Long noncoding RNA EGOT negatively affects the antiviral response and favors HCV replication

Elena Carnero, Marina Barriocanal, Celia Prior, Victor Segura, Elizabeth Guruceaga, Cristian Smerdou and Puri Fortes

Corresponding author: Puri Fortes, CIMA

Review timeline:

- Initial Submission: 17 November 2015
- Editorial Decision: 17 December 2015
- Revision received: 17 March 2016
- Editorial Decision: 07 April 2016
- Revision received: 03 May 2016
- Accepted: 12 May 2016

Editors: Martina Rembold, Achim Breiling

Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

1st Editorial Decision 17 December 2015

Thank you for the submission of your research manuscript to our journal. We have now received the full set of referee reports that is copied below.

As you will see, the referees acknowledge the potential interest of the findings. However, all three referees point out several major concerns and have a number of suggestions for how the study should be strengthened, and I think that all of them should be addressed. Looking at the reports it becomes clear that a number of concerns are shared by all referees. All referees request further experiments to address if the induction of EGOT is specific to HCV infection or if other PAMPS/stimuli can also induce it. Moreover, the absolute expression level and copy number of the CSRs should be determined. Concerning the presented patient data it should be tested if the viral load correlates with the induction of EGOT and what the contribution of patients with HCC is. Furthermore, it should be verified that overexpressed PKR is active and that the EGOT knockdown is effective.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as detailed above and in their reports) must be fully addressed and their suggestions taken on board. Please address all referee concerns in a complete point-by-point response. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.
REFEREE REPORTS

Referee #1:

Using RNAseq and microarray, Carnero et al identify CSRs (HCV stimulated lncRNAs) that are upregulated upon HCV infection. The authors then pick several CSRs based on their expression levels and degrees of induction. They show that many CSRs are stimulated by pIC, but are induced to a greater extent by HCV. The authors then focus on the lncRNA EGOT in more detail. Like other CSRs, EGOT is induced by IFN and pIC treatment, but more significantly induced upon infection with HCV. The authors hypothesize that EGOT is proviral and to this end, they knock down EGOT using gapmers (ASOs) and examine the effect on viral replication. Cells depleted of EGOT showed reduced levels of HCV replication compared with cells treated with a control gapmer. A similar phenotype was shown for influenza virus and Semliki Forest virus (SFV). Based on this, the authors hypothesize that EGOT is a negative regulator of the immune response, and show that knockdown of EGOT with gapmers increases expression of several ISGs. Seeing overlap in the stimuli and effects of EGOT and PKR, the authors then ask whether PKR has an effect on EGOT expression. They knock down or overexpress PKR and show induction of EGOT. Based on these results, the authors conclude that EGOT is upregulated by HCV-mediated activation of PKR. Furthermore, it is proposed that this activation is proviral due to the fact that EGOT depletion activates the innate immunity (ISGs) and inhibits viral replication. While this report is certainly interesting, there are several major concerns with the presentation of these results and the conclusions drawn.

Major Comments:

1) Generally, the methods and figure legends are not informative enough or carefully written. For example, it is nearly impossible for the reader to understand the timing/treatments of the experiments shown in Figure 9. In the legend for Figure 3, the authors refer to Pride, Lee and Bazini and refer the reader to the text for further details, yet I could not find any further details. It was often difficult to follow the timing/methodology of experiments.

2) The axes of the figures are not informative. There are no units given, nor any information as to what values are being used. As an example, in 5A, "Relative RNA levels" should say HCV copies/g tissue (or whatever it is) to give information on the actual measurements being divided/multiplied. Fold increase, relative RNA levels, relative expression, etc. are not sufficient. At the very least this information should be in the figure legends.

3) The conclusions made by the authors are overstated. Although it may be tempting to speculate that HCV is manipulating EGOT expression for its own benefit, this is not shown. Indeed, EGOT is induced by other PAMPs, suggesting its stimulation is NOT specific to HCV. The authors themselves highlight that the induction is probably due to an increase in PKR activation (although this is also not shown directly - see point on Figure 9 below). Treatments with different PAMPs/IFNs/cytokines should be done in tandem, and PKR activation and resulting EGOT expression should be measured. Statements such as: "Then, HCV and SFV may have evolved to induce the expression of EGOT and increase virus viability by limiting the IFN response" are not supported by the data.

4) Figure 2 shows the fold induction of lncRNAs upon HCV infection. However, many of the CSRs classified as expressed to "good levels" in Figure 2 (a term which should be defined) did not appear well-expressed in the heat map provided (Supp. Figure 2). Therefore, the copy number in Huh7 cells should be determined using an EGOT RNA standard. Basal expression becomes an issue at when the authors make the statement that CSR28 or 32 inductions precede the induction of most CSRs. First, other CSRs (7, 3, 19, 26) show the same trend and the apparent early or strong induction of CSR28 or CSR32 may be related to abundance (either one cannot see induction yet at early time points because the amplitude of the overall response is less, or, the lncRNA is expressed to a low level, and any induction appears high). Expression level is also an issue in Figure 3B (see comment below).
5) Figure 3A shows a summary of bioinformatic measures from which the authors draw conclusions about the amino acid coding potential of the CSRs they have identified. There should be a legend for this table, explaining the abbreviations used, explaining briefly to the reader what these measures are, and (to the extent possible) what they are based on/how they are obtained. In addition, the numerical cutoffs for each important value that contributes to the classification of lncRNA should be stated. Figure 3B demonstrates the cytoplasmic to nuclear distribution of several CSRs in mock or HCV-infected cells. The authors conclude that EGOT changes its cytoplasmic localization to be nuclear upon HCV infection. However, contrary to the authors' classification in Figure 2B, EGOT is expressed to very low levels within the liver (Supp. Fig. 2). Therefore, the relatively small difference in localization upon HCV infection could merely reflect increased transcription of EGOT.

6) Figure 4 demonstrates the induction of CSRs by HCV, IFN-alpha (IFN) and pIC. Again, HCV induces CSRs, and the combination of HCV and IFN did not further increase this induction. However, the timing of the experiment (IFN treatment/HCV infection) is unclear. Was a productive HCV infection established during treatment with IFN or at what time post infection did the IFN treatment start? Viral replication levels should be shown in the figure. Presumably, viral replication will be abrogated upon IFN signaling. Therefore it is likely that the viral RNA itself is able to trigger the induction of EGOT. To address this issue, the authors could infect cells that are pre-treated with HCV-DAAAs (e.g. sofosbuvir or daclatasvir). Under these conditions, there is no viral replication and only the incoming viral RNA and its initial translation can contribute to the upregulation of CSRs. Also, Huh-7.5 cells, which are defective in RIG-I mediated induction of ISGs, should be tested in comparison to Huh-7 cells. This would help to distinguish whether the induction of these CSRs is RIG-I mediated or via a different pathway (e.g. PKR).

7) Figure 5A shows EGOT levels in HCV-infected patients compared to a healthy control group. However, a number of factors make this rather small change difficult to interpret. If EGOT plays a role in innate immunity, inflammation in the liver may affect EGOT levels in an HCV-independent manner. In addition, could potential influx of immune cells into the infected liver influence measured EGOT abundance? How does the viral load correlate with the induction of EGOT and to what extent are ISGs upregulated in these liver samples? Finally, it was previously reported that EGOT is down regulated in prostate cancer. HCV infected patients was diagnosed with HCC should be identified on the graph. The cohort of 15 HCV patients had 5 individuals with HCC and there are 5 patients with highly elevated EGOT levels. If these patients with high EGOT levels had HCC, it is possible that upregulation of EGOT in patients is HCC-related, not caused by the HCV infection.

8) Figure 6 demonstrates knock down of EGOT and examines its effect on HCV and SFV replication. A major concern is that the gapmers will bind to EGOT, forming DNA-RNA hybrids. Such hybrids are recognized by cGAS and subsequently stimulate the innate immunity via STING/TBK1. This could lead to the upregulation of ISGs as shown in figure 8, independent of EGOT depletion. Would it be feasible to generate CRISPR KO cells that target the 5'UTR or the promoter region of EGOT (possibly inducible)? This would allow the authors to draw conclusions on the involvement of EGOT in viral replication and innate immunity. Alternatively, an inducible (TET-on/off) system for the expression of EGOT would be informative to determine its role in regulating the innate immunity. The authors should also overexpress EGOT and examine ISG expression and viral replication of HCV and SFV to support their conclusion that EGOT is a proviral negative regulator of the immune response. Additionally, ISG levels should be examined following EGOT knock down in the absence of HCV infection.

9) The authors make the statement several times that HCV activates PKR - it would be more compelling if the activation of PKR was shown in response to HCV infection e.g. by WB for phosphorylated PKR or downstream phosphorylation of eIF2α. In Figure 9, knockdown of PKR (siRNA) or overexpression of PKR influences EGOT levels negatively or positively, respectively. Although clearly exogenous expression of PKR has an effect on EGOT abundance, is the PKR expressed activated? This should be shown. Since upregulation of EGOT occurs during the sensing of cytoplasmic RNA (HCV, SFV, pIC), it is not surprising that PKR is involved in regulating EGOT expression. Usually, EGOT transcript levels rapidly increase following IL5 stimulation of CD34+ hematopoietic progenitors during eosinophil development. It is therefore debatable whether EGOT gets specifically upregulated in response to viral RNA or whether it is upregulated as a "byproduct" during activation of the innate immunity (see point 3 above).
Minor comments:
In Figure 9C in the minus PKR condition, the comparison of EGOT induction in mock versus HCV-infected cells is presumably comparable to the experiments performed previously (ie Figure 2, 4). However, in 9C the induction seems considerably less than that observed in Figure 2A or 4A. The change in axes from "fold increase" to "relative expression" and general lack of information on what is being measured probably exacerbates the issue (see 2nd major point). Is this an issue of the timing of the experiment? Can the authors comment on this in the text?

Would it be possible to identify common features of the promoter regions of all the identified CSRs? Can the authors comment on any unifying features?

Does the knockdown of EGOT have any cytotoxic effects? If knockdown of EGOT reduces cell viability, this could contribute to the reduction in HCV replication seen in Figure 6. Cell viability following EGOT gapmer treatment should be examined if cytotoxic effects/delayed cell growth were observed.

There is no mention of the length of these noncoding RNAs. Length of greater than 200 nt is a defining (although somewhat arbitrary) feature of lncRNAs and should be mentioned somewhere in the text.

There are several ambiguous sentences in the introduction:
1) "Most ISGs are antiviral genes that function to increase cell sensitivity to infection (...)." This sentence is ambiguous and reads as if cells are more easily infected due to ISGs.
2) "However, IncRNAs are more cell type-specific, less expressed (...)." Less expressed as in less abundant? Expressed in fewer cell types? Clarify.
3) "HIV and influenza are viruses that reach the nucleus and, therefore, gifted to alter the cell transcriptome." Many studies on transcriptomic changes have been conducted for non-nuclear viruses, so reaching the nucleus is not a requirement for altering the cellular transcriptome.

In Figure 1D, the names of hits are written but cannot be connected with the point to which they correspond - the point itself needs to be highlighted in some way. In addition, what is the rationale for the hits listed by name in Figure 1D? This should be clearly stated somewhere. It would also be useful if the top hits of the noncoding hits were also highlighted in some way.

Figure 2 has CSR37 shaded in a different color besides those used for the other CSRs, and there is no explanation for this in the figure legend. The reviewer understands that this CSR was removed from further analysis, but the differential shading for CSR37 still needs an explanation.

Provide some justification for continuing with NEURL3/CSR31 if it does not meet your requirements for a lncRNA.

The authors should be careful with broad statements claiming nuclear localization when discussing Figure 3B; they list two CSRs as having altered localizations following HCV infection, then describe them as having "nuclear localization."

The error bars in Figure 3B are not correctly placed.

In Figure 4B, CXCL10 should be treated the same as the other samples... is the increase shown significant? Not significant?

