated Huh7.25,CD81 cells (500 U/ml IFN-α2a; Cellsciences) after RT-PCR using the forward: 5'-ATGGCGAGAGCTGTGCCCCCT-3' and reverse 5'-CTACGTGGGAGCAGAATGG-3' primers. The pcDNA3.1(+) vector expressing 5'HA tagged-TRIM25 (provided by D. Garcin; University of Geneva, Switzerland) was used to generate the TRIM25 P530L construct by site-directed mutagenesis. The
Figure 5. HCV-dependent induction of ISG15 involves PKR, MAVS and TRAF3 and not RIG-I. (A–B). A-The Huh7.25.CD81 cells were transfected with 50 nM Control siRNA and the different Smartpool siRNAs (50 nM siPKR; 10 nM siRIG-I; 5 nM siMAVS; 50 nM siTRAF3; 50 nM siIRF3) for 48 hrs and infected with JFH1 (m.o.i = 6). (B) Huh7.5 or Huh7 cells were transfected with siRNA Control or siPKR (50 nM) for 48 hrs and infected with JFH1 (m.o.i = 0.2 for Huh7.5 or 10 for Huh7). At the times indicated, expression of endogenous ISG15 was determined by RTqPCR and expressed as fold induction. Error bars represent the mean ± S.D for triplicates. The expression level of ISG15 RNA at the start of infection in the siControl cells was 9.97 × 10^4 copies (Huh7.25.CD81), 1.31 × 10^4 copies (Huh7.5) and 1.28 × 10^4 copies (Huh7). (C–D) Huh7.25.CD81 cells, in 100 cm^2 plates, were infected with JFH1 (m.o.i = 0.2) alone (C) or in presence of PRI or C16 (D). At the times indicated, cell extracts (3.5 mg) were processed for immunoprecipitation of PKR or for incubation with mouse IgG as a control of specificity (asterisk). The detection of the proteins in the complexes and in the whole cell extracts (WCE) was revealed by immunoblot using the Odyssey procedure. (E) The Huh7.25.CD81 cells were incubated with PRI or C16 and infected with JFH1 (m.o.i = 0.2) for the times indicated. Expression of endogenous ISG15 was determined as in A–B. The ISG15 RNA levels were 3.81 × 10^4 copies in the siControl cells. (F) Human primary hepatocytes (HHP) were infected with JFH1 (m.o.i = 6). One set of cells was incubated with 30 mM of the PRI inhibitor during 8 hours. At the times indicated, expression of HCV RNA, ISG15 and IFNβ was determined by RTqPCR. The expression levels of ISG15 and IFNβ RNA at the start of infection was 1.05 × 10^5 copies and 1.11 × 10^4 copies, respectively. Inhibition of induction of ISG15 by PRI at 8 hr post-infection was statistically significant (**; p = 0.0001).

doi:10.1371/journal.ppat.1002289.g005
Figure 6. PKR both interacts with MAVS and TRAF3 and binds HCV RNA ahead of RIG-I. (A–B). Huh7.25.CD81 cells were transfected with 25 nM of siRNA Control (A) or 25 nM of siRNA ISG15 (B) for 48 hrs and infected with JFH1 (m.o.i = 0.2). At the times indicated, cell extracts (4.5 mg) were incubated with anti-MAVS antibodies. In addition, cell extracts prepared at 0 hr post-infection were incubated with mouse IgG as a control of specificity (asterisk). The immunoprecipitated complexes were run on three different NuPAGE gels and blotted using Mab 71/10, anti-MAVS, anti-RIG-I or anti-TRAF3 antibodies. The expression level of each protein was controlled in the total cell extracts. (C). Huh7.25.CD81 cells were incubated with JFH1 (m.o.i = 6) for 2 hrs at 37°C or at 4°C in the absence or presence of 30 μM of PRI. This drug was applied one hour before the end of the incubation time. After washing the cells twice with PBS, the cells were further incubated for 2, 4 or 6 hrs at 37°C or at 4°C in the absence or presence of PRI (added every hour). The cell extracts were processed for crosslinking of RNA to proteins before lysis, as described in Materials and Methods and different immunoprecipitations were performed with antibodies directed against PKR, RIG-I or eIF2α. After extensive washing, the presence of HCV RNA linked to the immunocomplexes was analysed by RTqPCR and the presence of the proteins was verified by Western blot. Measure of HCV RNA in
IFNβ-firefly luciferase (pGL2-IFNβ) and pRL-TK Renilla-luciferase reporter plasmids were described previously [8]. The pGL3 luciferase reporter construct containing the −3 to −654 nucleotides of the ISG56 promoter was provided by N. Grandvaux [52]. The Myc-HIS-Ubiquitin construct was provided by R. Kopito (Stanford University, CA). ISG15 was cloned from IFN-treated Huh7 cells using the forward: 5'-GGATCCATGGGGACCTGACGGTG-3' and reverse 5'-CTCGAGCTCCGCCCCGCAAGGCTCTGT-3' primers and inserted into the pcDNA3.1(+)+HA vector. The Ube1L, UbcH8 and HERC5 constructs were kindly provided by Jon M. Huibregtse [35]. The pcDNA1/AMP vector expressing PKR has been described previously [53].

