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DDX60 selectively reduces translation off viral type II internal ribosome entry sites

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I would thus like to invite you to revise your manuscript with the understanding that all referee points must be addressed in the revised manuscript or in the detailed point-by-point response (as suggested in your revision plan). Please also address the point of referee #2 mentioned in the significance statement ("However, as it stands, the overall conclusions are limited, and a major open question is how is DDX60 specific to the type II IRES, which was not addressed experimentally, although it was discussed a little bit in the discussion. For example, does it block recruitment of eIFs or host IRES transacting proteins?"). Please submit your revised manuscript together with a final p-b-p-response, addressing all referee concerns. Please address the points by the referees there in separate sections (one for each referee), not mixed as in your revision plan. This will make the re-assessment easier for the referees.

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Data availability

The datasets produced in this study are available in the following databases:

- RNA-Seq data: Gene Expression Omnibus GSE46843 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE46843)
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Yours sincerely,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

In this manuscript, Sadic and colleagues first addressed the mechanism by which DDX60, an interferon (IFN)-stimulated helicase, suppresses hepatitis C virus (HCV) replication. DDX60 antiviral mechanism has been previously attributed by others to various functions, including degradation of viral RNA or activation of the RIG-I signalling pathway. Here, the authors clarify that the previously effect observed on HCV replication was indirect and due to the viral bicistronic construct used in the study, in which expressions of HCV proteins and of fluorescent reporter protein were driven by two different internal ribosomal entry site (IRES). Specifically, DDX60 affected the translation driven by the encephalomyocarditis virus (EMCV). Using a variety of luciferase-based reporter systems whose translation was cap-dependent or driven by viral IRESs of different types, they found that the translation activity of type II IRESs, including EMCV and foot-and-mouth disease virus IRESs, but not type I (poliovirus) and type III (HCV) IRESs, were reduced in the presence of DDX60. This observation correlated with a reduced association of the luciferase reporter RNAs with polysomes. Finally, the impact of DDX60 was also observed during virus infection with viruses harbouring a type II IRES.

In general, this study is well organized and experiments are well executed and carefully controlled. More generally, however, conclusions must be tuned down in several parts of the manuscript, including in the title. Although specific for type II IRESs, DDX60 only moderately decreases the IRES-driven translation but does not inhibit it, and effects on the virus infection are mild. The following points need to be addressed and clarified to strengthen the study.

Major comments:

- Using tools and literature search, the authors identified four potential functional domains and motifs in DDX60 sequence based on which they generated a series of deletion and single point mutants. The antiviral activity of the mutants was on assessed HCV replication by ectopic expression in Huh7 cells, followed by flow cytometry analysis. How were the expression levels in Huh7 cells? Fig. 1B only show the expression in 293T cells.
- In Fig. 1B, some of the constructs, e.g. S918T and Q1321A show no RFP or reduced RFP signal. The authors comment on this point but still use RFP for FACS analysis. How were cells gated in this case?
- The authors generally conclude that each of these predicted domains is important for the antiviral activity of DDX60. However, results are contrasted. Only some residues in the selected domains, e.g. domains I and IV have an effect. Since the involvement of these domains in ATP binding, hydrolysis or RNA unwinding could not be validated in in vitro assays, the conclusion of this analysis should be tuned downwards or highlight in the main text that this is speculative. In addition, helicase mutant E890A is used from this figure on in all other assays as a control, it should be at least described in the result part of Fig. 1C.
- The specific impact of DDX60 on type II IRES is confirmed by infection assays, using an elegant chimeric poliovirus (Fig. 3). Does infection with these viruses induce endogenous expression of DDX60 over time in the selected target cell lines? Picornaviruses usually induce a high IFN-β or IFN-λ release and this might influence the results. HeLa were shown in Fig. S1 to be particularly responsive to IFN. In this case, how would silencing of DDX60 influence EMCV replication in parental cells?
- Cells stably expressing DDX60 were used for the infection assays. Viral genome translation is the first step after entry for positive single-stranded RNA viruses, so, it is surprising that effects become only noticeable after 24 hours. For example, for EMCV produces already 105 PFU/ml at 8 hours post infection and we would expect stronger effects in the first round of infection (Fig. S3D). In these assay, DDX60 expression levels are not shown. Would the effect be stronger at higher DDX60 levels? Is DDX60 degraded upon infection?
- Figure S4 and its description focusing on the potential RIG-I-DDX60 crosstalk are unclear. The panel A is hardly readable. Western blot quantifications are missing for the biological replicates. At a glance, there is no real difference in the expression of DDX60. Also effects might be better visible in an immune-competent cell type such as the A549 cells used in Fig. S1. This would not allow titrating DDX60 helicase mutant but would at least clarify effects of the wild type protein.
- RNA-immunoprecipitation using biotinylated reporter RNAs is an elegant assay to show direct protein-RNA interactions. The results in Figure S5B are very promising, but raise a technical question. It is important to spike the samples with an unrelated RNA to normalize for loss during RNA extraction. This influences the amount of RNA detected by qRT-PCR. Were the samples analysed in this manner? Furthermore, the data shown is representative of only 2 biological replicates. Such assays are not trivial and have high variability. Experiments should be performed at least 3 times and the variation should be shown to reinforce

this observation.
- Because of DDX60 ability to bind to the 5' cap reporter RNA in the pulldown assay, the authors propose that DDX60 is an RNA non-specific binder. Does DDX60 bind to endogenous GAPDH mRNA as well? The observed binding may be the result of transfection artifacts.
- The polysome profile analysis is an important assay when addressing translation efficiency. This part must be further substantiated. First, results in Fig. 5B represent the average of 2 biological replicates. Again, variability between technical replicates alone is often important. This analysis must be repeated at least three times and the significance of the results must be demonstrated, including control cells (expressing Fluc or GFP) that are missing from the current analysis. More generally, it is unclear which fraction belongs to the monosomal, light polysomal and heavy polysomal RNAs. Some mRNAs change distribution within the polysomal fractions (from heavy to light fractions) but their overall translation is not affected. Please provide a gel showing total RNA (18S and 28S) to help the reader. What is the percentage of reporter mRNA associated with polysomal fractions in the different conditions?
- To strongly support the claims on the role of DDX60 on type II IRES-driven translation, the authors should perform polysomal profile analyses in DDX60-expressing cells infected with poliovirus and chimeric poliovirus and measure changes in the association of the poliovirus genome with the poysomes. This would significantly strengthen the findings of the study.

Minor comments:

- Generally, the text would benefit from being shortened. One example is the DDX60 purification attempt described by more than 10 lines of text that could be shortened to one sentence.
- Calling out figure panels in their in numerical order would help the reader (e.g., S3A is cited after S3D). This occurs for several panels.
- Labelling the different constructs in panel Fig. 1A would help the reader. In the main text these are referred to as N and C-terminal mutants however, two N-terminal mutants were generated (Δ1-556 or Δ1-428) with different antiviral activity.
- Fig. 1B shows the Western blot analysis of DDX60 mutant expression levels. Relative intensity levels are quantified and shown for DDX60 and RFP. However, it is not clear which of the loading controls (β-actin, or GAPDH) was used as a reference and how or to which values this was normalized. This is apparently not normalized to the WT construct.
- The legend of the graphs shown in Fig. 4B is missing (blue vs green)
- Polysome occupancy is a misnomer. Polysomes are RNAs loaded with ribosomes, so we can speak of ribosome occupancy. It should be noted, however, that polysome profiles measure the association of a given mRNA with translating ribosomes, but do not provide information on the number of ribosomes loaded on the mRNA.