Referee #2:
In this manuscript, Carnero and co-workers are interested in the role of long noncoding RNAs (lncRNAs) in the control of the antiviral response induced by hepatitis C virus (HCV) infection. By using both arrays and deep sequencing approaches together with bioinformatics analyses, the authors identify lncRNAs which expression is modulated upon hepatitis C virus (HCV) infection of the human hepatoma-derived Huh7 cells. Quantitative RT-PCR was used to validate 37 candidates
which expression was upregulated and as well as their subcellular localization. The lncRNA EGOT was chosen for in-depth analysis. The authors showed that EGOT is upregulated in liver biopsies of HCV-positive patients and allows a better viral replication by regulating negatively the expression of numerous interferon-induced genes. Moreover, the induction of EGOT expression was shown to be PKR- and NF-kB-dependent.

The reported observations, which are made in an immune-incompetent cell culture system but corroborated by analyses in liver biopsies of HCV-positive patients, are most interesting, well documented and convincing. Growing evidence exists on a critical role of lncRNAs and noncoding RNAs in virus-host interactions, with several lncRNAs exhibiting important functions in the antiviral response. The work proposed in this manuscript is of strong relevance and helps on elucidating the new function associated with lncRNAs and modulation of the immune response to virus infection. Overall, the concept of virus-induced lncRNA might be more important for RNA viruses such as HCV and Influenza A virus rather than for DNA viruses, however, that advances the fields of HCV and innate immune biology. However several concerns should be addressed by the authors.

Major concerns:

- Figure 3B reports that EGOT cellular distribution is altered upon HCV infection and switches from the cytosol to nucleus. Since this observation was one of the strongest argument to focus on EGOT analysis, this result should be complemented immune-fluorescence analysis using RNA FISH.

- CRS19, 21, 26 and 34 are induced upon HCV infection but not upon IFN treatment, poly(I:C) transfection or "infection with other viruses tested". This allows the authors to claim that their expression is hence specific to HCV infection. However, this latter observation is not supported by data. The authors should provide this information as a complement of Figure 4B.

- The analysis of EGOT mRNA expression levels in livers of HCV-positive patients shown Figure 5A includes five samples of patients with hepato-cellular carcinoma (HCC). lncRNAs were also reported to be upregulated in hepatitis B virus-related HCC, and non-hepatitis-related HCC, and might hence be the consequence of pathogenesis establishment. Is the increase in EGOT levels observed in the liver of HCV-positive patients still significant if the HCC-samples are omitted from the analysis? Are EGOT mRNA expression levels correlated with HCV viral load or copy numbers? In addition, the authors should include another lncRNA, such as CSR4 which is not significantly modulated by HCV infection as shown in Figure 2A and expressed in the liver (Suppl. Figure 2), to provide information about the variability of lncRNA expression and thus the significance of EGOT expression.

- The difference in EGOT mRNA level induction kinetics and amplitude observed for Influenza virus and Semliki Forest Virus is really interesting. Recently, Li and coworkers (Nucl. Acid Research 2015) reported that Influenza A virus non-structural protein NS1 inhibitory effect on PKR activation is mediated by the induction of noncoding vault RNAs. Could this scenario could explain the observed decrease in EGOT mRNA levels at 24h post Influenza A virus infection?

- EGOT mRNA silencing in Huh7 cells results in almost 40% reduction in HCV RNA levels and 80% of the amount of HCV-infected cells (Figure 6 A-D). This result should include the measurement of the HCV viral titer using TCID50. Moreover, the multiplicity of infection used for this experiment (MOI of 0,01) is much lower than in the one used in the induction kinetics shown in Figure 2B (MOI of 0,3). Is the effect of EGOT silencing as pronounced on HCV viral titer when using a MOI of 0.3?

- Same comment as above, which MOI was used for the experiments shown in Figure 8? The authors should provide the corresponding data for EGOT silencing efficiency and HCV infection levels. The induction of ISGs in response to HCV infection occurs already 12h post infection. How is EGOT inhibitory effect on ISG mRNA levels regulated in the course of the infection, up to 96h?

- Results shown in Figure 9 are essential to understand the pathway by which EGOT expression is upregulated. The author should provide a western-blot analysis to confirm that in HCV infected cells upon PKR silencing PKR phosphorylation levels are inhibited. In addition, the authors should
clarify the discrepancy in EGOT expression levels in the control conditions shown in panel 9A and 9C: in panel 9A EGOT expression is increased over 200 fold in cells transfected with control siRNA and infected with HCV while only about 3 fold in panel 9C, in HCV-infected cells transfected with control plasmid.

- The authors schematized PKR functions in Figure 9D. However, an activation of NF-kB mediated by RIG-I cannot be excluded. An additional siRNA directed against RIG-I should be included in the experiments shown Figure 9A.

- The use of IRF3 dominant negative form and IKB(SA) is a nice complementary approach to dissect the signaling pathway. However, additional information is required to show the functionality of these constructs in Huh7 cells. Figure 9E, the inhibition of IRF3 should be validated by western-blot analyses of IRF3 phosphorylation or immune-fluorescence analysis of IRF3 translocation to the nucleus in both non-infected and infected DNIRF3-expressing cells. On the same line, Figure 9F, the repression of NF-kB activation in pIKB(SA)-transfected cells should be validated by western-blot analysis of p65 phosphorylation levels in non-infected and HCV-infected cells.

Minor concerns:

- It might be helpful to the readers that are not familiar with H3K4me1 and H3K4me3 to indicate that they correspond to mono or trimethylations of histones.

- Figure 2B misses the asterisk representing statistical analysis.

- In order to avoid misunderstanding, the term "mock" in the labeling of histogram axis shown in Figures 6, 8 and 9, which corresponds to control gapmer or siRNA transfections should be replaced by gaCtrl and siCtrl or similar. Mock is expected to be the non-infected control.

- The authors should provide the gapmer sequences used for EGOT silencing

Referee #3:

This manuscript explores the interesting field of lncRNAs mediating antiviral immunity. The authors use both microarrays and RNASeq to identify lncRNAs induced by Hepatitis C virus (HCV) in liver cells, and then focus their attention on one strongly HCV-inducible transcript EGOT. Knockdown of EGOT reduced viral replication of HCV and Semliki forest virus. Whereas this biological phenomenon is interesting, the work lacks mechanistic insights on how EGOT mediates this function and is quite correlative overall. In summary, this is an interesting story, but the findings are preliminary in this current form.

Major Comments:

1. This study has some problems especially with regards to the transcriptomic overlay of microarray vs. RNASeq. In Figure 1, the authors compare the protein coding transcriptome identified by either microarray or RNASeq analysis. While the authors state "similar coding genes were identified by microarray and RNASeq analysis... In fact, 18.7 % of the coding genes identified as altered in response to HCV infection after RNASeq analysis... were also identified in the microarray analysis." I disagree that this can be interpreted as a "similar" profile, since almost 4 out of 5 genes do not show the same differential expression pattern when comparing microarray with RNASeq (79.3%). This is a big concern since one would expect a higher overlap of differentially expressed protein-coding genes. Was the same RNA input used for the both experiments? How do the authors explain this very weak correlation?

2. In Figure 1F: According to the Venn diagram, only 383 protein-coding genes change following HCV infection (RNASeq). This seems like a small number, and the finding that the microarray detects almost 10fold the amount of differentially expressed genes (2842) is not understandable to
3. Throughout the manuscript, the authors use differential expression (i.e., fold change) as a measure for selecting candidates. This is a very reasonable approach for candidate selection, but at the same time it would be important to understand the absolute expression levels of the selected transcripts to be able to estimate the copy number per cell of those transcripts. For low-abundance transcripts like lncRNAs, a high fold change can be misleading.

4. Fig 4a: Given the kinetics of the type I interferon response on interferon-stimulated gene expression patterns, 72h of stimulation as the only time point is not ideal. It would be interesting to perform timecourse and dose response experiments.

5. Fig 4B: It would be interesting to see the differential expression of the selected lncRNAs after stimulation with a broader panel of TLR ligands and nucleic acids.

6. Fig 6: What is the effect of gapmer mediated knockdown of EGOT after stimulation? EGOT is almost 800-fold induced after HCV infection, so it would be important to assess the quality of the knockdown both basally and after induction.

7. Fig 6: How were the GAPmers target regions selected? There is no experimental data showing that knockdown of EGOT does not affect the expression of the gene ITPR1 gene, in which it is located. Given the incorrect annotation of some lncRNAs, the sequence of EGOT needs to be confirmed by RACE.

8. Page 7: "Our results indicate that sensing pIC or the viral RNA genome induces the expression of CSR32, which acts as a proviral factor by decreasing ISG expression." The authors do not show this causal link/mechanism between EGOT expression and ISG expression. Rather, the data they show is correlative.

9. Fig. 8: What do the authors mean by relative expression here—relative to what? Non-infected? Moreover, the asterisk for Mx1 ga2alpha is unclear. Why is ga2alpha "more" significant than ga1alpha, when the error bar is higher and the relative RNA level is identical to ga1alpha?

Minor Points:
1. In the Introduction the statement: "LncRNAs are functional transcripts with no identifiable protein coding capacity" Since lncRNAs are defined by length and lack of coding potential only, the fraction of functional transcripts among all lncRNAs is unclear to date. This statement should be revised.
2. "HIV and influenza are viruses that reach the nucleus and, therefore, gifted to alter the cell transcriptome." Clearly, dramatic transcriptional changes of host cells can also happen independently of nuclear entry of a virus (cytoplasmic nucleic acid signaling!), so this statement is not correct.
Previously described under Bioinformatic analyses (Materials and Methods), are now also defined in the figure legend (Fig. EV.1). The complete analysis of the coding potential of the CSRs is now also described in the figure legend.

2) The axes of the figures are not informative. There are no units given, nor any information as to what values are being used. As an example, in 5A, "Relative RNA levels" should say HCV copies/g tissue (or whatever it is) to give information on the actual measurements being divided/multiplied. Fold increase, relative RNA levels, relative expression, etc. are not sufficient. At the very least this information should be in the figure legends.

In the figure legends and in Material and Methods we indicated that “to calculate the relative levels of each transcript, GAPDH mRNA was quantified and used as a reference”. This is explained in more detail in the new version of the manuscript. To calculate the relative RNA levels, transcript levels were evaluated by quantitative RT-PCR and normalized to the expression level of an internal control. GAPDH mRNA was chosen as a reference because the levels of GAPDH mRNA were not altered by the treatments used. Furthermore, samples showing over one Ct value difference in the level of GAPDH mRNA compared to control samples were not used in the analysis. This occurred only in few cases. In the revised version of the manuscript, the axes of the figures have been changed from the general “relative RNA levels” to the more specific “$2^{ΔΔCt} \times 1000$” that was used in all cases, where $ΔΔCt = Ct$ of the gene of interest – Ct of the internal control. HCV levels have been translated to genome equivalents per µg of RNA, as has been described previously (Zhong et al., Proc Natl Acad Sci U S A 102: 9294-9299, 2005).

Absolute numbers of CSRs were not calculated in all cases, as the aim was to do a fast screening of the initial CSRs to select for further evaluation those that increase after HCV infection. The 37 CSR candidates are expressed to very different levels (Appendix Table S.2 and see below). A graph that would show the expression levels of all candidates in untreated and treated cells would not allow an easy evaluation of how the treatment affects expression. Therefore, fold induction was chosen to represent expression changes in treated versus control cells (Fig. 2). We believe that the reviewer will agree with this, as fold induction is a standard method to represent differences in gene expression regularly used in high impact journals such as EMBO Reports. The relative expression is also a widely accepted method to evaluate gene expression. However, as suggested by the reviewer, in the case of EGOT we have now quantified the absolute levels of EGOT. We agree with the reviewer that this is a relevant issue, as by only showing relative expression levels and fold inductions it is difficult for the reader to have an idea of the expression level of EGOT. The results have been included in the revised version of the manuscript. Please, see the answer to issue 4 for details.