RNA-mediated interference

The siRNAs directed against PKR, MAVS, RIG-I, TRAF3 and IRF3 which were used for the experiment described in figure 5A correspond to pools of siRNA (Smartpool) obtained from Dharmacon Research, Inc. (Lafayette, CO), as well as siRNAs directed against Ube1L used in Figure 2B. Control (scrambled) siRNA and siRNA directed against PKR or ISG15, used in all other experiments, were chemically synthesized by Dharmacon.
(scrambled and PKR) and by EUROFINS MWG Operon (ISG15) (Text S1). The siRNAs (final concentration 25 nM or 50 nM) were transfected for 48 h using jetPRIME reagent according to the manufacturer's instructions (PolyPlus transfection TM) before transfection with other plasmids or before infection.

**Antibodies**

Mab to ISG15 (clone 2.1) was a kind gift of E.Borden [54]. Mab to PKR was produced from the murine 71/10 hybridoma (Agrobio; Fr) with kind permission of A.G.Hovanessian [55]. Other antibodies were as follows: anti-mouse IgG (Santa Cruz), anti-TRAF5 (Santa Cruz), p14h451-PKR (Alexis), MAVS (Alexis), anti-actin (Sigma), anti-pSer10-Histone H3 (Millipore), anti-HCV NS3 (Chemicon), anti-HCV core (Thermo scientific), anti-RIG-I (Alexis Biochemical Inc.), anti-TRIM25 (6105710; BD Bioscience), anti-IRF3 (Santa Cruz), anti-HA (12CA5; Roche) and anti-Myc (Santa Cruz).

**Reporter assays**

Huh7.25.CD81 cells (80,000 cells/well; 24-well plates) were transfected with 40 ng of pRL-TK Renilla-luciferase reporter (Promega) and 150 ng of either pGL2-IFNβ-Flucifer luciferase reporter or pISG56-luciferase reporter and processed for dual-luciferase reporter assay as reported previously [8].

**Real-time RT-PCR analysis**

Total cellular RNA was extracted using the TRIZOL reagent (Invitrogen). HCV RNA was quantified by one-step RTqPCR. Reverse-transcription, amplification and real-time detection of PCR products were performed with 5 μl total RNA samples, using the SuperScript III Platinum one-step RTqPCR kit (Invitrogen) and an AbyPrism 7700 machine. For the sequence of the different amplicons were quantified by a two-step RTqPCR assay as described previously [8].

**Transcriptome analysis**

Cellular RNA was extracted and purified from the cells using RNAeasy mini kit (QIAGEN K.K., Tokyo, Japan). Comprehensive DNA microarray analysis was performed with 3D-GenHuman Oligo chip25k with 2-color fluorescence method by New Frontiers Research Laboratories, Toray Industries Inc, Kama kura, Japan as previously described [56]. In brief, each sample was hybridized with 3D-Gen chip. Hybridization signals were scanned using Scan Array Express (PerkinElmer, Waltham, MA). The scanned image was analyzed using GenePix Pro (MDS Analytical Technologies, Sunnyvale, CA). All the analyzed data were scaled by global normalization.

