

# Innate immunity induced by composition-dependent RIG-I recognition of hepatitis C virus RNA

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**Innate immune defences are essential for the control of virus infection and are triggered through host recognition of viral macromolecular motifs known as pathogen-associated molecular patterns (PAMPs)<sup>1</sup>. Hepatitis C virus (HCV) is an RNA virus that replicates in the liver, and infects 200 million people worldwide<sup>2</sup>. Infection is regulated by hepatic immune defences triggered by the cellular RIG-I helicase. RIG-I binds PAMP RNA and signals interferon regulatory factor 3 activation to induce the expression of interferon- $\alpha/\beta$  and antiviral/interferon-stimulated genes (ISGs) that limit infection<sup>3–10</sup>. Here we identify the polyuridine motif of the HCV genome 3' non-translated region and its replication intermediate as the PAMP substrate of RIG-I, and show that this and similar homopolyuridine or homopolyriboadenine motifs present in the genomes of RNA viruses are the chief feature of RIG-I recognition and immune triggering in human and murine cells<sup>8</sup>. 5' terminal triphosphate on the PAMP RNA was necessary but not sufficient for RIG-I binding, which was primarily dependent on homopolymeric ribonucleotide composition, linear structure and length. The HCV PAMP RNA stimulated RIG-I-dependent signalling to induce a hepatic innate immune response *in vivo*, and triggered interferon and ISG expression to suppress HCV infection *in vitro*. These results provide a conceptual advance by defining specific homopolymeric RNA motifs within the genome of HCV and other RNA viruses as the PAMP substrate of RIG-I, and demonstrate immunogenic features of the PAMP–RIG-I interaction that could be used as an immune adjuvant for vaccine and immunotherapy approaches.**

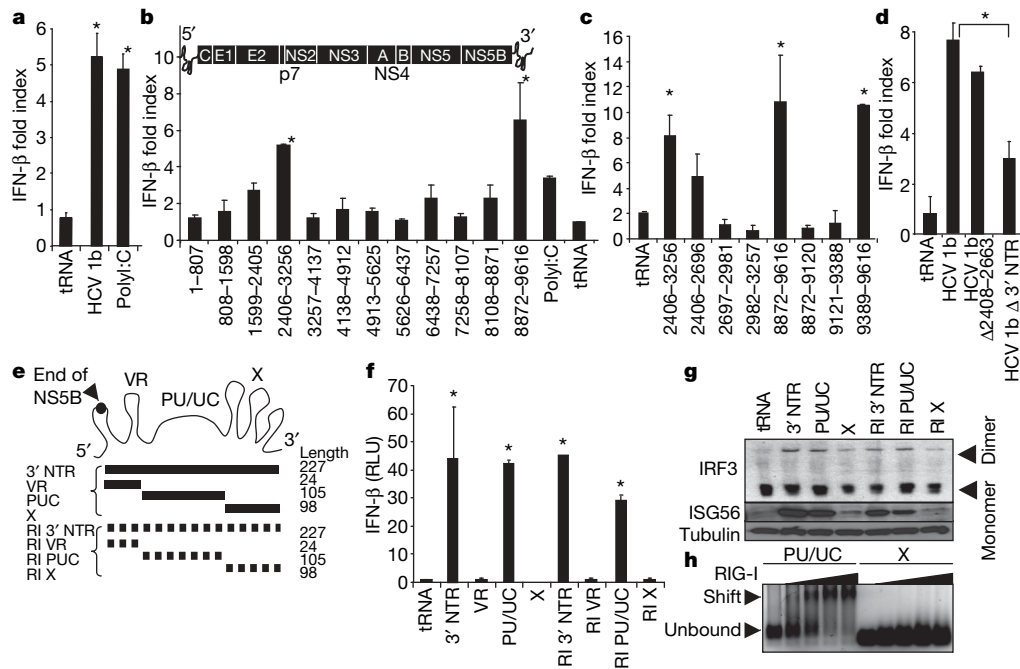
To determine the nature of the HCV PAMP RNA, we conducted a functional screen to identify HCV PAMP RNA motifs. We assessed the ability of 1  $\mu$ g of HCV genome RNA or contiguous subgenomic segments to trigger the interferon (IFN)- $\beta$  promoter in transfected human Huh7 cells. The full-length HCV genome triggered innate immune signalling to induce the IFN- $\beta$  promoter (Fig. 1a). Two regions of the HCV RNA, encoding nucleotides 2406–3256 and 8872–9616, significantly induced the IFN- $\beta$  promoter (Fig. 1b), with signalling activity respectively localized to nucleotides 2406–2696 of the open reading frame and 9389–9619 encoding the 3' non-translated region (NTR) (Fig. 1c). Deletion of the 3' NTR but not nucleotides 2408–2663 from the HCV genome significantly attenuated promoter signalling (Fig. 1d). PAMP motifs are typically conserved among strains of a pathogen<sup>1</sup>, and sequence comparison of multiple HCV genomes revealed global variability within nucleotides 2406–2696 among virus strains, but nucleotides 9389–9616 encoded motifs of high conservation (Supplementary Fig. 1)<sup>11</sup>. Thus, the viral 3' NTR might encode HCV PAMP motifs that trigger innate immune signalling in the host cell.