Significance

The existence of an interferon-induced effector that regulates genome translation in certain classes of viruses is a - -- very exciting finding. Furthermore, the involvement of DDX60 in the regulation of viral translation is novel and opens several avenues of research.
* Published knowledge: none about this topic
* Audience: This work will be of great interest to both the virology and IRES/translation communities.
* Expertise: virology, translation

Referee #2:

Viruses often use internal ribosome entry sites (IRES) to initiate their translation. Viral IRESs can have unique RNA structures and differential requirements for host proteins, and as such they are classified into different types. Importantly, the use of the IRES allows viruses to ensure their RNA gets translated in the face of a host defense program that often limits overall translation. Previously, the interferon-induced protein DDX60 had been shown to be antiviral to hepatitis C virus (HCV), in studies that using a bicistronic HCV reporter virus, in which expression was driven from a type II IRES. This study set out to show how DDX60 was antiviral to HCV, but found that actually DDX60 was not antiviral to HCV, which has a type I IRES, but instead was acting to inhibit translation from type II IRES elements. Using cell culture assays and various reporters, the results define amino acids and domains of DDX60 required to inhibit IRES-mediated translation. Importantly they also show that DDX60 acts only on the Type II IRES, but not Type I or Type III IRES. In a very elegant set of experiments, the results demonstrate that a type II IRES is sufficient to confer virus inhibition by DDX60, by showing that the addition of a type II IRES to an insensitive virus (poliovirus) made the virus susceptible to inhibition by DDX60. A number of experiments were done to describe how DDX60 inhibits type II IRES translation, and the results show that while DDX60 can bind to an IRES element, it only prevents polysome formation from a type II IRES element.

Major comments:

The key conclusions are convincing and the experiments were very well-controlled.

Minor comments:

* The results section in parts was challenging to read as so many caveats and limitations were presented, that it was hard to determine what parts of focus on. The authors might want to consider removing some of the experiments that have many caveats, such as Figure 2A (see below).
* Figure 1A shows a schematic of DDX60, with key domains indicated. It would be helpful if the amino acids that were mutated in Fig. 1C were numbered (in addition to their highlighting with the bold font), to make it easier to refer back and forth between the two figures.
* In Figure 1, amino acid substitutions were made in DDX60 that were designed to inactivate aspects of its function (ATP binding, helicase, ATP hydrolysis and unwinding) to determine if these suggested functions of DDX60 were important for its antiviral activity. The authors were not able to purify DDX60 to test these functions; however this was not described until much later in the manuscript. It would be helpful for the reader to put this section describing the purification issues in the text describing Figure 1, rather than on page 14, where it seems out of place.
* The first paragraph of the section at the end of page 7 and beginning of page 8, related to Figure 2, has a lot of information and is quite dense. It was also confusing because it mentioned wanting to use in vitro transcribed RNA, but then this wasn't actually done in Figure 2A, which comes after this long paragraph. It could be helpful to break this up into the relevant sections when describing the figure panels? For example, talk about the part that's relevant to Figure 2A when describing that section, and then give the limitations for why you next wanted to do the experiments in Figure 2B. Alternatively, since the experiments shown in Figure 2A seem to have so many caveats that limit their interpretation, the authors may want to consider only showing the data in Figure 2B.
* In Figure S2A, it is difficult to see that that the WT inhibits Type II IRES and the mutants do not as the scales go far beyond the data displayed. I would suggest making the Y-axis scale more limited to better see the data, and this comment stands for every figure.
* Related to Figure S4, the text states that cell were treated with PBS or poly(I:C), however the graph in Figure S4A only shows data as "Fold Change over Mock". As the text describes what RIG-I and DDX60 do in the absence of poly (I:C), I would suggest either changing the graph to show mock and poly (I:C) or relabeling the graph or rewriting the text to clarify what is being shown and the important points.
* For the polysome experiments in Figure 5, the fractions should be labeled to indicate the 40S, 60S, and 80S subunit peaks and the polysome peaks. Ideally, the ribosomal RNA bands from one typical run would be shown in the supplemental.

Significance

The conceptual advance of this study is showing that an antiviral factor (DDX60) acts only on specific IRES elements to limit translation from viruses that contain these IRES elements. Overall, the experiments were well-controlled, and I think that that there is novelty in showing that an antiviral factor has IRES-level antiviral specificity. However, as it stands, the overall conclusions are limited, and a major open question is how is DDX60 specific to the type II IRES, which was not addressed experimentally, although it was discussed a little bit in the discussion. For example, does it block recruitment of eIFs or host IRES transacting proteins?
* Viral RNA biologists or mRNA translation experts would be interested in the work.
* My expertise is viral RNA biologist.

Referee #3:

This manuscript by Sadic et al., entitled "Antiviral DExD/H-box helicase 60 selectively inhibits translation from type II internal 1 ribosome entry sites" sets out to understand the antiviral activity of DDX60 and hopefully clarify previous studies with conflicting roles for DDX60 as an antiviral host factor. First, they show with real-time PCR and westerns that IFN-beta upregulates both protein and mRNA levels of DDX60 consistent with it being an ISG. Next, they determined the effect of deletions or single point mutations in DDX60 on the replication of a bicistronic or monocistronic replicon. This analysis showed that the decrease in replication of the replicon due to DDX60 expression was likely due to the EMCV IRES and not the HCV IRES or HCV replicon replication. They confirmed this by performing translational reporter assays using Type I-III IRES bicistronic DNA or monocistronic RNA reporters. This all agrees well with their data showing overexpression of DDX60 decreased viral titers for viruses with type II IRESs but not those with type I IRESs. These were very well executed and convincing experiments. The second half of the manuscript describes a series of experiments to get at how DDX60 reduces translation of type II IRESs. They rule out effects on RNA stability (real-time RT-PCR), RIG-I (transfections of RIG-I and DDX60 WT or mutant at increasing concentrations), specific binding of DDX60 to type II IRESs (using RNA and DDX60 in vitro pull-down assays). They do find that DDX60 overexpression leads to reduced polysome association for type II IRESs, but not for a cap-dependent transcript providing an explanation for the decrease in type II IRES directed translation by DDX60. Overall this is a very interesting and well-executed set of experiments that provide a compelling role for DDX60 in inhibiting translation of type II IRESs. While there are some minor weaknesses they are minor and additional experiments may not change the final conclusions.

Major comments:
* In figure S2A, the authors claim "The findings from this analysis of mutants correlate with the EMCV IRES-driven RFP findings and bicistronic reporter HCV findings demonstrated in figures 1B and 1C. Future biochemical and structural studies comparing these mutants with wild-type DDX60 may elucidate the enzymatic activities responsible for the observed effects." First, the

EMCV bar graph is on a scale that makes it hard to compare the different mutants. Second, the EMCV luciferase units are surprisingly low as this IRES is one of the most active IRESs second only to perhaps EV71. Thirdly, there were no statistics applied to the graph. This reviewer would agree that S2B is consistent with DDX60 decreasing Type II IRESs, but from the data presented in S2A, it is hard to make any conclusion. Also, no stats were applied to S2B, either.
* It is not surprising that the DDX60 pull-downs are non-specific as this has been a problem plaguing the RNA protein binding field that has been greatly overcome by using zero distance cross-linking of proteins to RNA binding proteins prior to cell lysis. These types of in vitro binding experiments led to the misconception that RNA binding proteins bind to RNA non-specifically, when in fact there is specificity in binding targets before cellular compartmentalization is disrupted. It is possible that if an in vivo crosslinking approach was used there would be specificity in DDX60 association with the type II IRESs. At a minimum, it is worth considering this alternative explanation in the discussion.
* Typically, RNA turnover rates are not measured by comparing steady-state levels of an RNA by real-time PCR as differences in half-life may not be discernible. Also, there is some concern that these assays were carried out too long after transfection, see comments below.
* The methods describe the generation of HCV stocks, which wasn't presented in Figure 3. Data showing HCV titers are not affected by DDX60 would be nice data to add and would support the major conclusion.
* Assays following RNA transfections were carried out 24 hours post-transfection, which is fairly late and most of the protein signal from the transcription or RNA levels have diminished. A time-course following RNA transfection shows that protein expression from a transfected RNA is maximal between 2-6 hours post-transfection and is greatly diminished by 24 hours as one might expect given the half-life of mRNAs and the fact that in contrast to DNA transfections they do not have to be transcribed and exported to the cytoplasm but can be immediately translated. This also depends on the half-life of the protein. It is noted that the majority of the RNA transfection kits recommend assaying for protein expression at 24 hours post-transfection, which is the same time point recommended for DNA transfections. Thinking about how this might affect the interpretation of the results, it is possible that differences in effects on RNA turnover may be diminished by 24 hours compared to 4 hours post-transfection if the majority of the RNA is turned over in the first 2-8 hours post-transfection.