**Immunoprecipitation and immunoblot analysis**

Cells were washed once with PBS and scraped into lysis buffer 1 (50 mM TRIS-HCl [pH 7.5], 140 mM NaCl, 5% glycerol, 1% CHAPS) that contained phosphatase and protease inhibitors (Complete, Roche Applied Science). The protein concentration was determined by the Bradford method. For immunoprecipitation, lysates were incubated at 4°C overnight with the primary antibodies as indicated and then in the presence of A/G-agarose beads (Santa Cruz Biotechnology) for 60 minutes. The beads were washed three times, and the precipitated proteins were extracted at 70°C using NuPAGE LDS sample buffer. Protein electrophoresis was performed on NuPAGE 4–12% Bis TRIS gels (Invitrogen). Proteins were transferred onto nitrocellulose membranes (Biorad), and probed with specific antibodies. Fluorescent immunoblot images were acquired and quantified by using an Odyssey scanner and the Odyssey 3.1 software (Li-Cor Biosciences) as described previously [8]. For detection of ISG15, cells were lysed in RIPA buffer (50 mM TRIS-HCl [pH 8.0]; 200 mM NaCl; 1% NP-40; 0.5% Sodium Deoxycholate; 0.05% SDS; 2 mM EDTA) and protein electrophoresis was performed on 4–20% polyacrylamide gels (PIERCE).

**Nuclear/cytoplasmic extract**

Pellets from cells washed in ice-cold phosphate-buffered saline (PBS) were lysed in ice-cold cytoplasmic buffer (10 mM TRIS [pH 8.0], 5 mM EDTA, 0.5 mM EGTA, 0.25% Triton X-100) containing phosphatase and protease inhibitors. The suspension was centrifuged for 30 seconds at 14,000 g and the supernatant (cytoplasmic fraction) was transferred into microcentrifuge tubes. The nuclear pellet was resuspended in Urea buffer (8 M Urea, 10 mM TRIS [pH 7.4], 1 mM EDTA, 1 mM dithiothreitol) containing phosphatase and protease inhibitors, homogenized by vortex and boiled for 10 minutes. The protein concentration was determined by the Bradford method.

**Ubiquitination assay**

Huh7.25.CD81 cells were transfected for 48 hrs with 5 μg of Myc-His-Ubiquitin expression plasmid using jetPRIME reagent. The cells were then washed in ice-cold PBS containing 20 mM N-ethylmaleimide (Sigma-Aldrich), harvested directly in Gua8 phosphatase/protease and RNAse inhibitors (Promega), scraped in PBS and dispatched into three sets (J1, J2, J3) of the cell suspension. The three sets were centrifuged for 30 seconds at 14,000 g and the supernatant (cytoplasmic fraction) was transferred into microcentrifuge tubes. The nuclear pellet was resuspended in Urea buffer (8 M Urea, 10 mM TRIS [pH 7.4], 1 mM EDTA, 1 mM dithiothreitol) containing phosphatase and protease inhibitors, homogenized by vortex and boiled for 10 minutes. The protein concentration was determined by the Bradford method.

**Co-precipitation protein/HCV RNA**

Huh7.25.CD81 cells were incubated for 10 min in their culture medium containing 1/10 volume (Vol) of a crosslinking solution (11% Formaldehyde, 0.1 M NaCl, 1 mM Na-EDTA-[pH 8], 0.5 mM Na-EGTA-[pH 8], 50 mM NaH2PO4, 50 mM NaHPO4 [pH 8.0]), briefly sonicated, and centrifuged at 14,000 g for 15 min at 4°C. 1/10th of the lysate was subjected to precipitation with 10% trichloroacetic acid for protein analysis in whole cell extracts. The rest of the lysate was incubated for 2 hrs with 20 μl (packed volume) of Talon resin Ni-affinity beads (Clontech) on a rotating wheel. Bound proteins were washed four times in Gaue buffer, three times in Urea 6.3 buffer (8 M Urea, 10 mM TRIS, 0.1 M Na2HPO4, 20 mM Imidazole [pH 6.3]), and three times in cold PBS, after which they were eluted by boiling in NuPAGE LDS sample buffer. Electrophoresis was performed on 4–12% of acrylamide NuPAGE gels (Invitrogen).
RNA was quantified by one-step RTqPCR as described previously. The immunoprecipitated proteins from set 2 were extracted at 70°C using NuPAGE LDS sample buffer and analysed by immunoblot after electrophoresis on 4–12% of acrylamide NuPAGE gels (Invitrogen).