The HCV 3' NTR is comprised of three regions: a variable region with potential secondary structure; a non-structured poly-U/UC

region containing polyuridine with interspersed ribocytidine; and the terminal X region containing three conserved stem-loop structures (Fig. 1e)<sup>12</sup>. We evaluated the ability of RNA encoding the HCV 3' NTR or each of its regions to trigger intracellular signalling. Because HCV RNA replicates through a negative-sense replication intermediate<sup>2,13</sup>, we included analyses of signalling triggered by the replication intermediate counterparts of the 3' NTR and its composite regions. RNA encoding the genomic or replication intermediate poly-U/UC region was sufficient to trigger signalling to the IFN- $\beta$  promoter, but neither the variable nor X region genomic and replication intermediate RNA induced promoter signalling (Fig. 1f). The HCV 3' NTR and poly-U/UC, but not the X region, RNA motifs similarly stimulated signalling when introduced into HeLa cells (Supplementary Fig. 2a). Moreover, in Huh7 cells genomic or replication intermediate 3' NTR and poly-U/UC RNA each stimulated the formation of active interferon regulatory factor 3 (IRF3) dimers and expression of ISG56, an IRF3 target gene<sup>3</sup>, but X region RNA failed to trigger either (Fig. 1g). The poly-U/UC, but not X region, RNA formed a stable complex with purified RIG-I (Fig. 1h). These results define the 100-nucleotide poly-U/UC region of the HCV genome and replication intermediate RNA as the HCV PAMP motif and potential substrate of RIG-I signalling. We also found that the entire HCV 5' NTR, which contains four major stem-loop structures comprising the viral internal ribosome entry site<sup>14</sup>, was only a weak inducer of promoter signalling. However, prior treatment of Huh7 cells with IFN- $\beta$  to increase RIG-I levels<sup>4</sup> rendered them responsive to signalling triggered by the HCV 5' NTR or X region RNA (Supplementary Fig. 3). Thus, double-stranded (ds)RNA regions of the HCV RNA are not potent PAMPs but may confer signalling during the IFN response.

To determine the role of RIG-I or other pathogen recognition receptor (PRR) pathways in HCV PAMP signalling, we first examined IFN- $\beta$  promoter induction in Huh7.5 cells encoding non-functional RIG-I<sup>4</sup>. The cells were refractory to HCV RNA-induced signalling whereas their response was rescued and enhanced on over-expression of wild-type RIG-I (Fig. 2a). MDA5 is a PRR related to RIG-I that binds to dsRNA<sup>15</sup>, whereas MyD88 and TRIF are essential adaptor proteins used by Toll-like receptor (TLR) 7/8 and TLR3, respectively, which are PRRs that recognize endosomal single-stranded poly-U RNA or dsRNA<sup>1,16</sup>. We examined PAMP signalling in mouse embryo fibroblasts (MEFs) lacking RIG-I, MDA5, MyD88 or TRIF (Figs 2b–e, Supplementary Fig. 4). When introduced into RIG-I<sup>-/-</sup> MEFs the HCV RNAs did not trigger promoter activation, but 3' NTR and poly-U/UC RNA, but not X region RNA, stimulated signalling in wild-type, MDA5<sup>-/-</sup>, Myd88<sup>-/-</sup> or Trif<sup>-/-</sup> cells. In Huh7 cells, poly-U/UC RNA co-localized and mediated a specific interaction with RIG-I (Fig. 2f). Thus, RIG-I is the essential PRR that signals the innate immune response triggered by HCV poly-U/UC