Minor comments:
* Line 58: "IRESs assemble the translation initiation apparatus by interacting with a defined set of eIFs and host IRES transacting factor proteins (ITAFs) that assist in the recruitment of the 40S ribosomal subunit." There is no reference for this statement. Has there been any ITAFs that have been shown to be involved in 40S recruitment or do they mainly affect RNA structure or accessibility?
* Statement starting on line 92 needs to be referenced.
* Line 132: "and then decreasing back to baseline levels after 12 hours". This is confusing because the 0 hour appears much lower than the levels at 24 and 48 hours, but importantly most mRNA levels are peaking around 12 hours not returning to baseline. What is baseline, 0 hours? This needs to be clarified.
* There are multiple sentences that could have different interpretations, the authors should address these ambiguities: Lines 138, 246.
* Line 163. "Previous studies showed that DDX60 inhibits replication of a bicistronic reporter HCV". Are the authors referring to a replicon or a translational reporter? A diagram of precisely which reporter they are using would be helpful. This reviewer is assuming it is the one on the left in figure 1D.
* Line 173. "...yielding approximately 50% infected (Ypet+) cells in our negative control". What is the negative control? This needs to be clarified. Also, Line 176 "Firefly luciferase (Fluc) served as a negative control and normalization factor". Is this the same negative control? Was Fluc put on the ISG plasmid in place of the ISG? This is not clear at all and needs to be clarified how the experiment was performed. What is meant by the normalization factor?
* Line 177. When the authors say "virus inhibition" do they mean replicon inhibition? These are not the same and should be clarified.
* Figure legend S2: just state directly whether the reporter is the monocistronic RNA or the dicistronic DNA reporter.
* Figure S4A uses Mock to refer to a no DDX60 transfection control, this can be misinterpreted. The results discuss the control as an untransfected PBS treated control, which appears to be more appropriate. Also, the figure legend is insufficient. What are the amounts of RIG-I and DDX60 plasmids transfected? How much poly(I:C) and IFN? Also, data looks to be fold changes not RLUs. What are the white bars versus the gray bars? Also, the results state the differences are not significant, but there is no significance test mentioned in the legend or otherwise.
* The statement about how RNAs are trapped in endosomes... isn't this limited to naked RNAs? Transfected RNAs would more or less resemble lipid nanoparticles, which can pass through the membrane with high efficiency.
* The MIQE (minimum information for publication of quantitative real-time PCR experiments; PMID: 19246619) was not provided in the results. This is particularly, worrisome given that the RNA stability data relies entirely on real-time PCR analysis of transfected RNA. While this technique is really powerful for detecting very low levels of RNA or for determining differences in RNA levels over many logs, it is less accurate for determining differences in RNA levels in the range of 2-fold or less. The accuracy of this would greatly depend on the efficiency of the reactions. In particular, the essential information indicated in the above publication such as linear dynamic range, PCR efficiency, technical replicates, and the reaction conditions should be included in the methods.

Significance

Characterization of a specific RNA binding protein that is involved in the translation of specific IRESs is very timely and exciting

and will be of interest to virologists, RNA biologists, and the translational control field. As an expert in alternative mechanisms of translation initiation, RNA binding proteins, and RNA viruses this reviewer has significant enthusiasm for this study given their findings and experimental approaches.

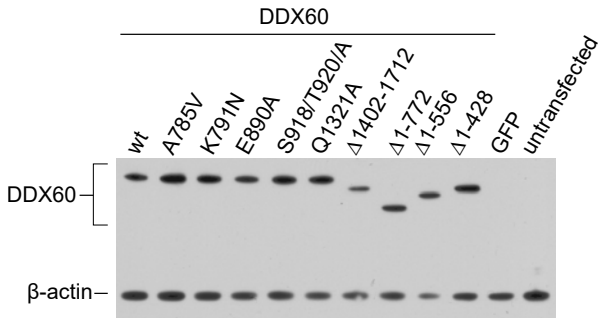
Point-by-point description of the revisions

Reviewer #1:

Major comments:

1. Using tools and literature search, the authors identified four potential functional domains and motifs in DDX60 sequence based on which they generated a series of deletion and single point mutants. The antiviral activity of the mutants was on assessed HCV replication by ectopic expression in Huh7 cells, followed by flow cytometry analysis. How were the expression levels in Huh7 cells? Fig. 1B only show the expression in 293T cells.

We thank the reviewer for bringing up this point. We chose to show expression of DDX60 in HEK293T cells as these are the cells used for the reporter assays and polysome profiling to establish the anti-type II IRES phenotype. In the absence of a functioning DDX60 antibody, equal transfection in Huh7 cells was previously verified by flow cytometry detecting RFP. Studies on Huh7 cells were performed spanning from 2011-2015 in the corresponding author's postdoctoral laboratory. Once a specific DDX60 antibody became available in 2015, a western blot was run using transfected Huh7 cells to verify equal DDX60 wild type and mutant construct expression. This western blot from 2015, shown below, is not publication quality, and we currently do not have ready access to Huh-7 cells.



We are happy to include this blot to the revised manuscript, should the reviewer deem this necessary.

2. In Fig. 1B, some of the constructs, e.g. S918T and Q1321A show no RFP or reduced RFP signal. The authors comment on this point but still use RFP for FACS analysis. How were cells gated in this case?

We apologize that this point was unclear. The reduction in RFP observed by western blot for some DDX60 constructs (e.g. S918/T920/A and Q1321A) reflects a reduction in mean fluorescence intensity of the RFP signal and not a reduction in % RFP-positive cells. Thus, our

gating strategy on RFP-positive cells works equally for all constructs. We clarified this point in the text (lines 200-203).

3. The authors generally conclude that each of these predicted domains is important for the antiviral activity of DDX60. However, results are contrasted. Only some residues in the selected domains, e.g. domains I and IV have an effect. Since the involvement of these domains in ATP binding, hydrolysis or RNA unwinding could not be validated in in vitro assays, the conclusion of this analysis should be tuned downwards or highlight in the main text that this is speculative. In addition, helicase mutant E890A is used from this figure on in all other assays as a control, it should be at least described in the result part of Fig. 1C.

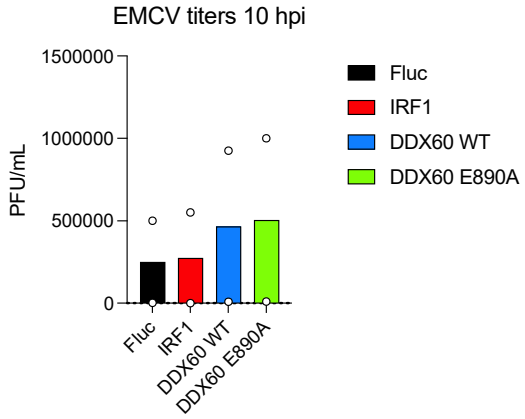
We agree and have revised this paragraph accordingly (lines 178-186).

4. The specific impact of DDX60 on type II IRES is confirmed by infection assays, using an elegant chimeric poliovirus (Fig. 3). Does infection with these viruses induce endogenous expression of DDX60 over time in the selected target cell lines? Picornaviruses usually induce a high IFN-β or IFN-λ release and this might influence the results. HeLa were shown in Fig. S1 to be particularly responsive to IFN. In this case, how would silencing of DDX60 influence EMCV replication in parental cells?