**Supporting Information**

**Figure S1** Efficient induction of TRIM25 by IFN in the Huh7.25.CD81 cells. Huh7.25.CD81 cells, seeded at 8 x 10⁴ cells in 24-well plates containing coverslips, were treated with 500 U/ml of IFNα for 24 hrs (IFN) or left untreated (Cont). Cells were fixed with 4% PFA and TRIM25 was detected using anti-TRIM25 antibodies (red). Nuclei are shown in blue after DAPI labelling. Microscope magnification was ×63.

(PDF)

**Figure S2** HCV controls RIG-I ubiquitination through ISG15 in the Huh7 cells. Huh7 cells were transfected for 24 hrs with 25 nM of siRNA (Control or ISG15) and for another 24 hr with 5 μg of a His-MyC-Ubiquitin plasmid in absence or presence of 5 μg of a plasmid expressing HA-TRIM25. The cells were infected with JFH1 (m.o.i = 0.2). At the times indicated, cell extracts were processed for analysis of RIG-I ubiquitination and the expression of the different proteins in the total cell extracts. Efficiency of infection by JFH1 in the Huh7 cells was 2 log less than in the Huh7.25.CD81 cells.

(PDF)

**Figure S3** Expression of ISG15 and ISG15 conjugating enzymes inhibit IFN induction in response to SeV. Huh7.25.CD81 cells were transfected with a plasmid expressing HA-ISG15 alone or in the presence of plasmids expressing the ISG15 conjugating enzymes Ubc1L (E1), UbcH8 (E2) and HERC5 (E3). The cells were then infected with JFH1 (m.o.i = 6) for the times indicated. Stimulation of endogenous IFNβ RNA expression was determined by RTqPCR and expressed as fold induction. The degree of statistical significance is indicated by stars after calculation of the p-values (from left to right: 0.0124 and 0.0058).

(PDF)

**Figure S4** Control of efficiency of siRNA Ube1L in the Huh7.25.CD81 cells. The Huh7.25.CD81 cells were transfected with 50 nM of siRNA directed against Ube1L for 48 hours and infected with HCV. RNA was prepared from the cells at different times post infection as indicated and expression levels of Ube1L were determined by RTqPCR.

(PDF)

**Figure S5** Modulation of PKR activation by ISG15. Huh7.25.CD81 cells, in 100 cm² plates, were transfected with siRNA Control or siRNA ISG15 or transfected with a plasmid expressing HA-ISG15 for 48 hrs and infected with JFH1 (m.o.i = 6). At the indicated times post-infection, cell extracts (2.2 mg) were processed for immunoprecipitation of PKR. The immunoprecipitated complexes were run on two different NuPAGE gels and blotted using Mab 71/10 or anti-phosphorylated PKR antibodies (PKR-P). The presence of PKR and PKR-P was revealed using the Odyssey procedure. The bands corresponding to total PKR and phosphorylated PKR were quantified using the Odyssey software and expressed as the ratio PKR-P/PKR in the absence (siISG15) and in the presence of ISG15 in the control cells (Control) or after transfection of the ISG15 expressing plasmid (HA-ISG15).

(PDF)

**Figure S6** Induction of ISG56 by Sendai virus in the Huh7.25.CD81 cells does not depend on PKR. Huh7.25.CD81 cells were either transfected with 25 nM of siRNA Control or 25 nM siPKR for 24 hrs and infected with SeV for the times indicated. The effect of PKR silencing on the stimulation of expression of endogenous ISG56 was determined by RTqPCR and expressed as fold induction. Error bars represent the mean ± S.D for triplicates. The expression levels of ISG56 RNA at the start of infection were respectively 1.15×10⁵ copies (siControl) and 1.16×10⁵ copies (siPKR).