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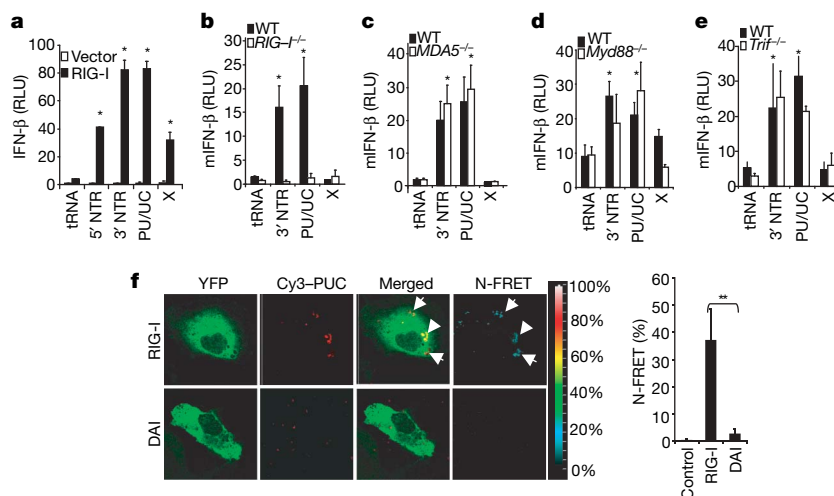
**Figure 1 | Identification of HCV PAMP RNA.** **a–d**, RNA-induced IFN- $\beta$  promoter luciferase activity in Huh7 cells, shown as mean fold index induction (compared to non-treated cells;  $\pm$ s.d.). Huh7 cells were transfected with 1  $\mu$ g (0.4 pmol) of HCV N (HCV 1b) genome RNA, 1  $\mu$ g of poly inosine:cytosine (polyI:C) RNA (control) or with 1  $\mu$ g of the indicated RNA species and harvested for dual luciferase assay 16 h later. HCV 1b refers to HCV genome RNA; tRNA, transfer RNA control. Nucleotide numbers encoded by HCV RNA constructs are shown in **b–d**. Bars are placed in their relative positions of each region within the HCV genome shown in **b**. The 5' NTR, protein coding regions and 3' NTR are indicated. **e**, The HCV 3' NTR motifs and respective RNA constructs. RI and broken lines denote

replication intermediate. PUC, PU/UC; VR, variable region. **f**, IFN- $\beta$  promoter activation, shown here and in the other figures as mean relative luciferase units (RLU;  $\pm$ s.d.), triggered by 1  $\mu$ g of the indicated RNA species in transfected Huh7 cells. **g**, The abundance of IRF3, ISG56 and tubulin (control) was measured by immunoblot. The upper panel shows the active IRF3 dimer and inactive monomer forms separated by non-denaturing PAGE. **h**, RNA binding/gel-shift analysis of purified RIG-I with poly-U/UC or X region RNA (6 pmol) reacted with 0, 10, 20, 40, or 60 pmol of RIG-I protein. All RNAs contain 5' ppp. Asterisks indicate significant difference ( $P < 0.01$ ) as determined by Student's *t*-test.

RNA independently of MDA5-, MyD88- or TRIF-dependent PRR pathways.

RIG-I binds to PAMP RNA containing 5' terminal triphosphate (5'ppp) through which the triphosphate end is proposed to anchor the RNA within charged residues of the RIG-I repressor domain,

causing a conformation change to displace the repressor domain and release signalling autorepression<sup>17–19</sup>. Gel-shift assays revealed that 5'ppp was required for poly-U/UC RNA binding by RIG-I but did not mediate stable RIG-I interaction with X region RNA (Fig. 3a). 5'ppp was required for IFN- $\beta$  promoter signalling by poly-U/UC