3) The conclusions made by the authors are overstated. Although it may be tempting to speculate that HCV is manipulating EGOT expression for its own benefit, this is not shown. Indeed, EGOT is induced by other PAMPs, suggesting its stimulation is NOT specific to HCV. The authors themselves highlight that the induction is probably due to an increase in PKR activation (although this is also not shown directly - see point on Figure 9 below). Treatments with different PAMPS/IFNs/cytokines should be done in tandem, and PKR activation and resulting EGOT expression should be measured. Statements such as: "Then, HCV and SFV may have evolved to induce the expression of EGOT and increase virus viability by limiting the IFN response" are not supported by the data.

We completely agree with the reviewer. The revised manuscript has been carefully reviewed to clarify this issue. We do not think that stimulation of EGOT is specific for HCV. Instead, our work shows that EGOT is induced in the cell in response to different stimuli. Following the suggestion of the reviewer, we have evaluated the effect of other PAMPs, IFNs and cytokines on EGOT expression. The results are shown in new Fig. 4 and/or indicated in the text. Most of the treatments performed (PAMPs, IFNs and TNFα, but not other cytokines such as IL6 or oncostatin) induce EGOT expression 2-6 fold (Fig. 3-4). However, EGOT levels are increased 100-800 fold in cells infected with Semliki Forest Virus (SFV) or HCV (Fig. 2 and 4B). Such a dramatic increase is not observed in cells infected with influenza virus, an RNA virus that, unlike SFV and HCV, replicates in the nucleus (Fig. 4A). We hypothesize that the increased levels of EGOT in cells infected with SFV or HCV depend on viral replication. In fact, we now show that infected cells treated with inhibitors of HCV replication show decreased levels of EGOT compared to untreated infected cells (new Figure 4C). The possibility that HCV could have evolved to increase EGOT levels for its own
benefit was just a speculation previously mentioned in the discussion.

4) Figure 2 shows the fold induction of IncRNAs upon HCV infection. However, many of the CSRs classified as expressed to "good levels" in Figure 2 (a term which should be defined) did not appear well-expressed in the heat map provided (Supp. Figure 2). Therefore, the copy number in Huh7 cells should be determined using an EGOT RNA standard. Basal expression becomes an issue at when the authors make the statement that CSR28 or 32 inductions precede the induction of most CSRs. First, other CSRs (7, 3, 19, 26) show the same trend and the apparent early or strong induction of CSR28 or CSR32 may be related to abundance (either one cannot see induction yet at early time points because the amplitude of the overall response is less, or, the IncRNA is expressed to a low level, and any induction appears high). Expression level is also an issue in Figure 3B (see comment below).

We agree with the reviewer that the paper requires a better definition of the expression levels. To simplify, we have now divided all CSRs from 1 to 35 into those expressed at high, intermediate or low levels in HCV infected cells (Appendix Table S.2). We now specify the range of Ct values that correspond to each category (low, Ct values > 30 (generally corresponding to one or fewer copies per cell); medium, Ct values 26-29 and high, Ct values ≤ 25. While qRT-PCR does not provide absolute measurements in the absence of a standard, we believe that Ct values may give a general idea of expression levels. As a reference, we mention that the abundant GAPDH mRNA (more than 1000 copies per cell) has an average Ct value of 17 using our qRT-PCR conditions while most coding genes evaluated in this work have Ct values of 21-25.

There are several reasons for the lack of correlation between the studies performed with the Human Body Map (Appendix Fig. S.2) and the expression levels found in HuH7 cells. Note that the Human Body Map evaluates expression in the whole liver, which includes mainly hepatocytes but also other cells. For instance, in HuH7 cells we detect low levels of CSR14 while this lncRNA is highly expressed in the liver according to the Human Body Map. We believe that CSR14 could be expressed from other cells different than hepatocytes or that the expression of CSR14 could be higher in human primary hepatocytes than in transformed HuH7 cells. Importantly, it should be noted that the expression levels shown in Appendix Table S.2 correspond to HCV infected HuH7 cells. This has been clarified in the new version of the manuscript. In the case of CSR32/EGOT, expression in the liver is low according to the Human Body Map (Appendix Fig. S.2). However, it is induced after infection with HCV.

To determine the copy number of EGOT in HuH7 cells, EGOT cDNA was cloned after a T7 promoter and EGOT RNA was transcribed in vitro and quantified by bioanalyzer. Then, it was used as a standard for a qRT-PCR reaction. An identical protocol was followed to evaluate the levels of the more abundant CSR34/UCA1. The results indicate that there are 8 - 15 molecules of EGOT mRNA and 60-90 molecules of UCA1 mRNA per HuH7 cell infected with HCV for 48h (page 5, paragraph 4). Similar results were obtained for CSR30/PINT, whose quantification resulted in 5-7 copies of PINT per cell. This correlates with what has been found by in situ hybridization (Martin-Bejar et al., Genome Biol 14:R104, 2013).

We agree with the reviewer that induction of CSR32 could be similar to the induction of CSR3 or CSR7. Therefore we have removed the statement indicating that the induction of CSR32 precedes the induction of most CSRs.

5) Figure 3A shows a summary of bioinformatic measures from which the authors draw conclusions about the amino acid coding potential of the CSRs they have identified. There should be a legend for this table, explaining the abbreviations used, explaining briefly to the reader what these measures are, and (to the extent possible) what they are based on/how they are obtained. In addition, the numerical cutoffs for each important value that contributes to the classification of IncRNA should be stated. Figure 3B demonstrates the cytoplasmic to nuclear distribution of several CSRs in mock or HCV-infected cells. The authors conclude that EGOT changes its cytoplasmic localization to be nuclear upon HCV infection. However, contrary to the authors' classification in Figure 2B, EGOT is expressed to very low levels within the liver (Supp. Fig. 2). Therefore, the relatively small difference in localization upon HCV infection could merely reflect increased transcription of EGOT.

In the revised version, the legend of Figure 3 (now Fig. EV1) describes the analyses performed in
more detail. Also, the table has been simplified. Basically, coding potential has been evaluated with the coding potential assessment tool (CPAT) and searching the LNCipedia database. CPAT measures ORF size and coverage and codon and hexamer score usage bias to yield a coding probability (Cod Prob). Values lower than 0.364 mark noncoding RNAs with high sensitivity and specificity. The LNCipedia database quantifies the number of times that each transcript has been found in the Pride proteomic database, a collection of peptides identified by proteomic analyses, or in the Lee or Bazzini lists of transcripts associated with ribosomes in ribosome profiling experiments. LNCipedia also calculates the Phylogenetic Codon Substitution Frequencies (PhyloCSF). PhyloCSF estimates coding potential after evaluation of a phylogenetic conservation score. As a rule, negative scores are likely to represent a conserved noncoding sequence. However, this method should not be used alone. The model is based on sequence evolution and makes many assumptions that are not fully satisfied in reality. This strongly affects pseudogenes. As pseudogenes are similar to their parental gene, they could score positive by PhyloCSF even if they are transcripts that have lost coding capacity. Thus, PhyloCSF values should be combined with other methods, as we have done. In our case we used CPAT and “lists”. Taking into consideration all the analyses performed, we describe the final coding probability under “label”.

Regarding the localization of the CSRs, we agree with the reviewer that we cannot exclude that the apparent nuclear relocalization of CSR32/EGOT results from increased transcription coupled to low baseline levels. Therefore, we have prepared a new figure showing the preferential accumulation of the CSRs only in infected cells, where expression levels are higher.

6) Figure 4 demonstrates the induction of CSRs by HCV, IFN-alpha (IFN) and pIC. Again, HCV induces CSRs, and the combination of HCV and IFN did not further increase this induction. However, the timing of the experiment (IFN treatment/HCV infection) is unclear. Was a productive HCV infection established during treatment with IFN or at what time post infection did the IFN treatment start? Viral replication levels should be shown in the figure. Presumably, viral replication will be abrogated upon IFN signaling. Therefore it is likely that the viral RNA itself is able to trigger the induction of EGOT. To address this issue, the authors could infect cells that are pre-treated with HCV-DAAs (e.g. sofosbuvir or daclatasvir). Under these conditions, there is no viral replication and only the incoming viral RNA and its initial translation can contribute to the upregulation of CSRs. Also, Huh-7.5 cells, which are defective in RIG-I mediated induction of ISGs, should be tested in comparison to Huh-7 cells. This would help to distinguish whether the induction of these CSRs is RIG-I mediated or via a different pathway (e.g. PKR).

This is a really interesting issue. In the IFN experiment, cells were treated with 10,000 units/ml of IFNα for 72 hours (previous Fig. 4, now Fig 3A). We have previously shown that under these conditions it is still possible to detect increased expression of genes that are induced early after IFN treatment but also increased levels of genes that respond to a secondary wave of the IFN response (Carnero et al., Front Immunol 5, 2014). Using these conditions only CSR31 and CSR32 exhibited a significant upregulation in IFN-treated cells. We have now analyzed IFN induction further to show that only CSR31 is induced at 6h post-IFN treatment (new Appendix Fig. S. 3A-B).

To find synergistic effects between IFN and HCV-infection, cells were first infected with a moi of 0.3 of HCV. Three days later, cells were treated or not with IFN as described above (10,000 units/ml). Cells were collected three days later to evaluate CSR expression. Under these conditions IFN treatment affects viral replication poorly. The HCV RNA levels are now shown in new Appendix Fig. S.3C.

To determine whether the viral RNA is able to trigger the induction of EGOT, the following experiments have been performed:
- Cells were infected with HCV and EGOT levels were evaluated at 5 hours post-infection (immediately after infection) (new Fig. 4D).
- Cells were infected with an UV-irradiated HCV and HCV and EGOT levels were evaluated 12 hours later (new Fig 4D). Controls cells were mock-treated or infected with a non UV-treated virus. The latter showed increased replication compared to the cells infected with the UV-treated HCV virus.
- Cells were incubated for 24 hours with media containing a cocktail of 1200 pM sofosbuvir, 500 pM daclatasvir and 100 µM ribavirin (DAAs). Then, treated cells and controls were infected with moi 0.3 of HCV and incubated for 48 hours in media containing the HCV
DAAs or normal media, respectively. EGOT and HCV RNA levels were evaluated (Rev.1 Fig. 1).

These experiments suggest that the incoming virus could increase the levels of EGOT by 2-3 fold.

![Image of graph showing HCV and EGOT levels](image)

**Rev. 1 Fig. 1.** HuH7 cells were incubated for 24 hours with media containing a cocktail of direct-acting antivirals (DAAs) (1200 pM sofosbuvir, 500 pM daclatasvir and 100 µM ribavirin). Then, cells were infected with moi 0.3 of HCV and incubated for 48 h in media containing the HCV DAAs. Control cells were non-infected (Mock) or infected and incubated with regular media for 48 h. EGOT and HCV RNA levels were evaluated by qRT-PCR. GAPDH mRNA was also quantified and used as a reference to calculate the relative levels of EGOT. The fold increase was calculated as the ratio of EGOT levels in infected versus non-infected cells. Genome equivalents (GE) of HCV RNA were calculated as described in materials and methods. The inhibitors decreased viral RNA ~1000 fold. EGOT levels were upregulated only 2-3 fold in the infected cells treated with DAAs and >100 fold in control infected cells.

Regarding RIG-I, we have evaluated EGOT induction in RIG-I deficient HuH7.5 cells and in HuH7 cells transfected with siRNAs targeting RIG-I. The results show that although RIG-I can also mediate EGOT induction, HCV can induce EGOT in the absence of RIG-I. These results are now shown in new Fig. 5A-B.