(PDF)

**Figure S7** Control of the efficiency of siRNA treatment in the Huh7.25.CD81 cells. The Huh7.25.CD81 cells were transfected for 48 hrs with 50 nM Control siRNA or with the different Smartpool siRNAs as shown (50 nM siPKR; 10 nM siRIG-I; 50 nM siIRF3; 50 nM siTRAF3; 5 nM siMAVS). Total cell extracts were prepared and the expression level of each protein, as well as that of actin used as control, was revealed by immunoblot and Odyssey procedure after a run on NuPAGE gels. Under each lane, the numbers represent the quantification of the different protein bands performed using the Odyssey software.

(TIF)

**Figure S8** HCV triggers nuclear translocation of IRF3 early after infection in the Huh7.25.CD81 cells. The Huh7.25.CD81 cells, seeded at 10⁵ cells in 24-well plates containing coverslips, were infected for different times (0, 4 and 6 hours) at 37°C with JFH1 (moi = 6) or with SeV (40 HAU/ml) in the absence or in the presence of 10 ng/ml Leptomycine B (LB; Sigma), which was used here as a convenient mean to enhance the nuclear detection of IRF3 since it can interfere with nuclear export [57]. Cells were fixed with 4% PFA and IRF3 was detected using anti-IRF3 antibodies (red). Nuclei are shown in blue after DAPI labelling. The arrows show the presence of IRF3 in the nucleus. Microscope magnification was ×63.

(PDF)

**Figure S9** Induction of ISG56 by HCV in the Huh7.5 and Huh7 cells depends on PKR. Huh7.5 or Huh7 cells were transfected with siRNA Control or siPKR (50 nM) for 48 hrs and infected with JFH1 (m.o.i = 0.2 for Huh7.5 or 10 for Huh7). At the times indicated, expression of endogenous ISG56 was determined by RTqPCR and expressed as fold induction. Error bars represent the mean ± S.D for triplicates. The expression levels of ISG56 RNA at the start of infection in the siControl cells was 1.37×10⁴ copies (Huh7.5 cells) and 1.28×10⁴ copies (Huh7 cells).

(PDF)

**Figure S10** Induction of ISG56 by HCV is specifically inhibited by the PKR inhibitor PRI. The Huh7.25.CD81 cells were incubated with PRI or C16 and infected with JFH1 (m.o.i = 0.2) for the times indicated. RTqPCR analysis of endogenous ISG56 was determined by RTqPCR and expressed as fold induction. The expression levels of ISG56 RNA at the start of infection in the control cells was 1.97×10⁴ copies. (TIF)

**Figure S11** The RNAse inhibitor RNAsin does not favour the formation of a RIG-I/PKR complex upon HCV infection. Two sets of Huh7.25.CD81 cells were plated into 100 cm² plates and infected with JFH1. At the times indicated, cell extracts (3.5 mg) from the two sets were processed similarly for immunoprecipitation of PKR or for incubation with mouse IgG as a control of specificity (asterisk), except that care was taken to add the RNase inhibitor RNAsin (1000 U/ml) at all steps for the second set (+RNAsin). Detection of RIG-I, MAVS, and PKR in
Acknowledgments

We thank Michael Gale Jr, Pierre-Olivier Vidalain and Christine Neuvet for critical reviews of the manuscript. We thank Adrien Six, Eric Batsche and Agata Budkowska for discussions. We thank Ernest Borden for the gift of anti-ISG15 antibodies, Jon Huibregste for the gift of plasmids expressing ISG15 conjugating enzymes, Dominique Garcin for providing the Senilad virus, Claire Gondev and Maritime Sanjait for their help in the preparation and infection of human primary hepatocytes with JFH1.