**Figure 2 | RIG-I-specific HCV RNA PAMP recognition and signalling.** **a–e**, Induction of the IFN- $\beta$  promoter in cells co-transfected with 1  $\mu$ g of tRNA control or the indicated HCV RNA species. **a**, Huh7.5 cells, lacking functional RIG-I, were co-transfected with a plasmid encoding vector alone or RIG-I. Promoter signalling in wild-type (WT) and RIG-I<sup>-/-</sup> (**b**), MDA5<sup>-/-</sup> (**c**), Myd88<sup>-/-</sup> (**d**) or Trif<sup>-/-</sup> (**e**) MEFs. Asterisks indicate a significant difference ( $P < 0.01$ ) from tRNA control. Similar results were obtained when cells were transfected with 30 pmol of each RNA (data not

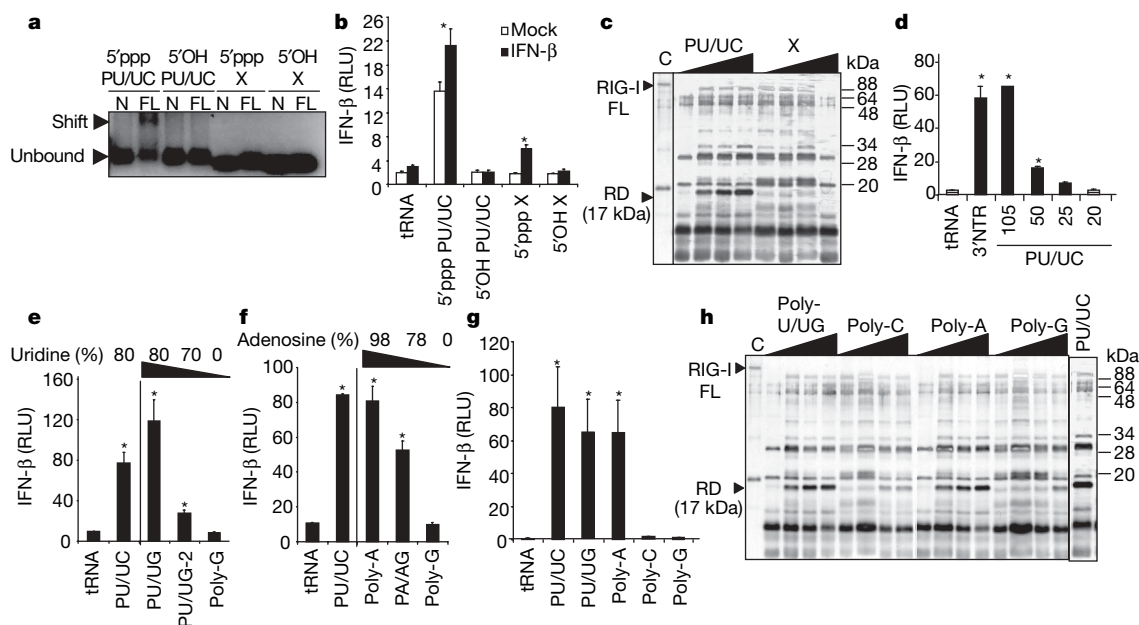
shown). **f**, FRET analysis of Cy3-labelled poly-U/UC RNA (Cy3-PUC) interaction with YFP-RIG-I or YFP-DAI protein in co-transfected Huh7 cells. Panels show representative images of YFP, Cy3, merged fluorescence and N-FRET (corrected FRET). The colour scale denotes N-FRET levels. The bar graph at the right shows the calculated values for RNA interaction with RIG-I or DAI (% N-FRET;  $\pm$ s.d.). Control values are from the image area that has no co-localization signal. All RNAs contain 5'ppp.

RNA, and supported low-level promoter induction triggered by X region RNA in IFN-treated cells (Fig. 3b). Because X region RNA failed to form a stable complex with RIG-I and only weakly triggered signalling, stable RIG-I–RNA interaction is probably required to release RIG-I autorepression. We therefore conducted limited trypsin digestion analysis of purified RIG-I alone or bound to poly-U/UC or X region RNA containing 5' ppp. This approach provides an assessment of RIG-I repressor domain displacement in response to PAMP RNA binding wherein the displaced repressor domain of signalling-active RIG-I presents as a protected 17-kDa fragment<sup>17–19</sup>. As shown in Fig. 3c, RIG-I binding of poly-U/UC but not X region RNA rendered the protected 17-kDa repressor domain fragment. These results demonstrate that 5' ppp is necessary but not sufficient for RNA binding by RIG-I wherein the HCV poly-U/UC RNA directs stable interaction with RIG-I in a 5' ppp-dependent manner that confers signalling activation. We used 5' ppp RNA in all further experiments.