We thank the reviewer for raising these interesting questions. To answer if infection with EMCV, poliovirus, and chimeric poliovirus induce endogenous expression of DDX60 over time, we posit that this may be true as these viruses all induce IFN-ß and IFN-λ as stated by this reviewer. We envision that performing an infection time series can address whether there are differences in DDX60 expression dynamics with infection of a type II IRES virus compared to a type I IRES virus, *and* possibly start to address whether these viruses differentially antagonize DDX60 RNA or protein expression. We believe this would be an interesting avenue to explore in a future study. The current scope of our manuscript is to establish DDX60's role as an antiviral factor that specifically decreases type II IRES translation.
To test how silencing DDX60 would influence EMCV replication in parental cells is experimentally challenging. DDX60 expression at both the RNA and protein levels are low without interferon treatment. Thus, one would first need to increase DDX60 expression with either IFN treatment or virus infection (and thus indirect interferon activation) and then simultaneously either silence DDX60 or not silence it. The second route is likely the most feasible experimentally as pre-treatment with IFN before virus infection may significantly limit initial infection. However, we also note that upon infection or interferon treatment, DDX60 would be among hundreds of other ISGs activated. The likelihood of DDX60 having a large impact on virus infection on its own is low given its already modest reduction of virus infection even with several fold higher DDX60 expression than IFN treatment. We attribute this to the fact that DDX60's mechanism specifically antagonizes a few viruses containing a type II IRES. Unlike DDX60, broad transcriptional regulators of antiviral genes like IRF1 (used as a positive control for many of the assays in this study) can activate its own set of antiviral genes that can inhibit a diverse group of viruses. Silencing broad regulators of antiviral genes or genes with more diverse mechanisms of virus inhibition will result in a more profound phenotype than silencing ISGs with a more specific mechanism of action.
We addressed questions related to virus-induced DDX60 expression and silencing experiments in the revised discussion, lines 502-519.

5. Cells stably expressing DDX60 were used for the infection assays. Viral genome translation is the first step after entry for positive single-stranded RNA viruses, so, it is surprising that effects become only noticeable after 24 hours. For example, for EMCV produces already 105 PFU/ml at 8 hours post infection and we would expect stronger effects in the first round of infection (Fig. S3D). In these assay, DDX60 expression levels are not shown. Would the effect be stronger at higher DDX60 levels? Is DDX60 degraded upon infection?

Given that inhibition of virus translation by the translational inhibitor PKR occurs within or shortly after the first cycle of virus replication[1,2], one may generalize to say that inhibitors of virus translation should demonstrate an early phenotype. However, we note that another well-known inhibitor of HCV translation, IFIT1 showed an inhibitory phenotype 96 hours post infection[3], even while active HCV replication can be observed as early as 48 hours post infection[4]. This example suggests that one cannot directly generalize to say that inhibitors of virus translation should always demonstrate an early phenotype. We attribute the observation that effects of DDX60 become noticeable only after 24 hours to DDX60's overall modest inhibition of virus translation that accumulates over time after multiple rounds of virus replication We performed assays looking at whether DDX60 inhibits EMCV infection early, after 8 – 10 hours post infection, but did not observe a significant effect (see below).



We therefore addressed this point in the revised discussion (lines 520-528).

While achieving higher DDX60 expression levels could result in a phenotype, we attempted producing stably expressing DDX60 cell lines in both HeLa and HFF1 and could achieve expression levels only as high as that shown in **Figure EV3A** in HeLa cells. The bottleneck is due to decreased lentivirus packaging because of the large size of DDX60 cDNA. We do not know if DDX60 is degraded upon infection, but this is an interesting point that we now added to the discussion in lines 617-625.

References cited in response to Reviewer #1 point #5

1. Meurs, E.F., et al., *Constitutive expression of human double-stranded RNA-activated p68 kinase in murine cells mediates phosphorylation of eukaryotic initiation factor 2 and partial resistance to encephalomyocarditis virus growth.* Journal of Virology, 1992. **66**(10): p. 5805-5814.
2. Lee, S.B., R. Bablanian, and M. Esteban, *Regulated expression of the interferon-induced protein kinase p68 (PKR) by vaccinia virus recombinants inhibits the replication of vesicular stomatitis virus but not that of poliovirus.* J Interferon Cytokine Res, 1996. **16**(12): p. 1073-8.
3. Raychoudhuri, A., et al., *ISG56 and IFITM1 Proteins Inhibit Hepatitis C Virus Replication.* Journal of Virology, 2011. **85**(24): p. 12881-12889.
4. Schoggins, J.W., et al., *A diverse range of gene products are effectors of the type I interferon antiviral response.* Nature, 2011. **472**(7344): p. 481-485.

6. Figure S4 and its description focusing on the potential RIG-I-DDX60 crosstalk are unclear. The panel A is hardly readable. Western blot quantifications are missing for the biological replicates. At a glance, there is no real difference in the expression of DDX60. Also, effects might be better visible in an immune-competent cell type such as the A549 cells used in Fig. S1. Also effects might be better visible in an immune-competent cell type such as the A549 cells used in Fig. S1. This would not allow titrating DDX60 helicase mutant but would at least clarify effects of the wild type protein.

Thank you for suggesting this. We increased the size of **Appendix Figure S1** and added quantifications to western blots for this assay. While concerted effects of RIG-I and DDX60 may be better visible in an immune-competent cells such as A549, previous studies by Miyashita et al., 2011 and Oshiumi et al., 2015 use HEK293 transfected cells to establish concerted RIG-I and DDX60 effects and a subsequent study by Goubau et al., 2015 used similar HEK293 transfected cells to refute such findings. We chose to employ a similar system as past studies to both determine whether DDX60 and RIG-I-mediated ISRE induction plays a role in type II IRES inhibition and to test whether we observe a concerted activation of downstream interferon signaling due to DDX60 and RIG-I expression in general. Our findings are consistent with Goubau et al., 2015. Additionally we are unable to use A549 cells for this particular assay as they do not tolerate transfection with plasmids to express DDX60 and RIG-I.

7. RNA-immunoprecipitation using biotinylated reporter RNAs is an elegant assay to show direct protein-RNA interactions. The results in Figure S5B are very promising, but raise a technical question. It is important to spike the samples with an unrelated RNA to normalize for loss during RNA extraction. This influences the amount of RNA detected by qRT-PCR. Were the samples analyzed in this manner? Furthermore, the data shown is representative of only 2 biological replicates. Such assays are not trivial and have high variability. Experiments should be performed at least 3 times and the variation should be shown to reinforce this observation.

We thank the reviewer for acknowledging the promising nature of the data presented and would be happy to address any concerns about the methodology. We did not spike the samples with an unrelated RNA to normalize for loss during RNA extraction; we assumed that loss during RNA extraction would be uniform for both the capped and EMCV IRES driven mRNA as only the presence of the IRES sequence differentiates the two mRNAs. While we agree that additional repetitions of the same experiment may aid the interpretation of highly variable data, such as that of revised **Figure EV4B**, we argue that an alternative complementary experiment, as provided by revised **Figure EV4A**, achieves the same purpose. We thus do not believe adding an additional replicate for either immunoprecipitation experiments would change the overall conclusions drawn from the figure.
We would certainly like to perform future RNA binding assays using the suggested methods involving in vivo crosslinking followed by RNA immunoprecipitation (as suggested by reviewer #3). However, these are not trivial assays and require extensive optimization that were not possible within the time scope of this revision. We now discuss these methods including their respective limitations in the revised discussion, lines 554-590.

8. Because of DDX60 ability to bind to the 5' cap reporter RNA in the pulldown assay, the authors propose that DDX60 is an RNA non-specific binder. Does DDX60 bind to

endogenous GAPDH mRNA as well? The observed binding may be the result of transfection artifacts.