7) Figure 5A shows EGOT levels in HCV-infected patients compared to a healthy control group. However, a number of factors make this rather small change difficult to interpret. If EGOT plays a role in innate immunity, inflammation in the liver may affect EGOT levels in an HCV-independent manner. In addition, could potential influx of immune cells into the infected liver influence measured EGOT abundance? How does the viral load correlate with the induction of EGOT and to what extent are ISGs upregulated in these liver samples? Finally, it was previously reported that EGOT is down regulated in prostate cancer. HCV infected patients was diagnosed with HCC should be identified on the graph. The cohort of 15 HCV patients had 5 individuals with HCC and there are 5 patients with highly elevated EGOT levels. If these patients with high EGOT levels had HCC, it is possible that upregulation of EGOT in patients is HCC-related, not caused by the HCV infection.

We thank the reviewer for raising this issue. We have now evaluated human samples in more detail in several ways:

1. The levels of EGOT have been evaluated in an independent cohort of patients (10 controls and 19 HCV-infected patients). Similar to what was obtained with the initial cohort, HCV-infected livers showed increased levels of EGOT compared to non-infected livers. This is now shown in new Fig 6A. However, we do not observe increased levels of CSR14 or CSR23 in HCV-infected livers (data not shown). These CSR14 have been taken as a control of lncRNAs that show good expression in the liver (new Appendix Fig. S. 2) and are not markedly increased after HCV infection in HuH7 cells (Fig. 2A).

2. We have quantified the levels of ISG15 and HCV RNAs in the livers of HCV-infected patients. As has been described, there is a significant correlation between ISG15 and HCV RNA in our human samples (Broering et al., Gut 59: 1111-1119, 2010). However, we do not observe a significant correlation between EGOT and HCV RNA (new Fig. 6B). Given that the proportion of HCV-infected hepatocytes per person ranges from 21-45% and that most infected hepatocytes express low levels of viral RNA (1-50 copies according to Kandathil et al., Gastroenterology 145: 1404-13, 2013; 1-8 copies according to Stiffler et al., PLoS One 4: e6661, 2009; and 7-64 copies
according to Chang et al., Am J Pathol 163: 433-444, 2003), we hypothesized that EGOT expression in HCV-infected livers could also be driven by TNFα, as shown in HuH7 cells (Fig. 5I). Therefore, we have quantified the levels of TNFα mRNA and we have observed that there is a significant correlation between TNFα levels and EGOT expression (new Fig. 6B). Our results are in agreement with the hypothesis that TNFα could be a major driver of EGOT expression in the liver of HCV-infected patients. Given the complexity of the HCV cell cycle and the antiviral response, it could be reasonable to think that the levels of HCV and ISG15 (and, probably, other ISGs) in patients could reflect the influence of several factors. We propose that EGOT could be one of these factors based on the experiments performed in HuH7 cells (Fig. 7-8).

4. TNFα may be secreted by several immune cells. Therefore, we agree with the reviewer that “immune cells into the infected liver influence measured EGOT abundance”. Further, as EGOT RNA has been originally described in eosinophils (Wagner et al., Blood 109: 5191-5198, 2007), we considered the possibility that EGOT could be expressed in the immune cells that infiltrate the HCV-infected liver. Therefore we quantified the levels of CD68, CD56, CD4 and CD8B mRNAs, which are specific markers for macrophages, NK cells and CD4, CD8 T lymphocytes, respectively. As we only have frozen liver samples we could not evaluate these populations by immunohistochemistry or by FACS. There was no correlation between the levels of EGOT and the levels of CD68, CD56, CD4 and CD8B mRNAs, suggesting that these cells are not contributing to the observed levels of EGOT. Finally, we obtained PBMCs from three healthy donors and we incubated them in control RPMI medium or RPMI with 2,500 units/ml of IFNα, 5ug/ml LPS and 15ug/ml pl:C for 8 hours. Then, RNA was extracted from these cells and EGOT levels were measured by qRT-PCR using GAPDH mRNA as a reference. The levels of EGOT in controls and treated cells were very similar and close to background levels (Page 10 paragraph 1).

5. As suggested by the reviewer, we have identified in the graphs those HCV-infected patients with HCC (new Fig. 6A). Furthermore, we have evaluated the role of EGOT on proliferation in more detail. The results obtained do not support a role of EGOT in the proliferation of liver cells because:

a) HuH7 cells in which EGOT expression has been decreased with specific gapmers showed growth rates similar to cells treated with control gapmers (new Appendix Fig. S. 4).

b) We have compared EGOT expression in the liver of different patients infected with HCV. EGOT levels were similar in patients with and without HCC. Most of the samples have been obtained recently and we cannot evaluate yet whether those patients with higher levels of EGOT have a different survival compared to patients with lower EGOT levels.

c) We have quantified the levels of a putative mouse homologue of EGOT (mEGOT) in different samples. We have observed that mEGOT is expressed in mouse liver and in primary hepatocytes. We did not observe differences in the expression levels of mEGOT between healthy mouse livers (n=7), and peritumor (n=17) or tumor tissue (n=23) obtained from HCCs developed in mice 9 months after treatment with DEN (page 7, paragraph 3). In turn, the mouse homologue of PVT1 was significantly increased in tumor samples compared to healthy livers (data not shown).

All these results are now mentioned/shown in the revised version of the manuscript.

8) Figure 6 demonstrates knock down of EGOT and its effects on HCV and SFV replication. A major concern is that the gapmers will bind to EGOT, forming DNA-RNA hybrids. Such hybrids are recognized by cGAS and subsequently stimulate the innate immunity via STING/TBK1. This could lead to the upregulation of ISGs as shown in figure 8, independent of EGOT depletion. Would it be feasible to generate CRISPR KO cells that target the 5'UTR or the promoter region of EGOT (possibly inducible)? This would allow the authors to draw conclusions on the involvement of EGOT in viral replication and innate immunity. Alternatively, an inducible (TET-on/off) system for the expression of EGOT would be informative to determine its role in regulating the innate immunity. The authors should also overexpress EGOT and examine ISG expression and viral replication of HCV and SFV to support their conclusion that EGOT is a proviral negative regulator of the immune response. Additionally, ISG levels should be examined following EGOT knock down in the absence of HCV infection.

We thank the reviewer, as we were not aware of this issue. We have experience in the lab in the generation of CRISPR KO cells that target the promoter of IncRNAs. In our experience, it is not feasible to produce, select and evaluate these cells in the timing allowed by the editor of EMBO.
Reports to provide a revised version of the manuscript (three months). Instead we have performed the following approaches:

- We have evaluated the effect of the siRNA previously described to target EGOT (Wagner et al., Blood **109**: 5191-5198, 2007). However, we did not observe significantly decreased levels of EGOT after transfection of this siRNA into HuH7 cells (fold change 1.2). It has been reported that siRNAs are less effective in targeting structured or nuclear RNAs.

- We have designed 9 new sequences that should guide the CRIPSR-Cas9 protein to the promoter of EGOT (sgRNAs). Six of the sgRNAs were designed manually by looking for NGG nucleotides within -50 to +300 nucleotides from the transcription start site of the EGOT gene, where sgRNA efficiency is higher according to high throughput tiling screens (Gilbert et al., *Cell* **159**: 647-661, 2014; Lawhorn et al., *PLoS One* 9: e113232, 2014). Three additional sgRNAs were designed using the CRISPR-ERA online software (Liu et al., *Bioinformatics* **31**: 3676-3678, 2015). Then, we transfected the plasmids expressing these sgRNAs with a plasmid expressing a CRISPR-Cas9 fused to the KRAB repressor. It has been reported that with this system, a library of 10 sgRNAs should be sufficient to obtain 2 or more highly active sgRNAs (Gilbert et al., *Cell* **159**: 647-661, 2014). In the case of lncRNAs, the design of up to 3 sgRNAs led to the effective knockdown of 5 out of 6 non-coding genes with over 80% repression at the RNA level, as measured by qRT-PCR and then confirmed by FISH (Gilbert et al., *Cell* **159**: 647-661, 2014). However, we did not observe decreased levels of EGOT after transfecting the plasmids into control or HCV-infected cells.

- Finally, we have studied whether control gapmers that target other cellular lncRNAs can upregulate ISGs. We have chosen UCA1, which is expressed at higher levels than EGOT (Appendix Table S.2 and page 5 paragraph 4). HuH7 cells were transfected with control gapmers or a specific gapmer that targets UCA1 and then infected with HCV for 48 h. Gapmers targeting UCA decreased the expression of UCA 6-10 fold, while the levels of ISG15 and GBP1 mRNAs were not significantly altered (new Appendix Fig. S.5). Using the same conditions, we obtained similar results when a gapmer targeting ISR8 was used (Carnero et al., *Front Immunol* **5**, 2014). As cGAS is a cytoplasmic sensor (Sun et al., *Science* **339**: 786-791, 2013) and transfected gapmers reach the cell nucleus efficiently (Bennett et al., *Mol Pharmacol* **41**: 1023-1033, 1992), we propose that gapmer/target DNA RNA hybrids could be formed in the nucleus, away from the cytoplasmic cGAS sensor, and could be degraded very rapidly by RNase H.

To overexpress EGOT in the cell, we cloned the full-length transcript described in Encode, RefSeq, and ENSEMBL databases, first, under a constitutive promoter. Then, we transfected increasing concentrations of the plasmid (or a control plasmid) in HuH7 cells to obtain levels similar to or higher than those observed in HCV-infected cells. Some cells were infected at 24 hours post-transfection to evaluate the effect of EGOT overexpression in HCV replication. Control and HCV-infected cells were collected at 48h post-transfection (and 24hpi) or at 72h post-transfection (and 48hpi). Under these conditions we evaluated the levels of HCV, GBP1 and ISG15 RNAs. We did not observe differences in the expression of HCV RNA or GBP1 and ISG15 mRNAs between controls and cells transfected with the plasmid expressing EGOT. As expected, EGOT levels increased compared to mock transfected cells. There are several possibilities to explain this negative result. As has been described for several lncRNAs, EGOT could work in cis. Therefore, functionality should be reconstituted by overexpressing EGOT close to the position where the endogenous gene is transcribed. Also, we cannot rule out that when expressing EGOT from a plasmid we are adding extra sequences at the 5’ or 3’ ends (to guarantee polyadenylation, for instance) that interfere with EGOT structure and disrupt functionality.

Finally, we have evaluated ISG levels when EGOT is silenced for 72 hours in non-infected cells. The result also shows increased levels of ISG15 and GBP1 after EGOT depletion (new Fig. 8B).

9) The authors make the statement several times that HCV activates PKR - it would be more compelling if the activation of PKR was shown in response to HCV infection e.g. by WB for phosphorylated PKR or downstream phosphorylation of eIF2α. In Figure 9, knockdown of PKR (siRNA) or overexpression of PKR influences EGOT levels negatively or positively, respectively. Although clearly exogenous expression of PKR has an effect on EGOT abundance, is the PKR expressed activated? This should be shown. Since upregulation of EGOT occurs during the sensing
of cytoplasmic RNA (HCV, SFV, pIC), it is not surprising that PKR is involved in regulating EGOT expression. Usually, EGOT transcript levels rapidly increase following IL5 stimulation of CD34+ hematopoietic progenitors during eosinophil development. It is therefore debatable whether EGOT gets specifically upregulated in response to viral RNA or whether it is upregulated as a "byproduct" during activation of the innate immunity (see point 3 above).

We have evaluated by Western-blot the levels of phosphorylated PKR in control cells, cells infected with HCV, cells treated with pIC and cells transfected with a plasmid that expresses PKR. The results show increased PKR phosphorylation in treated versus control cells. These results are now shown in new Fig. EV.3B and agree with what has been previously observed: HCV infection induces PKR (Arnaud et al., PLoS Pathogens 7: e1002289, 2011; Garaigorta et al., Cell Host Microbe 6: 513-522, 2009).

As mentioned above (see issue 3), we have reviewed the manuscript to clarify that EGOT is induced in response to TLRs, RIG-I and PKR activation and it is not exclusively induced by viral RNA.