The HCV polyU/UC region is a flexible motif among HCV strains and is essential for viral replication<sup>13</sup>. In the HCV genotype 1b strain used in these experiments, the poly-U/UC region is comprised of 100 nucleotides containing 78% uridine and 22% ribocytosine (Supplementary Fig. 5). A reduction of length through progressive 3' truncation to 50 nucleotides or fewer attenuated signalling in Huh7 cells (Fig. 3d), consistent with reduced RIG-I binding of short RNAs<sup>20</sup>. Replacement of ribocytosine with riboguanine (poly-U/UG) had no impact on PAMP signalling, but progressive replacement of uridine for riboguanine to below 80% uridine or 100% polyriboguanine (poly-G) reduced or abrogated PAMP signalling (Fig. 3e). Because the replication intermediate poly-U/UC RNA contains high polymeric riboadenine content, we also examined the impact of

poly-A composition and length on RIG-I signalling. One-hundred-nucleotide poly-A RNA and poly-U/UC RNA equally induced signalling to the IFN- $\beta$  promoter, whereas reduced riboadenine content of the former through progressive nucleotide replacement to poly-G attenuated or ablated signalling (Fig. 3f). In side-by-side analyses we found that poly-U/UC, poly-U/UG and poly-A RNA, but neither poly-C nor poly-G RNA, could trigger signalling to the IFN- $\beta$  promoter in Huh7 (Fig. 3g) and HeLa cells (Supplementary Fig. 2b). Truncation of the poly-A RNA to 50 nucleotides or fewer attenuated signalling to the IFN- $\beta$  promoter to the same extent as truncation of poly-U/UC (Supplementary Fig. 2c). Whereas poly-C and poly-G RNA bound negligibly to RIG-I, poly-U/UC, poly-U/UG and poly-A RNA formed a stable complex with RIG-I (Supplementary Fig. 5e) that released the RIG-I repressor domain to the active conformation (Fig. 3h). These results define polymeric uridine or riboadenine motifs of 50 nucleotides or greater—including the PU/UC and replication intermediate PU/UC motif of HCV—as the PAMP signature within 5' ppp RNA that is efficiently recognized by RIG-I to trigger the immune response.

To determine whether RIG-I recognition of the HCV poly-U/UC PAMP motif triggers hepatic innate immune defences *in vivo*, we conducted RNA signalling analysis in wild-type and RIG-I<sup>-/-</sup> mice. Intravenous administration of full-length HCV 1b genome stimulated hepatic *Ifnb* messenger RNA expression within 8 h in wild-type mice but not in RIG-I<sup>-/-</sup> mice, and signalling was significantly attenuated on deletion of the HCV 3' NTR (Fig. 4a). Moreover, the poly-U/UC RNA motif, but not the X region RNA motif, was sufficient to trigger the hepatic IFN- $\beta$  expression in wild-type but not in RIG-I<sup>-/-</sup> mice. In time course studies we found that the poly-U/UC RNA motif



**Figure 3 | Polyuridine and polyriboadenine ribonucleotides are RIG-I ligands.** **a**, Gel-shift analysis of complex formation between 25 pmol of purified N-RIG (RIG-I amino acids 1–228, control) or full-length RIG-I (FL) and 10 pmol of poly-U/UC (PU/UC) or X region RNA containing 5' ppp or 5' OH as indicated. Arrows denote position of unbound RNA and RNA–RIG-I complexes. **b**, Effect of 5' ppp on IFN- $\beta$  promoter activity. Huh7 cells were either mock-treated or treated with IFN- $\beta$  8 h before transfection with 1  $\mu$ g (30 pmol) of RNA. **c**, Effect of poly-U/UC or X region RNA on RIG-I activation. The silver-stained gel image shows trypsin digestion products of RIG-I that was pre-incubated with increasing amounts poly-U/UC or X region RNA. Arrows indicate positions of full-length (FL) RIG-I and the 17-kDa trypsin-resistant repressor domain (RD) from RIG-I–RNA complexes. **d**, Effect of nucleotide length of 1  $\mu$ g poly-U/UC 3' truncation products on IFN- $\beta$  promoter signalling in Huh7 cells. IFN- $\beta$  promoter activation is