We thank the reviewer for raising this point. We had previously performed qPCR to detect if *GAPDH* mRNA is also pulled down with DDX60 immunoprecipitation and we observed that indeed it is. We now discuss this in lines 554-590 and explain that due to cell compartment mixing during cell lysis, and higher DDX60 expression in transfected cells, we may be observing DDX60 binding to RNA that it may not physiologically bind. We also discuss the necessity for using in vivo crosslinking before cell lysis to mitigate these possible artifacts as suggested by reviewer #3.

9. The polysome profile analysis is an important assay when addressing translation efficiency. This part must be further substantiated. First, results in Fig. 5B represent the average of 2 biological replicates. Again, variability between technical replicates alone is often important. This analysis must be repeated at least three times and the significance of the results must be demonstrated, including control cells (expressing Fluc or GFP) that are missing from the current analysis. More generally, it is unclear which fraction belongs to the monosomal, light polysomal and heavy polysomal RNAs. Some mRNAs change distribution within the polysomal fractions (from heavy to light fractions) but their overall translation is not affected. Please provide a gel showing total RNA (18S and 28S) to help the reader. What is the percentage of reporter mRNA associated with polysomal fractions in the different conditions?

We thank the reviewer for suggesting this additional experiment – we have performed an additional replicate of polysome profiling with reporter mRNAs (**revised Figure 5**). The third replicate substantiated our previous findings. In all our analyses, we compare wild type DDX60 to a DDX60 E890A mutant, which differs from wild type by only one amino acid. We believe this mutant control is superior to using GFP or Fluc; we show in revised **Figure 2B** and **Figure 3** that this mutant behaves like GFP or Fluc expressing cells in terms of relative luciferase activity from our mRNA reporters and viral titers from our virus infections. To help the reader, and as suggested, we now provide an RNA agarose gel to show the distribution of 18S and 28S RNA among the polysome fractions (**revised EV Figure 5**). We are unsure about what the reviewer means by the question "What is the percentage of reporter mRNA associated with polysomal fractions in the different conditions?" as the graph shown in **Figure 5B** reports percentage of Fluc reporter mRNA distributed amongst the different polysome fractions in the different conditions. We are happy to revise the manuscript accordingly in case this point requires further clarification.

10. To strongly support the claims on the role of DDX60 on type II IRES-driven translation, the authors should perform polysome profile analyses in DDX60-expressing cells infected with poliovirus and chimeric poliovirus and measure changes in the association of the poliovirus genome with the polysomes. This would significantly strengthen the findings of the study.

We absolutely agree with the reviewer that showing the DDX60 phenotype during a viral infection strengthens our study. We have now successfully performed this experiment (**new Fig. 6**), and show that DDX60 indeed reduces type II IRES-driven translation during viral infections.

11. Generally, the text would benefit from being shortened. One example is the DDX60 purification attempt described by more than 10 lines of text that could be shortened to one sentence.

We agree with this reviewer and have shortened this part of the manuscript and other parts of the text.

12. Calling out figure panels in their in numerical order would help the reader (e.g., S3A is cited after S3D). This occurs for several panels.

We thank the reviewer for this comment – this issue has now been fixed.

13. Labelling the different constructs in panel Fig. 1A would help the reader. In the main text these are referred to as N and C-terminal mutants however, two N-terminal mutants were generated (Δ1-556 or Δ1-428).

Thank you for this suggestion - this has been addressed in **Figure 1A**, its corresponding legend (lines 1595-1613), and in the corresponding results section (lines 156-205).

14. Fig. 1B shows the Western blot analysis of DDX60 mutant expression levels. Relative intensity levels are quantified and shown for DDX60 and RFP. However, it is not clear which of the loading controls (β-actin, or GAPDH) was used as a reference and how or to which values this was normalized.

Thank you for catching this. We clarify in the methods section (lines 808-818) how western blots were quantified and clarify in the figure legend for **Figure 1B** (lines 1595-1613) that GAPDH was used as the loading control during quantification.

15. The legend of the graphs shown in Fig. 4B is missing (blue vs green).

Thank you - this has been addressed in **Figure 4B**.

16. Polysome occupancy is a misnomer. Polysomes are RNAs loaded with ribosomes, so we can speak of ribosome occupancy. It should be noted, however, that polysome profiles measure the association of a given mRNA with translating ribosomes, but do not provide information on the number of ribosomes loaded on the mRNA.

Thank you so much for pointing this out. This has been addressed throughout the manuscript.

Significance:

The existence of an interferon-induced effector that regulates genome translation in certain classes of viruses is a --- very exciting finding. Furthermore, the involvement of DDX60 in the regulation of viral translation is novel and opens several avenues of research.

- Published knowledge: none about this topic
- Audience: This work will be of great interest to both the virology and IRES/translation communities.
- Expertise: virology, translation

We thank the reviewer for this enthusiastic assessment and for the provided constructive criticism. We hope that the points raised above were addressed to the reviewer's satisfaction.

1. The key conclusions are convincing and the experiments were very well-controlled.

We thank the reviewer for this positive and only major comment.

Minor comments:

2. The results section in parts was challenging to read as so many caveats and limitations were presented, that it was hard to determine what parts of focus on. The authors might want to consider removing some of the experiments that have many caveats, such as Figure 2A (see below).

We thank the reviewer for this input. This has been addressed in the revised results section, lines 209-261. More specifically, we now discuss the part relevant to **Figure 2A**, and then give the limitation as a rationale for experiments in **Figure 2B**.

3. Figure 1A shows a schematic of DDX60, with key domains indicated. It would be helpful if the amino acids that were mutated in Fig. 1C were numbered (in addition to their highlighting with the bold font), to make it easier to refer back and forth between the two figures.

Thank you - this has been addressed in **Figure 1A**.

4. In Figure 1, amino acid substitutions were made in DDX60 that were designed to inactivate aspects of its function (ATP binding, helicase, ATP hydrolysis and unwinding) to determine if these suggested functions of DDX60 were important for its antiviral activity. The authors were not able to purify DDX60 to test these functions; however this was not described until much later in the manuscript. It would be helpful for the reader to put this section describing the purification issues in the text describing Figure 1, rather than on page 14, where it seems out of place.

Thank you for this suggestion, we now moved this information up to lines 178-186.

5. The first paragraph of the section at the end of page 7 and beginning of page 8, related to Figure 2, has a lot of information and is quite dense. It was also confusing because it mentioned wanting to use in vitro transcribed RNA, but then this wasn't actually done in Figure 2A, which comes after this long paragraph. It could be helpful to break this up into the relevant sections when describing the figure panels? For example, talk about the part that's relevant to Figure 2A when describing that section, and then give the limitations for why you next wanted to do the experiments in Figure 2B. Alternatively, since the experiments shown in Figure 2A seem to have so many caveats that limit their interpretation, the authors may want to consider only showing the data in Figure 2B.

We thank the reviewer for these suggestions. This has been addressed in lines 209-261. More specifically, we now discuss the part relevant to **Figure 2A**, and then give the limitation as a rationale for experiments in **Figure 2B**.

6. In Figure S2A, it is difficult to see that that the WT inhibits Type II IRES and the mutants do not as the scales go far beyond the data displayed. I would suggest making the Y-axis scale more limited to better see the data, and this comment stands for every figure.

Thank you. The Y-scales have been changed for **Figure EV2** and statistical tests were added as further suggested by reviewer #3.

7. Related to Figure S4, the text states that cell were treated with PBS or poly(I:C), however the graph in Figure S4A only shows data as "Fold Change over Mock". As the text describes what RIG-I and DDX60 do in the absence of poly (I:C), I would suggest either changing the graph to show mock and poly (I:C) or relabeling the graph or rewriting the text to clarify what is being shown and the important points.

Thank you for this suggestion. **Figure EV4** has been modified to show untransfected (mock) and poly(I:C) only transfected controls and raw luciferase units rather than relative fold changes are plotted. Legends in the graph were included to clarify the difference between grey, white, and blue bars. The figure legend now also clarifies the different conditions. Additionally statistical tests have been included to show that the results are not significant.