Minor comments:
1. In Figure 9C in the minus PKR condition, the comparison of EGOT induction in mock versus HCV-infected cells is presumably comparable to the experiments performed previously (ie Figure 2, 4). However, in 9C the induction seems considerably less than that observed in Figure 2A or 4A. The change in axes from "fold increase" to "relative expression" and general lack of information on what is being measured probably exacerbates the issue (see 2nd major point). Is this an issue of the timing of the experiment? Can the authors comment on this in the text?

The reviewer is right. The data included in this figure for HCV-infected cells were wrong (now Fig. 5E). The data corresponded to cells that had been first infected with HCV, then transfected with a plasmid that expresses PKR and collected 24 hours later. We have now included the data obtained with cells that were first transfected (with control plasmid or plasmid expressing PKR) and then infected with HCV, as has been done with the controls. In this case, the fold increase is similar to what has been described in other figures.

2. Would it be possible to identify common features of the promotor regions of all the identified CSRs? Can the authors comment on any unifying features?

Appendix Table S.3 includes a column with transcription factors that bind to CSR promoters according to conservation analysis or ChIP-Seq performed by ENCODE. The promoters of several CSRs contain transcription factor binding sites for myc, NFKB, IRFs or STATs. This is mentioned in the discussion (page 9, paragraph 3).

3. Does the knockdown of EGOT have any cytotoxic effects? If knockdown of EGOT reduces cell viability, this could contribute to the reduction in HCV replication seen in Figure 6. Cell viability following EGOT gapmer treatment should be examined if cytotoxic effects/delayed cell growth were observed.

We have not observed cytotoxic effects with the concentration of gapmers used in our experiments. We have measured cell growth after transfection with the gapmers that target EGOT. No significant differences in cell growth were observed at 72 hours post-transfection (new Appendix Fig. S.4).

4. There is no mention of the length of these noncoding RNAs. Length of greater than 200 nt is a defining (although somewhat arbitrary) feature of lncRNAs and should be mentioned somewhere in the text.

Appendix Table S.3 includes a column with this information. As several transcripts have been described for some CSR genes, the length of the shorter and the longest form is indicated. All described transcripts are longer than 200 nts.

5. There are several ambiguous sentences in the introduction:
1) "Most ISGs are antiviral genes that function to increase cell sensitivity to infection (...)." This sentence is ambiguous and reads as if cells are more easily infected due to ISGs.
2) "However, lncRNAs are more cell type-specific, less expressed (...)." Less expressed as in less
abundant? Expressed in fewer cell types? Clarify.
3) "HIV and influenza are viruses that reach the nucleus and, therefore, gifted to alter the cell transcriptome." Many studies on transcriptomic changes have been conducted for non-nuclear viruses, so reaching the nucleus is not a requirement for altering the cellular transcriptome.

These ambiguous sentences have been modified.

6. In Figure 1D, the names of hits are written but cannot be connected with the point to which they correspond - the point itself needs to be highlighted in some way. In addition, what is the rationale for the hits listed by name in Figure 1D? This should be clearly stated somewhere. It would also be useful if the top hits of the noncoding hits were also highlighted in some way.

This has been corrected (see new Appendix Fig. S.1). As suggested by another reviewer, the RNASeq data is less relevant in the revised version of the manuscript.

7. Figure 2 has CSR37 shaded in a different color besides those used for the other CSRs, and there is no explanation for this in the figure legend. The reviewer understands that this CSR was removed from further analysis, but the differential shading for CSR37 still needs an explanation.

The reviewer is right. This is now mentioned in the legend of Fig. 2. CSR37 has been renamed CSR28.

8. Provide some justification for continuing with NEURL3/CSR31 if it does not meet your requirements for a lncRNA.

This has been done in the revised version of the manuscript. NEURL3/CSR31 is described as a non-coding pseudogene by Encode and as a coding gene that encodes for an E3 ubiquitin-protein ligase by the RefSeq and Ensembl databases. NEURL3/CSR31 could be non-coding as peptides derived from this gene have not been identified in proteomic analyses and the NEURL3/CSR31 RNA has not been found associated with the ribosome in the ribosome profiling studies performed by Lee and Bazzini (Fig. EV.1B). However, CPAT and PhyloSCF analyses predict that NEURL3/CSR31 could be a coding gene. It should be taken into consideration that analysis of the coding potential of pseudogenes using PhyloSCF is tricky, as this program evaluates conservation to predict coding capacity. By definition, noncoding pseudogenes are similar to their parental coding genes. Therefore, we believe that further experiments should be performed to evaluate whether NEURL3/CSR31 is indeed a coding gene. If this is not the case, our studies describing the high increase of NEURL3/CSR31 after HCV infection, and IFN or pI:C treatments, may be of interest for other scientists.

9. The authors should be careful with broad statements claiming nuclear localization when discussing Figure 3B; they list two CSRs as having altered localizations following HCV infection, then describe them as having "nuclear localization."

This has been corrected in the revised version of the manuscript (now Fig. EV.1C).

10. The error bars in Figure 3B are not correctly placed.

This has been corrected in the revised version of the manuscript (now Fig. EV.1C).

11. In Figure 4B, CXCL10 should be treated the same as the other samples... is the increase shown significant? Not significant?

This has been corrected in the revised version of the manuscript. The increase is significant (now Fig. 3B).

Referee #2:
Major concerns:

1. Figure 3B reports that EGOT cellular distribution is altered upon HCV infection and switches
from the cytosol to nucleus. Since this observation was one of the strongest arguments to focus on EGOT analysis, this result should be complemented immune-fluorescence analysis using RNA FISH.

We have removed from the revised version of the manuscript the putative relocation of EGOT after infection to address an issue raised by reviewer 1. The levels of EGOT in HuH7 cells are low (less than one copy per cell). According to reviewer 1, the relative small difference in localization upon HCV infection could merely reflect increased transcription of EGOT. In the new version we now highlight other strong arguments to focus on EGOT.

2• CRS19, 21, 26 and 34 are induced upon HCV infection but not upon IFN treatment, poly(I:C) transfection or "infection with other viruses tested". This allows the authors to claim that their expression is hence specific to HCV infection. However, this latter observation is not supported by data. The authors should provide this information as a complement of Figure 4B.

We strongly agree with the reviewer. We now include a figure showing that there are no significant differences in the levels of CSR19, 21, 26 or 34 in control cells and in cells infected with influenza virus or SFV for 8 hours (new Appendix Fig. S.3D). We thank the reviewer for raising this issue. We have now evaluated human samples in more detail. First, we have evaluated EGOT levels in an independent cohort of patients (10 controls and 19 HCV-infected patients). Similar to what was obtained with the initial cohort, HCV-infected livers showed increased levels of EGOT compared to non-infected livers. This is now included in new Fig 6A. Furthermore, in the revised version of the figure, we identified in the graphs those samples from HCV-infected patients with HCC (new Fig. 6A). The levels of EGOT are similar in HCV-infected livers with or without HCC.

We have also obtained new evidences suggesting that EGOT is not involved in the proliferation of liver cells. In the first place we observed that HuH7 cells in which EGOT expression has been decreased with specific gapmers showed similar growth rates than cells treated with control gapmers. We have also quantified the levels of a putative mouse homologue of EGOT (mEGOT) in different samples from murine liver and spontaneous HCCs developed in these animals. We did not observe differences in the expression levels of mEGOT comparing healthy mouse livers (n=7), and peritumor (n=17) or tumor tissue (n=23) obtained from HCCs developed in mice 9 months after treatment with DEN. In turn, the mouse homologue of PVT1, an oncogenic IncRNA, was significantly increased in tumor samples compared to healthy livers (data not shown).

We have also quantified the levels of HCV RNAs in the second cohort of patients. We do not observe a significant correlation between EGOT and HCV RNA (new Fig. 6B). Given that the proportion of HCV-infected hepatocytes per person ranges from 21-45% and that most infected hepatocytes express low levels of viral RNA (1-50 copies according to Kandathil et al., Gastroenterology 145: 1404-13, 2013; 1-8 copies according to Stiffler et al., PLoS One 4: e6661, 2009; and 7-64 copies according to Chang et al., Am J Pathol 163: 433-444, 2003), we hypothesized that EGOT expression in HCV-infected livers could also be driven by TNFα, as shown in HuH7 cells (Fig. S1). Therefore we have quantified the levels of TNFα mRNA and we have observed that there is a significant correlation between TNFα levels and EGOT expression (new Fig. 6B). Our results are in agreement with the hypothesis that TNFα could be a major driver of EGOT expression in the liver of HCV-infected patients. Given the complexity of the HCV cell cycle, it seems reasonable to think that the levels of HCV in patients could reflect the influence of several factors.
We propose that EGOT could be one of these factors based on the experiments performed in HuH7 cells (Fig. 7).

Finally, we have evaluated the levels of CSR4, CSR14 and CSR23 in HCV-infected livers and controls. As suggested by the reviewer, these CSRs have been taken as a control of lncRNAs that show good expression in the liver (Appendix Fig. S2) and are not highly increased after HCV infection in HuH7 cells (Fig. 2A). We did not detect good levels of CSR4 in human liver. The levels of CSR14 and CSR23 were similar in HCV-infected livers and controls (Rev. 2. Fig. 1).

All these issues are now mentioned/shown in the revised version of the manuscript.

Rev. 2 Fig. 1. Expression levels of CSR23 and CSR14 were evaluated by qRT-PCR in RNA samples obtained from livers from HCV-negative (n=10) and HCV-positive (n=8) patients. GAPDH mRNA was also quantified and used as a reference to calculate the relative levels (2^ΔΔCt x 1000, where ΔΔCt = Ct of the gene of interest − Ct of the control). Statistical significance was calculated using a two-tailed non-parametric Mann-Whitney U-test. Non-significant (ns) differences were observed.

The difference in EGOT mRNA level induction kinetics and amplitude observed for Influenza virus and Semliki Forest Virus is really interesting. Recently, Li and coworkers (Nucl. Acid Research 2015) reported that Influenza A virus non-structural protein NS1 inhibitory effect on PKR activation is mediated by the induction of noncoding vault RNAs. Could this scenario could explain the observed decrease in EGOT mRNA levels at 24h post Influenza A virus infection?

This is a very interesting possibility. We hypothesized that RNA viruses that replicate in the cytoplasm, such as HCV or SFV, produce high levels of dsRNAs and therefore, they activate PKR (or other cytoplasmic sensors) more strongly than RNA viruses such as influenza, that replicate in the nucleus. Therefore, EGOT levels should be higher in cells infected with HCV or SFV than in cells infected with influenza virus. However, the reviewer is right. Inhibition of PKR activation by NS1 could result in decreased levels of EGOT. Interestingly, infection with HCV and SFV proceeds with activated PKR, as translation of HCV or SFV viral proteins is resistant to the inhibitory action of eIF2a phosphorylation (Garaigorta et al., Cell Host Microbe 6: 513-522, 2009; Ventoso et al., Genes Dev 20: 87-100, 2006). Then, infections with viruses that activate PKR, such as SFV and HCV, could lead to higher levels of EGOT compared to infection with viruses that block PKR activation. To address this possibility we have evaluated EGOT levels in control cells or cells infected for 4 or 24 hours with a wild-type influenza virus or with a virus that lacks NS1 protein. EGOT levels were very similar at 4 hours post infection. At 24 hours, EGOT levels were significantly higher in cells infected with the virus that does not express NS1 than in control cells or cells infected with the wild-type influenza virus (Rev. 2 Fig. 2). However, EGOT levels were low compared with those observed in cells infected with SFV or HCV. Therefore, cytoplasmic viral replication could be important for EGOT induction. In agreement with this hypothesis we now show that EGOT levels decrease when viral replication is inhibited (new Fig. 4C).
Rev. 2 Fig. 2. HuH7 cells were mock-infected or infected for 4 or 24 hours with a wild-type influenza virus (Flu) or with a virus that lacks NS1 protein (ΔNS1). The, EGOT levels were evaluated by qRT-PCR. GAPDH mRNA was also quantified and used as a reference to calculate the relative levels of EGOT (2^ΔΔCt x 1000). Statistical significance was calculated using a two-tailed non-parametric Mann-Whitney U-test. Non-significant (ns) differences or significant (*, p< 0.05; **, p<0.01) differences are indicated. The average fold increase versus mock infected cells is indicated at the top of each bar.