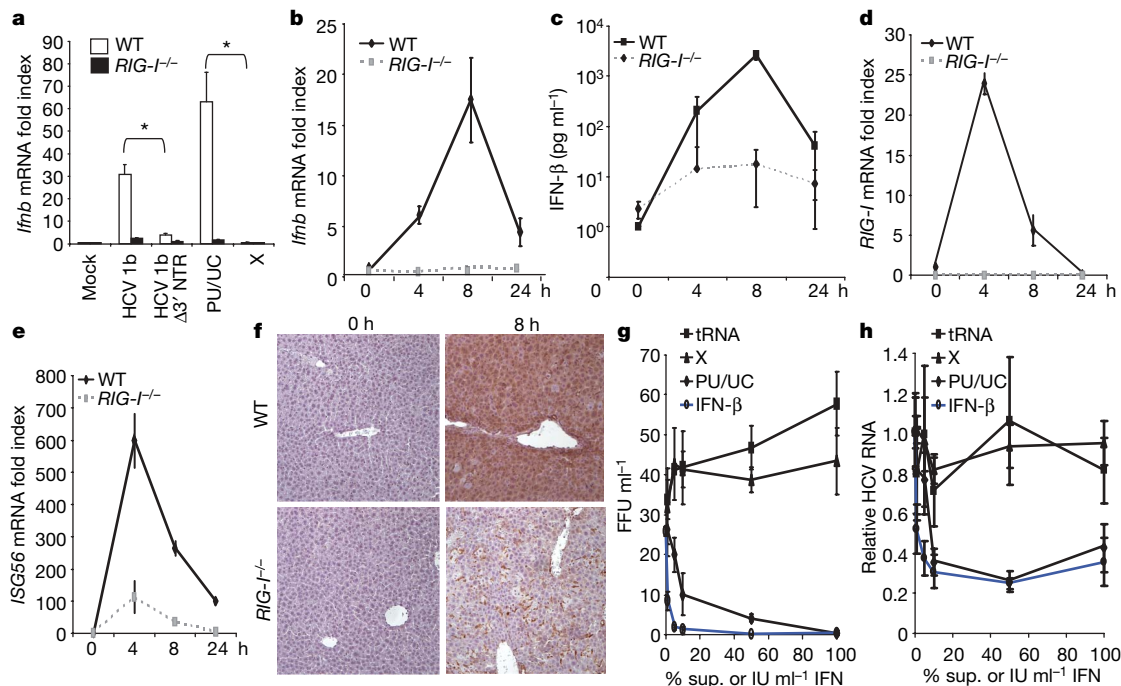
shown as mean relative luciferase units (RLU;  $\pm$ s.d.). Similar results were obtained when cells were transfected with 30 pmol of each RNA (data not shown). **e–g**, Effect of nucleotide composition on IFN- $\beta$  promoter signalling in Huh7 cells transfected with 1  $\mu$ g (30 pmol) of RNA. **h**, Effect of nucleotide composition on RIG-I activation. The silver-stained gel image shows trypsin digestion products of RIG-I that was pre-incubated with increasing amounts poly-U/UG, poly-C, poly-A, or poly-G RNA. Arrows indicate positions of full-length RIG-I and the 17-kDa trypsin-resistant repressor domain. We confirmed the 17-kDa fragment as the RIG-I repressor domain by immunoblot analysis of the digestion products using an antiserum specific to the RIG-I carboxy terminus (not shown), as previously described<sup>17</sup>. Asterisks indicate significant difference ( $P < 0.01$ ) as determined by Student's *t*-test.