Author Contributions

Conceived and designed the experiments: NA TW EFM. Performed the experiments: NA DA MF FS-O. Analyzed the data: NA TW EFM. Contributed reagents/materials/analysis tools: JH DA MF TW. Wrote the paper: NA EFM.
40. Shimoike T, McKenna SA, Lindhout DA, Puglisi JD (2009) Translational insensitivity to potent activation of PKR by HCV IRES RNA. Antiviral Res 83: 228–237.
41. Nallagatla SR, Hwang J, Toroney R, Zheng X, Cameron CE, et al. (2007) 5′-triphosphate-dependent activation of PKR by RNAs with short stem-loops. Science 318: 1455–1458.
42. Gil J, Garcia MA, Gomez-Puertas P, Guerra S, Rullas J, et al. (2004) TRAF family proteins link PKR with NF-kappa B activation. Mol Cell Biol 24: 4302–4312.
43. Oganesyan G, Saha SK, Guo B, He JQ, Shahangian A, et al. (2006) Critical role of TRAF3 in the Toll-like receptor-dependent and -independent antiviral response. Nature 439: 288–291.
44. Zhang P, Samuel CE (2008) Induction of protein kinase PKR-dependent activation of interferon regulatory factor 3 by vaccinia virus occurs through adapter IPS-1 signaling. J Biol Chem 283: 34580–34587.
45. McAllister CS, Samuel CE (2009) The RNA-activated protein kinase enhances the induction of interferon-beta and apoptotic mediated by cytoplasmic RNA sensors. J Biol Chem 284: 1644–1651.
46. McAllister CS, Toth AM, Zhang P, Devaux P, Cattaneo R, et al. (2010) Mechanisms of protein kinase PKR-mediated amplification of beta interferon induction by C protein-deficient measles virus. J Virol 84: 380–386.
47. Strahle L, Mayer JB, Briti A, Hausmann S, Kolakofsky D, et al. (2007) Activation of the beta interferon promoter by unnatural Sendai virus infection requires RIG-I and is inhibited by viral C proteins. J Virol 81: 12227–12237.
48. Bitoun-Andreani C, Raulet E, Pichard-Garcia L, Maurel P (2010) Use of human hepatocytes to investigate blood coagulation factor. Methods Mol Biol 640: 431–445.
49. Strahle L, Garcia D, Kolakofsky D (2006) Sendai virus defective-interfering genomes and the activation of interferon-beta. Virology 353: 101–111.
50. Jammi NV, Whitty LR, Beal PA (2003) Small molecule inhibitors of the RNA-dependent protein kinase. Biochem Biophys Res Commun 308: 50–57.
51. Nekhai S, Bottozo GP, Woldchawariat G, Spellerberg A, Petryshyn R (2000) A cell-permeable peptide inhibits activation of PKR and enhances cell proliferation. Peptides 21: 1449–1456.
52. Grandvaux N, Servant MJ, ten Oever B, Sen GC, Balachandran S, et al. (2002) Transcriptional profiling of interferon regulatory factor 3 target genes: direct involvement in the regulation of interferon-stimulated genes. J Virol 76: 5532–5539.
53. Bonnet MC, Daurat C, Ottone C, Meurs EF (2006) The N-terminus of PKR is responsible for the activation of the NF-kappaB signaling pathway by interacting with the IKK complex. Cell Signal 18: 1865–1875.
54. Malakhov MP, Kim KI, Malakhova OA, Jacobs BS, Borden EC, et al. (2003) High-throughput immunoblotting. Ubiquitin-like protein ISG15 modifies key regulators of signal transduction. J Biol Chem 278: 16608–16613.
55. Laurent AG, Krust B, Galabru J, Scab J, Hovanessian AG (1985) Monoclonal antibodies to interferon induced 68,000 Mr protein and their use for the detection of double-stranded RNA dependent protein kinase in human cells. Proc Natl Acad Sci USA 82: 4341–4345.
56. Iwano S, Ichikawa M, Takizawa S, Hashimoto H, Miyamoto Y (2010) Identification of AhR-regulated genes involved in PAH-induced immunotoxicity using a highly-sensitive DNA chip, 3D-Gene Human Immunity and Metabolic Syndrome 9k. Toxicol In Vitro 24: 85–91.
57. Wolff B, Sanglier JJ, Wang Y (1997) Leptomycin B is a inhibitor of nuclear export: inhibition of nucleo-cytoplasmic translocation of the human immunodeficiency virus type 1 (HIV-1) Rev protein and Rev-dependent mRNA. Chem Biol 4: 139–147.