5• EGOT mRNA silencing in Huh7 cells results in almost 40% reduction in HCV RNA levels and 80% of the amount of HCV-infected cells (Figure 6 A-D). This result should include the measurement of the HCV viral titer using TCID50. Moreover, the multiplicity of infection used for this experiment (MOI of 0.01) is much lower than in the one used in the induction kinetics shown in Figure 2B (MOI of 0.3). Is the effect of EGOT silencing as pronounced on HCV viral titer when using a MOI of 0.3?

We want to apologize because the moi of HCV that we used was in fact 0.3, and was not indicated in the legend of former Fig. 5. This has been corrected in the revised version of the manuscript. We have tried to calculate TCID50 with HCV. We have successfully used this method to calculate adenovirus titers with 293 cells in the past. However, when we use HuH7 cells to calculate HCV TCID50s, we have background from uninfected cells grown on 96-well plates. Therefore, we have evaluated Focus-Forming Units per millilitre (FFU/ml). Several laboratories use this method to titer HCV (Zhong et al., PNAS102:9294-9, 2005). We used a modification of the method previously described. In brief, cell supernatants are serially diluted 10-fold in complete DMEM media and used to infect 10^5 naïve HuH7 cells grown onto coverslips in 24-well plates. The inoculum is incubated with cells for 4 h at 37°C and then supplemented with fresh complete DMEM. Cells are fixed and permeabilized two days post-infection and HCV-infected cells are visualized by immunofluorescence staining of the core protein. Total and core-positive cells are counted in the highest possible dilutions (with at least one core-positive cell per field). Fields continue to be evaluated until a stable average number of core-positive vs total cells is obtained. This value is used to calculate the focus-forming units per milliliter of supernatant. Fig. 7C (in the new version) has been modified in the revised version to show FFU/ml.

6• Same comment as above, which MOI was used for the experiments shown in Figure 8? The authors should provide the corresponding data for EGOT silencing efficiency and HCV infection levels. The induction of ISGs in response to HCV infection occurs already 12h post infection. How is EGOT inhibitory effect on ISG mRNA levels regulated in the course of the infection, up to 96h?

We have also clarified this in the revised version of the manuscript. Actually, the measurements performed in Fig. 8 were made with the samples used in former Fig. 6 (now Fig. 7). Therefore the moi was 0.3. The new Fig. 8C also shows the results of silencing EGOT in cells infected with HCV for 12 or 24 h. The results obtained at 48 h post-infection were similar to those shown in Fig. 8A. At later times post infection we did not observe efficient EGOT silencing and therefore those samples were not evaluated. Interestingly, the results show that EGOT depletion leads to increased levels of ISG15 and GBP1 already at 12 hpi (Fig. 8C and data not shown). However at this time point we do not observe differences in the levels of HCV RNA between EGOT-depleted cells and controls. EGOT effect on HCV RNA is observed at 24 hpi. Therefore, EGOT effect on ISGs precedes the effect on viral replication.

7• Results shown in Figure 9 are essential to understand the pathway by which EGOT expression is upregulated. The author should provide a western-blot analysis to confirm that in HCV infected cells upon PKR silencing PKR phosphorylation levels are inhibited. In addition, the authors should clarify the discrepancy in EGOT expression levels in the control conditions shown in panel 9A and 9C: in panel 9A EGOT expression is increased over 200 fold in cells transfected with control siRNA and infected with HCV while only about 3 fold in panel 9C, in HCV-infected cells transfected with control plasmid.
It has been reported that HCV RNA interacts with PKR early after infection (Arnaud et al., *PLoS Pathogens* 7: e1002289, 2011). Binding occurs on the first dsRNA binding domain located at the N terminus of PKR and it is independent of its kinase activity. Non-phosphorylated PKR can induce NFKB by binding members of the TRAF family or MAVS. This results in a preferential induction of ISGs, such as ISG15. Therefore, rather than measuring PKR phosphorylation (which is not required for PKR-induced transcriptional activity) we have evaluated the levels of ISG15 mRNA in HCV-infected control cells and cells in which PKR had been inhibited. The results are shown in Fig. EV.3C. As has been described previously, decreasing PKR results in reduced levels of ISG15 in HCV-infected cells.

Regarding former figure 9C, we now realize that the labeling of the figure was slightly confusing. The 3 fold increase in EGOT levels is between cells infected with HCV and transfected with a control plasmid (HCV –) and cells infected with HCV and transfected with a PKR expressing plasmid (HCV +). This is the only place where such a comparison is shown. We believe that the reviewer interpreted the data as EGOT levels in non-infected cells versus infected cells. Looking at the figure closely to understand the concern raised by the reviewer, we realized (and probably also the referee?) that in these data, the increase in EGOT levels after infection is only around 20 fold (compare the first bar, Mock – PKR with the fifth bar, HCV – PKR). We went back to the original experiments and realized that the data included in this figure for HCV-infected cells were not the correct ones (the data corresponded to cells that had been first infected with HCV, then transfected and collected 24 hours later). We have now included the data obtained with cells that were first transfected (with control plasmid or plasmid expressing PKR) and then infected with HCV, as has been done with the controls. In this case, the fold increase is similar to what has been described in other figures (around 200 fold).

8. The authors schematized PKR functions in Figure 9D. However, an activation of NF-kB mediated by RIG-I cannot be excluded. An additional siRNA directed against RIG-I should be included in the experiments shown Figure 9A.

This is a very important issue. We have now evaluated EGOT induction in RIG-I deficient HuH7.5 cells and in HuH7 cells treated with siRNAs targeting RIG-I. The results show that although RIG-I can also mediate EGOT induction, HCV can induce EGOT in the absence of RIG-I. These results are now shown in new Fig. 5A-B.

9. The use of IRF3 dominant negative form and IKB(SA) is a nice complementary approach to dissect the signaling pathway. However, additional information is required to show the functionality of these constructs in HuH7 cells. Figure 9E, the inhibition of IRF3 should be validated by western-blot analysis of IRF3 phosphorylation or immune-fluorescence analysis of IRF3 translocation to the nucleus in both non-infected and infected DNIRF3 expressing cells. On the same line, Figure 9F, the repression of NF-kB activation in pIKB(SA)-transfected cells should be validated by western-blot analysis of p65 phosphorylation levels in non-infected and HCV-infected cells.

The dominant negative of IRF3 used has a deletion of the 133 residues in the NH-terminal region, where the DNA binding domain locates. This deletion mutant contains the carboxy-terminal regions, with the inhibitory domain (ID) and the IRF association domain (IAD), required for phosphorylation and dimerization, respectively. Therefore, in the presence of the dominant negative mutant, IRF3 phosphorylation, dimerization and subsequent transport to the nucleus should not be affected. Therefore, to provide additional information showing the functionality of these constructs, we have evaluated their effect in luciferase reporter constructs that respond to IRF3 ((PRDIII-I)4-Luc; Ehrhardt et al., *FEBS Lett* 567: 230-238, 2004) or NFKB (NFκB 3xLuc; Abad et al., *Nucleic Acids Res* 38: e136, 2010). The results showing decreased expression of the reporters in the presence of the dominant negatives of IRF3 and NFKB are now shown in Fig. EV.3F-G.

Minor concerns:

1. It might be helpful to the readers that are not familiar with H3K4me1 and H3K4me3 to indicate that they correspond to mono or trimethylations of histones.
This correction has been made

2. **Figure 2B misses the asterisk representing statistical analysis.**

This correction has been made

3. **In order to avoid misunderstanding, the term "mock" in the labeling of histogram axis shown in Figures 6, 8 and 9, which corresponds to control gapmer or siRNA transfections should be replaced by gaCtrl and siCtrl or similar. Mock is expected to be the non-infected control.**

This correction has been made in new Figs 7, 8 and in Appendix Fig. S.5.

4. *The authors should provide the gapmer sequences used for EGOT silencing*

Gapmer sequences are provided in Materials and Methods

**Referee #3:**

**Major Comments:**

1. This study has some problems especially with regards to the transcriptomic overlay of microarray vs. RNASeq. In Figure 1, the authors compare the protein coding transcriptome identified by either microarray or RNASeq analysis. While the authors state "similar coding genes were identified by microarray and RNASeq analysis... In fact, 18.7 % of the coding genes identified as altered in response to HCV infection after RNASeq analysis.... were also identified in the microarray analysis." I disagree that this can be interpreted as a "similar" profile, since almost 4 out of 5 genes do not show the same differential expression pattern when comparing microarray with RNASeq (79.3%). This is a big concern since one would expect a higher overlap of differentially expressed protein-coding genes. Was the same RNA input used for the both experiments? How do the authors explain this very weak correlation?

   and

2. In Figure 1F: According to the Venn diagram, only 383 protein-coding genes change following HCV infection (RNASeq). This seems like a small number, and the finding that the microarray detects almost 10 fold the amount of differentially expressed genes (2842) is not understandable to me.

We have reevaluated the data regarding the intersection between the differentially expressed genes (DEG) obtained in the microarray and the RNASeq analyses. We agree with the reviewer that the overlap should be better. We believe that the results (18.7% of the coding genes were identified in both analyses) could be explained by the differences in the design and the statistical power of the analyses performed in both cases. The absence of replicates in the RNASeq experiment makes the application of a robust statistical analysis very difficult. Although by using cuffdiff software it is possible to obtain a p-value for each detected transcript, the statistical significance of the result and the estimation of the FDR should be interpreted with caution. In contrast, the statistical analysis of the microarray experiment is robust and reliable.

Given these circumstances, in the revised version of the manuscript we have decreased the relevance of the RNASeq analyses. Actually, most of the CSRs evaluated in our work were identified with the microarray data. In the new version we mention that the RNASeq was performed and that the expression of the candidates identified in the microarray analysis was visualized using the RNASeq data. Former CSR27 and 28, identified only by RNASeq, have been now deleted from the revised version of the manuscript. Cufflink structures are only shown in a supplementary figure to comply with a request made by reviewer 1. Long non-coding DEGs identified in the RNASeq data are not shown. This should help to diminish the concerns regarding the RNASeq analysis.

3. Throughout the manuscript, the authors use differential expression (ie fold change) as a measure for selecting candidates. This is a very reasonable approach for candidate selection, but at the same time it would be important to understand the absolute expression levels of the selected transcripts to be able to estimate the copy number per cell of those transcripts. For low-abundance transcripts like lncRNAs, a high fold change can be misleading.
We strongly agree with the reviewer about this point. In fact, the highest fold changes (Fig. 2) are observed with transcripts that are expressed to low levels (Appendix Table S.2). In the revised version of the manuscript we have divided all CSRs from 1 to 35 into those expressed to high, intermediate or low levels in HCV infected cells (Appendix Table S.2). We now specify the range of Ct values that correspond to each category (low, Ct values ≥ 30 (generally corresponding to one or less copies per cell); medium, Ct values 26-29 and high, Ct values ≤ 25. While qRT-PCR does not provide absolute measurements in the absence of a standard, we believe that Ct values may give a general idea of expression levels. As a reference, we mention that the abundant GAPDH mRNA (more than 1000 copies per cell) has an average Ct value of 17 using our qRT-PCR conditions while most coding genes evaluated in this work have Ct values of 21-25.