induced a peak of hepatic *Ifnb* mRNA expression and IFN- $\beta$  serum levels at 8 h after injection in wild-type mice (Fig. 4b, c). This response was associated with induced hepatic expression of *RIG-I* and *ISG56* mRNA and tissue-wide expression of hepatic ISG54 (Fig. 4d–f), similar to the hepatic response in HCV-infected patients<sup>9,10</sup>. *RIG-I*<sup>-/-</sup> mice expressed only a low level of *Ifnb* and *ISG56*. The tissue-wide nature of hepatic ISG54 expression in wild-type mice suggests that paracrine signalling of IFN- $\beta$  could have an important role in hepatic defences against HCV. To test this idea we measured HCV production in infected Huh7 cells that were treated with IFN- $\beta$  or supernatants collected from cultures transfected with HCV poly-U/UC RNA, X-region RNA, or tRNA (control). Poly-U/UC RNA triggered IFN- $\beta$  expression in the transfected cells (data not shown), and only treatment with IFN- $\beta$  or supernatant from the poly-U/UC-transfected cells induced a response that suppressed HCV infection (Fig. 4g, h). Thus, the poly-U/UC RNA is an HCV genome PAMP that is necessary and sufficient to trigger RIG-I signalling of the hepatic innate immune response. The actions of RIG-I signalling can induce an antiviral response directly (Fig. 1g), as well as through indirect, paracrine actions of IFN produced from HCV PAMP signalling (Fig. 4g, h).

Our results provide new insights into the features of PAMP specificity of RIG-I wherein RNA virus genome sequences consisting of poly-U and respective replication intermediate poly-A motifs of length >50 nucleotides are the determinants that confer efficient RIG-I binding and signalling. 5' ppp was necessary but not sufficient for stable binding of HCV PAMP RNA by RIG-I. In terms of the HCV genome, well defined internal RNA interactions of the 5' and 3' ends<sup>21,22</sup> could provide 5' ppp and PAMP motif proximity for stable RIG-I binding. The poly-U/UC motif is an essential determinant of

HCV replication fitness<sup>21</sup>. Thus, as the virus must maintain this motif for its viability, the host takes advantage of this requirement and targets the poly-U/UC region as a discriminator of PAMP RNA through RIG-I interaction. Poly-U and/or poly-A motifs are present in localized regions in the genome of RNA viruses known to trigger RIG-I signalling (Supplementary Table 1)<sup>5,8,19</sup>. We found that 5' ppp genomic poly-U/A-rich RNA motifs within the rabies virus leader sequence, Ebola virus 3' region, or the measles virus leader sequence each triggered signalling to the IFN- $\beta$  promoter in Huh7 cells, but GC-rich RNA motifs from each viral genome did not trigger a significant response (Supplementary Fig. 7a, b). We also found that pppT3-63, Tri-GFP and EGFP 2 T7 RNAs, previously described as RIG-I substrates and comprised of 50% or fewer A/U nucleotides<sup>5,18,23</sup>, could induce only weak signalling to the IFN- $\beta$  promoter compared to the poly-U/UC RNA. Thus, A/U composition and poly-U motifs are the major determinants of viral PAMP RNA recognition by RIG-I. Cellular RNAs also contain poly-U and poly-A motifs but mRNAs are typically capped and are bound by poly-A binding proteins<sup>24</sup>, whereas ribosomal RNAs are 'masked' as ribonucleoprotein complexes<sup>25</sup>. These features and the context of 5' ppp with viral poly-U and poly-A motifs serve to identify self from non-self RNA by governing RIG-I recognition, wherein, non-self recognition of the HCV PAMP RNA triggers a hepatic innate immune response. These observations provide a possible explanation of why 25% or more of all HCV-exposed people clear acute infection<sup>2</sup> and why HCV needs to evade innate immunity through viral NS3/4A protease targeting of the RIG-I pathway<sup>26,27</sup>. RIG-I substrates such as the poly-U/UC RNA or structurally similar compounds could provide therapeutic application as immune adjuvants similar to TLR agonists<sup>28</sup>, and offer innate immune stimulatory properties that