8. For the polysome experiments in Figure 5, the fractions should be labeled to indicate the 40S, 60S, and 80S subunit peaks and the polysome peaks. Ideally, the ribosomal RNA bands from one typical run would be shown in the supplemental.

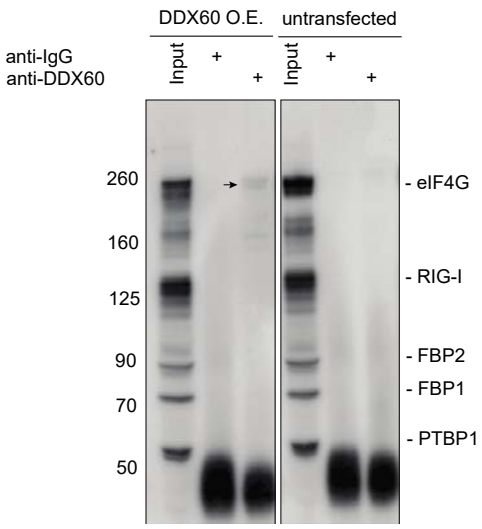
We thank the reviewer for bringing up this point, which was also suggested by Reviewer #1. We now provide an RNA agarose gel to show the distribution of 18S and 28S RNA among the polysome fractions (**Figure EV5**).

Significance:

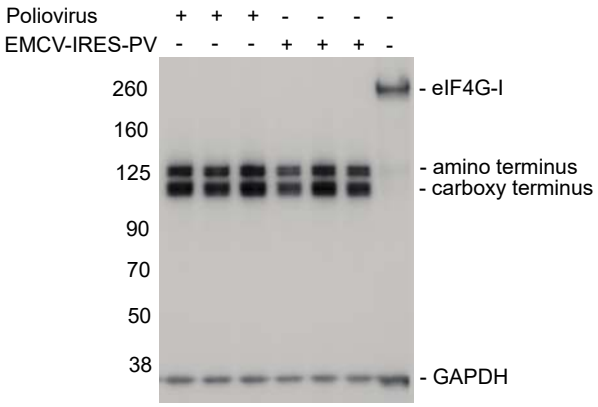
The conceptual advance of this study is showing that an antiviral factor (DDX60) acts only on specific IRES elements to limit translation from viruses that contain these IRES elements. Overall, the experiments were well-controlled, and I think that that there is novelty in showing that an antiviral factor has IRES-level antiviral specificity. However, as it stands, the overall conclusions are limited, and a major open question is how is DDX60 specific to the type II IRES, which was not addressed experimentally, although it was discussed a little bit in the discussion. For example, does it block recruitment of eIFs or host IRES transacting proteins?

- Viral RNA biologists or mRNA translation experts would be interested in the work.
- My expertise is viral RNA biologist.

We thank the reviewer for this assessment of significance and agree that this is an open question. We had previously performed experiments on this point. We performed pulldown experiments with anti-DDX60 antibody and analyzed protein-protein interactions between DDX60 and eIF4G, FBP1 + FBP2, and PTBP1. We did indeed observe an interaction between DDX60 and eIF4G as shown below:



At the same time, we were unable to detect DDX60-mediated protection from eIF4G cleavage in experiments using parental poliovirus or the chimeric EMCV-IRES-PV poliovirus (shown below are samples from n=3 independent experiments on a western blot probed for eIF4G and GAPDH):



Thus, we felt it was difficult to interpret the significance of the observed interaction during exogenous DDX60 expression, as we cannot state that DDX60 blocks eIF4G recruitment to the type II IRES during a viral infection. We did not read this reviewer's significance statement as a request for new experiments, and thus, in the current manuscript, addressed this point by expanding on the discussion (lines 574-590). However, we are more than happy to include the above data in the manuscript, should the reviewer feel it raises the significance of our study.

Reviewer #3:

Major comments:

1. In figure S2A, the authors claim "The findings from this analysis of mutants correlate with the EMCV IRES-driven RFP findings and bicistronic reporter HCV findings demonstrated in figures 1B and 1C. Future biochemical and structural studies comparing these mutants with wild-type DDX60 may elucidate the enzymatic activities responsible for the observed effects." First, the EMCV bar graph is on a scale that makes it hard to compare the different mutants. Second, the EMCV luciferase units are surprisingly low as this IRES is one of the most active IRESs second only to perhaps EV71. Thirdly, there were no statistics applied to the graph. This reviewer would agree that S2B is consistent with DDX60 decreasing Type II IRESs, but from the data presented in S2A, it is hard to make any conclusion. Also, no stats were applied to S2B, either.

We thank the reviewer for this comment. The Y-scales have been changed for **Figure EV2** and statistical tests were added.

2. It is not surprising that the DDX60 pull-downs are non-specific as this has been a problem plaguing the RNA protein binding field that has been greatly overcome by using zero distance cross-linking of proteins to RNA binding proteins prior to cell lysis. These types of in vitro binding experiments led to the misconception that RNA binding proteins bind to RNA non-specifically, when in fact there is specificity in binding targets before cellular compartmentalization is disrupted. It is possible that if an in vivo crosslinking approach was used there would be specificity in DDX60 association with the type II IRESs. At a minimum, it is worth considering this alternative explanation in the discussion.

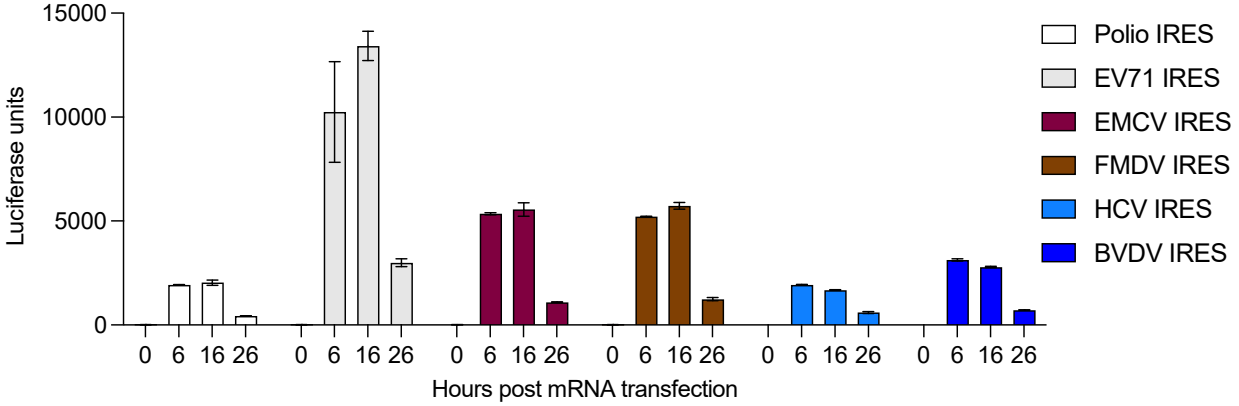
We are grateful for this suggestion. We have included this alternative explanation in the revised discussion, in lines 567-569.

3. Typically, RNA turnover rates are not measured by comparing steady-state levels of an RNA by real-time PCR as differences in half-life may not be discernible. Also, there is some concern that these assays were carried out too long after transfection, see comments below.

   Assays following RNA transfections were carried out 24 hours post-transfection, which is fairly late and most of the protein signal from the transcription or RNA levels have diminished. A time-course following RNA transfection shows that protein expression from a transfected RNA is maximal between 2-6 hours post-transfection and is greatly diminished by 24 hours as one might expect given the half-life of mRNAs and the fact that in contrast to DNA transfections they do not have to be transcribed and exported to the cytoplasm but can be immediately translated. This also depends on the half-life of the protein. It is noted that most of the RNA transfection kits recommend assaying for protein expression at 24 hours post-transfection, which is the same time point recommended for DNA transfections. Thinking about how this might affect the interpretation of the results, it is possible that differences in effects on RNA turnover may be diminished by 24 hours compared to 4 hours post-transfection if most of the RNA is turned over in the first 2-8 hours post-transfection.