Furthermore, we have determined the copy number of EGOT in infected cells. To this aim, EGOT cDNA was cloned after a T7 promoter and EGOT RNA was transcribed in vitro and quantified by bioanalyzer. Then, it was used as a standard for a qRT-PCR reaction. An identical protocol was followed to evaluate the levels of the more abundant CSR34/UCA1. The results indicate that there are 8 - 15 molecules of EGOT mRNA and 60-90 molecules of UCA1 mRNA per HuH7 cell infected with HCV for 48h (Page 5, paragraph 4). Similar results were obtained for CSR30/PINT, whose quantification resulted in 5-7 copies of PINT per cell. This correlates with what has been found in situ hybridization (Martin-Bejar et al., Genome Biol 2013; 14:R104).

4. Fig 4a: Given the kinetics of the type I interferon response on interferon-stimulated gene expression patterns, 72h of stimulation as the only time point is not ideal. It would be interesting to perform timecourse and dose response experiments.

We evaluated the expression levels of CSRs at 72 hours post-IFN treatment as we have previously shown that at this time point it is still possible to detect increased expression of genes that are induced early after IFN treatment but also increased levels of genes that respond to a secondary wave of the IFN response (Carrero et al., Front Immunol 5, 2014). As suggested by the reviewer, in the revised version we have evaluated expression levels of CSRs at 6 hours post-IFN treatment. Only CSR31 is induced at that time point. In this case, we have evaluated the effect of several doses of IFN incubated for 6 or 72 hours in the expression of CSR31. The results are now shown in Appendix Fig. S.3.

5. Fig 4B: It would be interesting to see the differential expression of the selected lncRNAs after stimulation with a broader panel of TLR ligands and nucleic acids

We believe that this is a very relevant issue in the case of CSR32/EGOT. HuH7 cells do not express all TLRs, but they do express TLR4 (LPS sensor) and TLR7 (ssRNA and Imiquinod sensor). Therefore, we tested the expression levels of EGOT after incubating the cells with LPS or Imiquinod. Further, we tested the effect of several cytokines such as TNFα, IL6 or oncostatin. The results show a significant increase in the levels of EGOT after treatment with pI:C, Imiquinod, LPS and TNFα. However, treatment with IL6 or oncostatin did not affect EGOT levels. These results are shown in Fig. 4E and 5I of the revised version of the manuscript.

6. Fig 6: What is the effect of gapmer mediated knockdown of EGOT after stimulation? EGOT is almost 800 fold induced after HCV infection, so it would be important to assess the quality of the knockdown both basally and after induction.

The gapmers reduce EGOT levels efficiently. The levels of EGOT are induced 100-200 fold at 48 hours post-HCV infection. Depending on the experiments, gapmers reduce the levels of EGOT in infected cells 5-30 fold (Fig. 7 A and E; see also new Fig.8C) and in non-infected cells 3-30 fold (new Fig. 8B). Gapmers lose efficiency at later times post-infection, when EGOT levels increase more than a 100 fold. EGOT depletion results first in increased levels of ISGs and later in decreased replication (new Fig.7-8).

7. Fig 6: How were the GAPmers target regions selected? There is no experimental data showing that knockdown of EGOT does not affect the expression of the gene ITPR1 gene, in which it is located. Given the incorrect annotation of some lncRNAs, the sequence of EGOT needs to be confirmed by RACE
Exiqon selected five target sequences for EGOT using algorithms developed by the company. They synthesized the gapmers that match these target sequences. We evaluated the efficacy and toxicity of the 5 gapmers and selected 2 able to inhibit EGOT efficiently with non-detectable toxicity (Fig. 7A and new Appendix Fig. S.4).

Regarding ITPR, all analyses performed between ITPR1 and EGOT indicate that there is no expression correlation (see Page 9). In fact, cells infected with HCV show very high levels of EGOT compared to normal cells while the levels of ITPR1 do not change (R3. Fig. 1A). Therefore, we were surprised to find that gapmers that target EGOT, but not control gapmers, reduce the levels of ITPR1 significantly (R3. Fig. 1B). Then, we speculated that low levels of EGOT may be required to induce ITPR1 expression. As the EGOT region is classified as an enhancer according to chromatin marks (Fig. EV.2), we believe that ITPR expression could be regulated by EGOT acting as an enhancer RNA. Future studies will be done in the laboratory to address this issue.

R3. Fig. 1. Analysis of the regulation between CSR32 and ITPR1. RNA was isolated from control cells or cells infected with HCV for 48 hours (A) or from infected cells treated with control gapmers or gapmers that target CSR32/EGOT (ga·1αEGOT and ga·2αEGOT). Expression of CSR32 (A) and ITPR (A and B) was evaluated by qRT-PCR. GAPDH levels were also measured and used as a reference.

Finally, we have confirmed EGOT sequence by RACE. Compared to the sequence of EGOT annotated in public databases (ENCODE, RefSeq and Ensemble), we find that EGOT 5´end starts 32 nucleotides upstream (new Fig. EV. 2). EGOT exon junction has been confirmed by cloning and sequencing EGOT cDNA (page 5 paragraph 4).

Our work shows that EGOT levels are increased in response to pI:C. We now show that EGOT is also increased in cells infected with UV-irradiated HCV, deficient for replication or in HCV-infected cells treated with replication inhibitors (Fig. 4D), although at much lower levels. This suggests that sensing the viral genome induces the expression of EGOT.

Then, we show that inhibition of EGOT leads to an increase in the levels of several ISGs and to a decrease in viral replication (Fig. 7 and 8). We believe that the decreased viral replication is the result of the increased levels of ISGs, because:
- We now show that depletion of EGOT causes first an increase of several ISGs (observed at 12 hours post-infection) and then a decrease in viral replication (observed at 24 hours post-infection).

However, this is a hypothesis. We now mention that we cannot exclude the possibility that EGOT affects viral replication also in an ISG-independent manner.
9. Fig. 8: What do the authors mean by relative expression here-re- relative to what? Non-infected? Moreover, the asterisk for Mx1 ga2alpha is unclear. Why is ga2alpha "more" significant than ga1alpha, when the error bar is higher and the relative RNA level is identical to ga1alpha?

This has been corrected and the term “relative expression” has been clarified. To calculate the relative RNA levels, transcript levels were evaluated by quantitative RT-PCR and normalized to the expression of an internal control. GAPDH mRNA was chosen as a reference because the levels of GAPDH mRNA were not altered by the treatments used. Furthermore, samples showing over one Ct value difference in the level of GAPDH mRNA compared to control samples were not used in the analysis. This occurred only in few cases. In the revised version of the manuscript, the axes of the figures have been changed from the general "relative RNA levels" to the more specific “2ΔCt x 1000” that was used in all cases, where ΔCt = Ct of the gene of interest – Ct of the internal control.

Minor Points:
1. In the Introduction the statement: "LncRNAs are functional transcripts with no identifiable protein coding capacity" Since lncRNAs are defined by length and lack of coding potential only, the fraction of functional transcripts among all lncRNAs is unclear to date. This statement should be revised.

The reviewer is right. This has been corrected.

2. "HIV and influenza are viruses that reach the nucleus and, therefore, gifted to alter the cell transcriptome." Clearly, dramatic transcriptional changes of host cells can also happen independently of nuclear entry of a virus (cytoplasmic nucleic acid signaling!), so this statement is not correct.

This has been deleted.

2nd Editorial Decision 07 April 2016

Thank you for the submission of your revised manuscript to our editorial offices. We have now received the enclosed reports on it. As you will see, all three referees find the manuscript suitable for publication in EMBO reports. Nevertheless, referees #1 and #2 have raised several minor points and suggestions that should be addressed, before we can proceed to formal acceptance. I would therefore like to ask you for further minor revisions (see also below), before we can proceed with the formal acceptance of your manuscript. Please also address all referee points (as detailed in their reports) in a complete point-by-point response.

We would also ask you to replace the capital letters in the title and several subtitles with normal letters.

The figures look blurred when looking at them in full size (100%). Could you provide better quality or higher resolution versions?

-----------------------------

REFEREE REPORTS

Referee #1:

The authors addressed many of the concerns raised in response to the first submission. Importantly, they now calculate the copy number of EGOT in uninfected and HCV-infected cells, showing that EGOT is essentially not expressed in uninfected cells, but in response to HCV induction, there are 8-15 copies of EGOT/cell. The authors also examined the specificity of EGOT induction, showing EGOT is upregulated in response to many different triggers e.g. TLRs, interferon, TNF-a, PKR and
also to viral RNA during infections with HCV, SFV and influenza virus. Overall, explanations of the experiments are much clearer.

The manuscript is greatly improved, but some results are puzzling and require either additional experiments or tempering conclusions:

1) The authors now show that ~8-15 molecules per cell of EGOT are induced by HCV infection. Given the 200-fold upregulation relative to baseline levels, this shows that most cells at baseline do not express even a single EGOT transcript. It is therefore difficult to understand how EGOT gapmer treatment at baseline can upregulate ISG expression. Either there are a few "jackpot" cells in the culture where EGOT is expressed and important for suppressing ISG expression or the gapmer effects are off target.

2) Given the 2-fold effect on IPTR1 expression, a gene that harbors EGOT between exons 43 and 44, one worries that some or all of the gapmer effects could be due to downregulation of IPTR1 and effects on Ca2+ homeostasis. This concern could be mitigated by either by examining the effect of selective downregulation or knock out of IPTR1, without affecting EGOT levels or induction, or demonstrating EGOT activity in gain-of-function experiments (which were attempted but failed). Transfection of in vitro transcribed EGOT RNA might be an alternative method worth trying. However, it is possible that EGOT RNA is necessary but not sufficient for restoring the suppressive effect under these experimental conditions.

3) When testing HCV infected liver samples, the authors did not find significant correlation between HCV RNA levels and EGOT levels. This would seem to argue against EGOT being directly induced by HCV in vivo. What should we take from these results, which appear to conflict with the cell culture data? It is certainly possible that chronic HCV infection and inflammation in the liver leads to other EGOT-inducing stimuli that obscure any direct effects of HCV.

Referee #2:

Carnero and colleagues provide a substantially improved manuscript addressing all my concerns and the ones of the other reviewers. I particularly appreciate the efforts to clarify the possible involvement of RIG-I in EGOT induction and the more in depth analysis of patient data. Altogether, the final manuscript benefits from the new rearrangement. The section with bioinformatics data has been simplified and is much clearer, experimental procedure, figure legends and axe annotations more accurate.

Minor comments that should be improved for publication:
1) Result section paragraph "Identification of LncRNAs regulated by HCV": We selected the probes that showed a significantly altered expression by HCV infection and that were described in the array as LncRNAs (505 probes with B>2) (Fig. 1A). Fig. 1A should be replaced by Fig. 1C.
2) Result section paragraph "Some CSR respond to the antiviral pathway ...": with HCV for 6 days at moi 0.03 (Fig. 3A). According to the authors comments, in this paragraph moi 0.03 should be replaced by 0.3.
3) Result section paragraph "EGOT is induced by RIG-I and...": It has been reporter, replace by reported.
4) Figure EV2: the letter "C" is missing to annotate panel C.
5) Figure EV3 panel B: the use of pPKR for PKR plasmid shown in the annotation of the western-blot lanes is misleading since the staining for phospho-PKR protein levels is also annotated pPKR. Please make it consistent with Figure 5D and E in which transfection of the PKR plasmid is annotated with "PKR". The fact that transfection of PKR plasmid alone induces such an autophosphorylation (lane 4 of the Western-blot) is surprising and not discussed. Was this sample infected with HCV?
6) Figure EV3 panel G: same comment as for PKR plasmid, pIKB misses "(SA)" in the legend annotation.
Referee #3:

The authors have addressed to my satisfaction the majority of the issues raised. As a result the manuscript is improved. I have no further issues.

2nd Revision - authors’ response 03 May 2016

Complete point-by-point response to referees:
Referee #1:
The manuscript is greatly improved, but some results are puzzling and require either additional experiments or tempering conclusions:

1) The authors now show that ~8-15 molecules per cell of EGOT are induced by HCV infection. Given the 200-fold upregulation relative to baseline levels, this shows that most cells at baseline do not express even a single EGOT transcript. It is therefore difficult to understand how EGOT gapmer treatment at baseline can upregulate ISG expression. Either there are a few "jackpot" cells in the culture where EGOT is expressed and important for suppressing ISG expression or the gapmer effects are off target.