**Figure 4 | HCV PAMP RNA triggers the hepatic innate immune response and anti-HCV defences.** **a–f**, Wild-type or *RIG-I*<sup>-/-</sup> mice ( $n = 3$ ) were hydrodynamically transfected intravenously with HCV RNA. **a**, Mice received 100  $\mu$ g of HCV 1b genome, HCV 1b genome lacking the 3' NTR (HCV 1b  $\Delta$ 3' NTR), PU/UC RNA or X region RNA. Hepatic *Ifnb* mRNA expression was measured 8 h later. **b–f**, Wild-type or *RIG-I*<sup>-/-</sup> mice ( $n = 3$ ) received 200  $\mu$ g of poly-U/UC RNA or buffer control, and were killed 4, 8 or 24 h later for comparative measurement of mRNA and protein expression. **b**, Liver-specific expression of *Ifnb* mRNA. **c**, Serum IFN- $\beta$  protein levels. **d**, Liver-specific expression of *RIG-I* mRNA. **e**, Liver-specific expression of *ISG56* mRNA. **f**, Immunohistochemical stain of ISG54 protein expression in liver tissue sections. **g, h**, Paracrine antiviral effect of the innate immune

response triggered by HCV PAMP RNA. **g**, Inhibition of HCV infection in pre-treated cells. Triplicate cultures of Huh7.5 cells were treated with DMEM containing increasing concentrations of IFN- $\beta$  or conditioned media collected from Huh7 cells transfected with the indicated RNA species for 12 h before HCV infection. The graph shows the number of infected cells ( $\pm$ s.d.) as determined by focus-forming unit (FFU) assay at 48 h after infection. **h**, Huh7.5 cells were infected with HCV for 48 h and then were treated with increasing international units per ml concentrations of IFN- $\beta$  (IU ml<sup>-1</sup> IFN) or the indicated amount of conditioned media (% sup.) for an additional 48 h. Intracellular HCV RNA levels relative to *GAPDH* were determined and are plotted as mean HCV RNA index ( $\pm$ s.d.) relative to infected, untreated cells.

may improve IFN-based therapy for HCV through paracrine immune actions that limit infection<sup>2</sup>.

## METHODS SUMMARY

**RNA.** RNA constructs and quality control analysis are shown in Supplementary Fig. 5. 5' ppp RNA was synthesized using the T7 Megascript kit (Ambion). Full-length and subgenomic HCV RNA were produced from plasmid DNA or T7 promoter-linked PCR products generated from cloned HCV N (A gift from S. Lemon) or Con1 genome (HCV genotype-1b)<sup>29</sup>. 5'OH RNAs were purchased from Fidelity Systems. RNA transfection was performed using 1 µg of RNA per 1 × 10<sup>5</sup> cells with the Transmessenger reagent (Qiagen). One microgram of RNA mass approximates to the following picomoles: full-length HCV 1b genome, HCV 1b Δ2408–2663 and HCV 1b Δ3' NTR, 0.4 pmol; HCV 1b subgenomic RNA constructs, 5 or 15 pmol; HCV genotype 1b (Con1) 5' NTR, 3' NTR (20 pmol), and all other RNA constructs, 30–150 pmol. RNA concentrations in the transfection mix were 5–10 µg ml<sup>-1</sup>. Additionally, in experiments to assess signalling induced by subgenomic HCV RNA, we also measured promoter expression triggered by equimolar amounts of RNA. RNA delivery was assessed as described in Supplementary Fig. 6. mRNA expression was determined by quantitative polymerase chain reaction with reverse transcription assay. DNA oligonucleotides used in this study are described in Supplementary Table 2.

**RNA signalling analysis.** IFN-β promoter luciferase analyses of transfected cells were conducted as described<sup>17</sup>. Protein expression and the abundance of IRF3 dimer and monomeric forms in cells were determined by immunoblot analysis<sup>17</sup>.

**RIG-I purification and RNA binding analysis.** Full-length RIG-I or RIG-I amino acids 1–228 (N-RIG) were expressed in *Escherichia coli* and purified. RIG-I–RNA complexes were assessed by gel-shift assay and SYBR green staining (Lonza). RIG-I activation/conformation shift was analysed using the limited trypsin digestion method<sup>17</sup>. FRET analysis of Cy3-poly-U/UC RNA interaction with YFP–RIG-I or YFP–DAI proteins was conducted using N-FRET on a Zeiss confocal microscope<sup>30</sup>. Serum IFN-β levels were measured by ELISA (PBL).

**Mice.** Mice<sup>15</sup> were from S. Akira<sup>15</sup> and were transfected using lipid-based *in vivo* RNA transfection reagent (Altogen).

**Statistical analysis.** Data were compared using the Student's *t*-test.

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**Supplementary Information** is linked to the online version of the paper at [www.nature.com/nature](http://www.nature.com/nature).

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