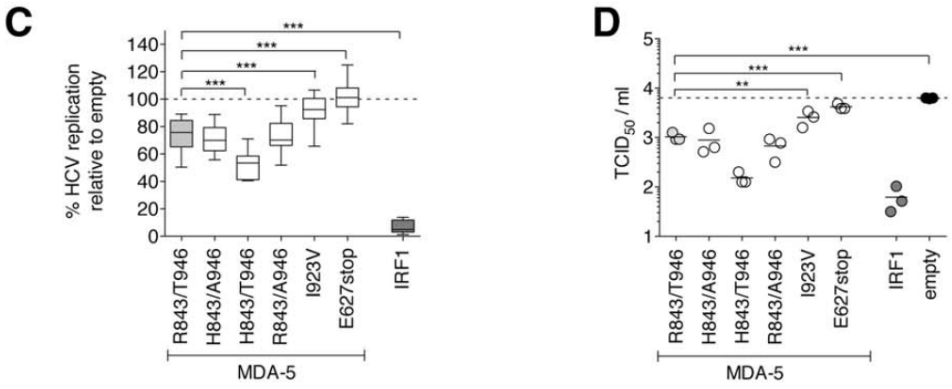
We thank the reviewer and understand their concerns about real-time PCR not being able to discern differences in half-life of the RNA if effects on RNA abundance are modest. When we

tested our PCR primers for PCR efficiency, we were able to detect five-fold differences at Ct values as high as 18 and as low as 6, and ten-fold differences at Ct values as high as 29 and as low as 13. The Ct values for our RNA reporters ranged from 14-16 in our RNA abundance assay in figure 4A. Our PCR efficiency value was calculated to be 5.46 – 5.53, which is greater than the maximum value of 1 using the formula Efficiency=10^(-1/slope of Ct vs. Log2 cDNA copies graph). Functionally, this tells us that our generated primers are extremely efficient at amplifying copies of Fluc cDNA. We have included the information about PCR efficiency and linear range of detection in the methods section (lines 989 - 996). Given that we did not test for being able to detect RNA abundance differences for changes less than five-fold, we included this in our revised results (lines 324-329) and discussion (line 532).
To address the second part, we performed a time series of luciferase activity following mRNA transfection alone and found that there is no significant decrease in luciferase activity between 6 and 16 hours post RNA transfection, but that there was a decrease in activity as late as 26 hours post RNA transfection (see below). We must clarify and correct that all our assays using mRNA reporters were stopped 16, not 24 hours post transfection. Any mistakes in the manuscript made referring to a 24-hour time point post RNA transfection has been corrected.



4. The methods describe the generation of HCV stocks, which wasn't presented in Figure 3. Data showing HCV titers are not affected by DDX60 would be nice data to add and would support the major conclusion.

We agree with the reviewer that this would be nice data to add. Unfortunately, we do not currently have HCV infections established in the corresponding author's laboratory. However, our previous work demonstrated that findings from the HCV reporter assay tightly correlate with HCV infectious titers (below from Hoffmann et al., Hepatology, 2014, Figure 3):



In the above example from Hoffmann et al., a 50% reduction of replication in the reporter assay equates to a 1 log reduction in infectious titers. We therefore would expect infectious titers for the bicistronic reporter HCV to be reduced by 5-10-fold $TCID_{50}$ upon DDX60 expression, but would expect the monocistronic reporter HCV titers to remain unchanged.

Of note, previous work showing DDX60-mediated inhibition of HCV did not determine infectious titers either, but used the flow-cytometry with a bicistronic HCV reporter (Schoggins et al., Nature, 2011), HCV RNA transfection and RT-qPCR or HCV bicistronic replicon systems and luciferase readout (both approaches in Oshiumi et al., Cell Reports, 2015). We feel confident that our data support our major conclusion that DDX60 does not inhibit HCV through inhibiting the HCV IRES, as we use a complementary reporter virus solely relying on the endogenous HCV IRES, which is resistant to DDX60's antiviral activity.

Minor comments:

5. Line 58: "IRESs assemble the translation initiation apparatus by interacting with a defined set of eIFs and host IRES transacting factor proteins (ITAFs) that assist in the recruitment of the 40S ribosomal subunit." There is no reference for this statement. Has there been any ITAFs that have been shown to be involved in 40S recruitment or do they mainly affect RNA structure or accessibility?

We thank the reviewer for raising this point. ITAFs have been shown to affect RNA structure and accessibility as this reviewer mentions, but some are required for translation of particular IRESs (e.g. PCBP2 for type I IRESs). This has been clarified and a citation added (line 57-58).

6. Statement starting on line 92 needs to be referenced.

Thank you for pointing this out - we now referenced "The interferon-induced protein with tetratricopeptide repeats (IFIT) family members and interferon-induced transmembrane protein (IFITM) family members bind specific eIFs to restrict global protein synthesis or recognize structures absent in viral 5' caps such as 2'O-methylation (Diamond & Farzan, 2013; Schoggins, 2019)" in line 101.

7. Line 132: "and then decreasing back to baseline levels after 12 hours". This is confusing because the 0 hour appears much lower than the levels at 24 and 48 hours, but importantly most mRNA levels are peaking around 12 hours not returning to baseline. What is baseline, 0 hours? This needs to be clarified.

We apologize for causing confusion. The definition of "baseline" has been clarified in line 142.

8. There are multiple sentences that could have different interpretations, the authors should address these ambiguities: Lines 138, 246

Thank you for pointing out these ambiguities. The statement starting in line 138 ("Unlike primary HFF1, HEK293T, A549…") has been removed as it is not necessary and not the focus of the study. In referring to line 246, we assume that the reviewer is referring to the statement "We chose to perform the reporter assays in HEK293T cells due to their low DDX60 expression in the absence of type I IFN stimulation (**Figure EV1A, E**) and ease of transfectability due to the absence of the DNA sensing pathway protein, STING (Sun et al., Science, 2013). This allowed us to simulate the effects of DDX60 upregulation in the absence of endogenous DDX60…" We removed the statement attributing ease of HEK293T transfectability to loss of STING.

9. Line 163. "Previous studies showed that DDX60 inhibits replication of a bicistronic reporter HCV". Are the authors referring to a replicon or a translational reporter? A diagram of

precisely which reporter they are using would be helpful. This reviewer is assuming it is the one on the left in figure 1D.

Thank you for raising this question and apologies for not being clear on this front. The reporter is a live virus with a fluorescent protein inserted in the virus genome as depicted in **Figure 1D**. The technology is referred to as "virus inhibition assay" in 163 for clarification. Additionally, a schematic of a virus has been added to the workflow depicted in **Figure 1C** to emphasize that the reporter is a live virus, and Figure legend updated (lines 1596-1613).

10. Line 173. "...yielding approximately 50% infected (Ypet+) cells in our negative control". What is the negative control? This needs to be clarified. Also, Line 176 "Firefly luciferase (Fluc) served as a negative control and normalization factor". Is this the same negative control? Was Fluc put on the ISG plasmid in place of the ISG? This is not clear at all and needs to be clarified how the experiment was performed. What is meant by the normalization factor?

Thank you for raising this question. We clarified that the negative control referred to in the sentence cited is Fluc (lines 165-166) and that this plasmid is the ISG plasmid backbone with the ISG replaced with Fluc (methods section, line 718). We use Fluc to relatively compare all our experimental conditions to each other. We clarify this in the legend of figure 1 (lines 1595-1613).

11. Line 177. When the authors say "virus inhibition" do they mean replicon inhibition? These are not the same and should be clarified.

Thank you for asking this - Figure 1 uses a live virus with a fluorescent reporter added into the virus genome. This has been clarified as stated above in point #9.

12. Figure legend S2: just state directly whether the reporter is the monocistronic RNA or the dicistronic DNA reporter.

All the reporters in this figure are monocistronic RNA reporters.This has now been clarified in heading of Figure EV2 (line 1631).