As suggested by the reviewer, the results can be explained by the existence of few cells in the culture that express EGOT and suppress ISG expression. Alternatively, if EGOT is not very stable, it could be expressed transiently in many cells. In this situation, only a small population of cells would express EGOT at a given time (this is what we observed in the quantification experiments, where for example, ~5% of the cells could express a single molecule/cell). Then, a different subpopulation could express EGOT at later time points. Preliminary experiments suggest that the half-life of EGOT is only ~2 hours and it should be noted that the inhibition experiments were performed during three days. Then, most cells may have transiently expressed EGOT during this time. If this is the case, the inhibition of EGOT should occur in most cells and should impact on a regulator that controls ISG expression for long term in most cells. Both possibilities (a few expressing cells or many cells expressing transiently) are very appealing and we will try to address which one is correct in the future. This is now discussed in the revised version of the manuscript (page 10, last paragraph). We do not believe that our results are due to off-target effects as two inhibitors against EGOT and several negative controls have been used.

2) Given the 2-fold effect on IPTR1 expression, a gene that harbors EGOT between exons 43 and 44, one worries that some or all of the gapmer effects could be due to downregulation of IPTR1 and effects on Ca2+ homeostasis. This concern could be mitigated by either by examining the effect of selective downregulation or knock out of IPTR1, without affecting EGOT levels or induction, or demonstrating EGOT activity in gain-of-function experiments (which were attempted but failed). Transfection of in vitro transcribed EGOT RNA might be an alternative method worth trying. However, it is possible that EGOT RNA is necessary but not sufficient for restoring the suppressive effect under these experimental conditions.

We have tried to perform the experiment suggested by the reviewer without success. Therefore we have tempered our conclusion by indicating in the revised version that “we cannot rule out the possibility that EGOT affects viral replication in an ISG-independent manner, for instance through regulation of ITPR” (page 10, last paragraph). This is something that we need to address in the future.

3) When testing HCV infected liver samples, the authors did not find significant correlation between HCV RNA levels and EGOT levels. This would seem to argue against EGOT being directly induced by HCV in vivo. What should we take from these results, which appear to conflict with the cell culture data? It is certainly possible that chronic HCV infection and inflammation in the liver leads to other EGOT-inducing stimuli that obscure any direct effects of HCV.

This is what we think. In HCV patients, the number of infected hepatocytes is low and most infected hepatocytes express low levels of viral RNA. However, liver inflammation will lead to increased TNFα that should induce EGOT expression in most hepatocytes. In fact, we do observe a correlation between TNFα and EGOT in vivo. We mention in the manuscript that “we find a significant
correlation between the levels of TNFα mRNA and EGOT, suggesting that this cytokine could be the major driver of EGOT expression in the liver of HCV-infected patients”.

Referee #2:
Minor comments that should be improved for publication:
1) Result section paragraph "Identification of LncRNAs regulated by HCV": We selected the probes that showed a significantly altered expression by HCV infection and that were described in the array as lncRNAs (505 probes with B>2) (Fig. 1A). Fig. 1A should be replaced by Fig. 1C.
This has been corrected.

2) Result section paragraph "Some CSR respond to the antiviral pathway ...": with HCV for 6 days at moi 0.03 (Fig. 3A).
According to the authors comments, in this paragraph moi 0.03 should be replaced by 0.3.
The reviewer is right. This has been corrected.

3) Result section paragraph "EGOT is induced by RIG-I and...": It has been reporter, replace by reported.
This has been corrected.

4) Figure EV2: the letter "C" is missing to annotate panel C.
This has been corrected.

5) Figure EV3 panel B: the use of pPKR for PKR plasmid shown in the annotation of the western-blot lanes is misleading since the staining for phospho-PKR protein levels is also annotated pPKR. Please make it consistent with Figure 5D and E in which transfection of the PKR plasmid is annotated with “PKR”. The fact that transfection of PKR plasmid alone induces such an autophosphorylation (lane 4 of the Western-blot) is surprising and not discussed. Was this sample infected with HCV?
This has been corrected. Regarding PKR, we agree with the reviewer that the levels of autophosphorylation obtained after plasmid transfection are high. This sample (lane 4) was not infected with HCV. We have observed a similar autophosphorylation of PKR in cells infected with HCV and transfected with the PKR plasmid. We consider that the overexpression of PKR could induce autophosphorylation.

6) Figure EV3 panel G: same comment as for PKR plasmid. pIKB misses "(SA)" in the legend annotation.
This has been corrected.

Referee #3:
The authors have addressed to my satisfaction the majority of the issues raised. As a result the manuscript is improved. I have no further issues.

3rd Editorial Decision 12 May 2016
I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.
**A- Figures**

1. **Data**
   - The data shown in figures should satisfy the following conditions:
     - The data were obtained and processed according to the method(s) and are presented to reflect the results of the experiments in an accurate and unbiased manner.
     - Figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
     - Graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
     - If n ≥ 2, the individual data points from each experiment should be plotted and any statistical test employed should be justified.
     - Source data should be included to report the data underlying graphs. Please follow the guidelines set out in the authorship guidelines.

2. **Captions**
   - Each figure caption should contain the following information, for each panel where they are relevant:
     - A description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
     - A statement of how many times the experiment shown was independently replicated in the laboratory.
     - Definitions of statistical methods and measures:
       - Common tests, such as t-test (please specify whether paired or unpaired), simple p-test, Williams and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section.
       - Are tests one-sided or two-sided?
       - Are there adjustments for multiple comparisons?
       - I was statistical test results, e.g., F-value > x test if F-value < x.
       - Definition of “center values” as median or average.
       - Definition of error bars as ± or n.r.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models, and human subjects.

**B- Statistics and general methods**

1a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?

Sample size was chosen using biomath (http://www.biostat.wisc.edu/power/index.htm) to have high possibilities of achieving statistical significance with the n used.

1b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.

See above.

2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?

Samples were excluded for analysis. Criteria with 13 months were included.

3. Were any steps taken to minimize the effects of subjective bias when allocating samples or treatments to experiments (e.g. randomization procedure)? If yes, please describe.

Steps were taken randomly.

4. For animal studies, include a statement about randomization even if no randomization was used.

Animals were taken randomly.

5. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes, please describe.

No steps were taken to minimize these effects.

6a. For animal studies, include a statement about blinding even if no blinding was done.

No blinding method was done.

6b. For every figure, are statistical tests justified as appropriate?

Statistical tests were justified as appropriate.

7. In the data table, are the assumptions of the tests (e.g., normal distribution) described in the methods section? If yes, please describe.

Statistical analysis was performed using GraphPad. Statistical significance of unpaired or paired versus two independent non-sampled data was calculated using a two-tailed non-parametric Mann-Whitney U-test for samples that do not follow a normal distribution. When the samples followed a normal distribution according to the Shapiro-Wilk test a two-tailed Students t-test was used. Correlation was assessed by Spearman’s correlation coefficients. All data show means ± standard deviation. If values lower than 0.05 were deemed as significant. In all data shown, * denotes p<0.05, ** p<0.01, *** p<0.001 and **** p<0.0001.

8. Is there an estimate of variation within each group of data?

Data samples were homogeneous, although some outliers were found in some experiments with patient samples.

9. Is the variance similar between the groups that are being statistically compared?

Variance was similar between groups when compared.
C - Reagents

1. Antibodies: Antibodies were profiled for use in the system under study (assay and species), to provide a chassis, culture number and/or clone number, supplementary information or reference to an antibody validation profile, e.g., Antibodypedia (see link list at top right), Ixiom (see link list at top right).

2. Reagents: Antibodies were profiled for use in the system under study (assay and species), to provide a chassis, culture number and/or clone number, supplementary information or reference to an antibody validation profile, e.g., Antibodypedia (see link list at top right), Ixiom (see link list at top right).

3. Identify the source of cell lines and report if they were recently authenticated (e.g., by SRM profiling) and tested for mycoplasma contamination.

4. Antibodies: Antibodies were profiled for use in the system under study (assay and species), to provide a chassis, culture number and/or clone number, supplementary information or reference to an antibody validation profile, e.g., Antibodypedia (see link list at top right), Ixiom (see link list at top right).

D - Animal Models

1. Antibodies: Antibodies were profiled for use in the system under study (assay and species), to provide a chassis, culture number and/or clone number, supplementary information or reference to an antibody validation profile, e.g., Antibodypedia (see link list at top right), Ixiom (see link list at top right).

2. Animal models: Antibodies were profiled for use in the system under study (assay and species), to provide a chassis, culture number and/or clone number, supplementary information or reference to an antibody validation profile, e.g., Antibodypedia (see link list at top right), Ixiom (see link list at top right).

E - Human Subjects

1. Antibodies: Antibodies were profiled for use in the system under study (assay and species), to provide a chassis, culture number and/or clone number, supplementary information or reference to an antibody validation profile, e.g., Antibodypedia (see link list at top right), Ixiom (see link list at top right).

2. Antibodies: Antibodies were profiled for use in the system under study (assay and species), to provide a chassis, culture number and/or clone number, supplementary information or reference to an antibody validation profile, e.g., Antibodypedia (see link list at top right), Ixiom (see link list at top right).

3. Antibodies: Antibodies were profiled for use in the system under study (assay and species), to provide a chassis, culture number and/or clone number, supplementary information or reference to an antibody validation profile, e.g., Antibodypedia (see link list at top right), Ixiom (see link list at top right).

F - Data Accessibility

1. Data deposition in a public repository is mandatory for:
   - Protein, DNA, RNA sequences
   - Biomolecular structures
   - Crystallographic data for small molecules
   - Functional genomics data
   - Proteomics and molecular interactions

2. Please provide accession codes for deposited data. See author guidelines, under "Data Deposition".

3. Data deposition in a public repository is mandatory for:
   - Protein, DNA, RNA sequences
   - Biomolecular structures
   - Crystallographic data for small molecules
   - Functional genomics data
   - Proteomics and molecular interactions

4. Access to human, animal, and clinical datasets should be provided with a few restrictions as possible, while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGaP (see link list at top right) or EGA (see link list at top right).

G - Dual use research of concern

1. Cell study framework: Under what research conditions? Please check biosecurity references (see link list at top right) and list of select agents and toxins (APHL/OSA/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.

2. Cell study framework: Under what research conditions? Please check biosecurity references (see link list at top right) and list of select agents and toxins (APHL/OSA/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.

3. Cell study framework: Under what research conditions? Please check biosecurity references (see link list at top right) and list of select agents and toxins (APHL/OSA/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.

4. Cell study framework: Under what research conditions? Please check biosecurity references (see link list at top right) and list of select agents and toxins (APHL/OSA/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.

5. Cell study framework: Under what research conditions? Please check biosecurity references (see link list at top right) and list of select agents and toxins (APHL/OSA/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.

6. Cell study framework: Under what research conditions? Please check biosecurity references (see link list at top right) and list of select agents and toxins (APHL/OSA/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.

7. Cell study framework: Under what research conditions? Please check biosecurity references (see link list at top right) and list of select agents and toxins (APHL/OSA/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.

8. Cell study framework: Under what research conditions? Please check biosecurity references (see link list at top right) and list of select agents and toxins (APHL/OSA/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.

9. Cell study framework: Under what research conditions? Please check biosecurity references (see link list at top right) and list of select agents and toxins (APHL/OSA/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.

10. Cell study framework: Under what research conditions? Please check biosecurity references (see link list at top right) and list of select agents and toxins (APHL/OSA/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.

11. Cell study framework: Under what research conditions? Please check biosecurity references (see link list at top right) and list of select agents and toxins (APHL/OSA/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.