13. Figure S4A uses Mock to refer to a no DDX60 transfection control, this can be misinterpreted. The results discuss the control as an untransfected PBS treated control, which appears to be more appropriate. Also, the figure legend is insufficient. What are the amounts of RIG-I and DDX60 plasmids transfected? How much poly(I:C) and IFN? Also, data looks to be fold changes not RLUs. What are the white bars versus the gray bars? Also, the results state the differences are not significant, but there is no significance test mentioned in the legend or otherwise.

Thank you for pointing this out – this was a concern by reviewer #2 as well. **Figure EV4** has been modified to show untransfected (previously "mock") and poly(I:C) only transfected controls and raw luciferase units rather than relative fold changes are plotted. Legends in the graph were included to clarify the difference between grey, white, and blue bars. The EV4 figure legend (lines 1681-1702) now also clarifies the different conditions. Additionally statistical tests have been included to show that the results are not significant. Information about amounts of RIG-I, DDX60, poly(I:C) transfection, and IFN treatment are included in the methods section in lines 998-1023.

14. The statement about how RNAs are trapped in endosomes... isn't this limited to naked RNAs? Transfected RNAs would more or less resemble lipid nanoparticles, which can pass through the membrane with high efficiency.

This reviewer is correct in that naked RNAs are the ones taken up by cells and trapped in endosomes. This concern comes from the field of using siRNA for therapeutic purposes, for which endosomal escape is a problem in the field (Johannes, L., & Lucchino, M. (2018). Current Challenges in Delivery and Cytosolic Translocation of Therapeutic RNAs. Nucleic acid therapeutics, 28(3), 178–193. https://doi.org/10.1089/nat.2017.0716) . While our mRNAs are not all that similar to siRNAs, we included it to be as comprehensive as possible about potential caveats of this experiment. This sentence has been removed as it does not greatly change the conclusions drawn from the study.

15. The MIQE (minimum information for publication of quantitative real-time PCR experiments; PMID: 19246619) was not provided in the results. This is particularly, worrisome given that the RNA stability data relies entirely on real-time PCR analysis of transfected RNA. While this technique is really powerful for detecting very low levels of RNA or for determining differences in RNA levels over many logs, it is less accurate for determining differences in RNA levels in the range of 2-fold or less. The accuracy of this would greatly depend on the efficiency of the reactions. In particular, the essential information indicated in the above publication such as linear dynamic range, PCR efficiency, technical replicates, and the reaction conditions should be included in the methods.

We thank the reviewer for raising this point. We have included information about PCR primers, PCR efficiency, linear range of detection, technical replicates, and reaction conditions using our qPCR primers for Fluc in the methods section (lines 989 - 996).

Significance:

Characterization of a specific RNA binding protein that is involved in the translation of specific IRESs is very timely and exciting and will be of interest to virologists, RNA biologists, and the translational control field. As an expert in alternative mechanisms of translation initiation, RNA binding proteins, and RNA viruses this reviewer has significant enthusiasm for this study given their findings and experimental approaches.

We thank the reviewer for this enthusiastic assessment of our study and hope that their comments have been addressed to their satisfaction.

Dear Dr. Dittmann,

Thank you for the submission of your revised manuscript to our editorial offices. I have now received the reports from the three referees that were asked to re-evaluate your study that I have already forwarded to you, you will also find below. As you will see, referees #2 and #3 now support the publication of your study. Referee #2 has two remaining concerns or suggestions to improve study, I ask you to address during a final revision of the study. Referee #3 has also several suggestions I ask you to address. Please also provide a point-by-point response regarding these remaining issues.

Moreover, I have these editorial requests I also ask you to address:

- Please change 'off' to 'of' in the title.
DDX60 selectively reduces translation of viral type II internal ribosome entry sites

- We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policy https://www.embopress.org/competing-interests and update your competing interests if necessary. Please name this section 'Disclosure and Competing Interests Statement' and put it after the Acknowledgements section.

- We now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section. Please use the free text box to provide more detailed descriptions. Thus, please remove the author contributions section from the manuscript text file. See also guide to authors:
https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines

- The "Data Availability section" (DAS - placed after Materials and Methods) should list deposited primary datasets, accession numbers and links to the database. It seems no such datasets have created in this study and deposited? In that case, please state this in this section (e.g. 'No primary datasets have been generated and deposited'). Please also remove the paragraph 'New material availability statement'. Authors publishing in EMBO press journals always agree to make available reagents used in the study upon request.

- Please order the manuscript sections like this (using these names):
Title page - Abstract - Keywords - Introduction - Results - Discussion - Materials & Methods - DAS - Acknowledgements - Disclosure and Competing Interests Statement - References - Figure legends - Expanded View Figure legends (please separate main and EV figure legends).

- Please remove the point from all panel labels in the figures (it should be A, B, C ... not A., B. and C. ...).

- Please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (main, EV and Appendix figures), and that statistical testing has been done where applicable. Please avoid phrases like 'independent experiment', but clearly state if these were biological or technical replicates. Please add complete statistical testing to all diagrams (main, EV and Appendix figures). Please also indicate (e.g. with n.s.) if testing was performed, but the differences are not significant. In case n=2, please shoe the data as separate datapoints without error bars and statistics (see also the first point of referee #1 - please also apply this to the data in figures EV1 and EV3 where necessary).

- Please make sure that all the funding information is also entered into the online submission system and that it is complete and similar to the one in the acknowledgement section of the manuscript text file. Please include the funding information in the acknowledgements and remove the separate paragraph.

- I wonder if the Appendix Figure could be included in one of the main or EV figures? Otherwise, we need a formal Appendix file. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

- Please upload the information provided in 'Appendix Table S1' as 'Reagents and Tools table'. I have attached templates for that in word or excel format. Please upload the filled in table to the manuscript tracking system as a 'Reagent Table' file. The example linked below shows how the table will display in the published article and includes examples of the type of information that should be provided for the different categories of reagents and tools. Please list your reagents/tools using the categories provided in the template and do not add additional subheadings to the table. Reagents/tools that do not fit in any of the specific categories can be listed under "Other":
https://www.embopress.org/pb%2Dassets/embo-site/msb_177951_sample_FINAL.pdf

- As they are significantly cropped, please provide the source data for the Western blots shown in the manuscript (including the

EV figures). The source data will be published in separate source data files online along with the accepted manuscript and will be linked to the relevant figures. Please submit scans of entire gels or blots together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure (main and EV).

- You indicate in the author checklist that your study could fall under dual use research restriction. Please detail this in your point-by-point response and in a dedicated paragraph termed 'biosafety' in the methods section (see below). Dual Use Research of Concern (DURC) is life sciences research that, based on current understanding, can be reasonably anticipated to provide knowledge, information, products, or technologies that could be directly misapplied to pose a significant threat with broad potential consequences to public health and safety, agricultural crops and other plants, animals, the environment, materiel, or national security. If this is really the case here, we need a detailed explanation to decide if we proceed with publication. See also: http://www.embopress.org/page/journal/14693178/authorguide#biosecurity

- Please add a paragraph detailing biosafety measurements taken during the study. This should list safety regulations, biosafety levels and institutional or governmental approval of the experiments with viruses.

- Finally, please find attached a word file of the manuscript text (provided by our publisher) with changes we ask you to include in your final manuscript text, and some queries, we ask you to address. Please provide your final manuscript file (using the attached file as basis) with track changes, in order that we can see any modifications done.

In addition, I would need from you:
- a short, two-sentence summary of the manuscript (not more than 35 words).
- two to four short bullet points highlighting the key findings of your study (two lines each).
- a schematic summary figure (in jpeg or tiff format with the exact width of 550 pixels and a height of not more than 400 pixels) that can be used as a visual synopsis on our website.

I look forward to seeing the final revised version of your manuscript when it is ready. Please let me know if you have questions regarding the revision.

Please use this link to submit your revision: https://embor.msubmit.net/cgi-bin/main.plex

Yours sincerely,

Achim Breiling
Senior Editor
EMBO Reports

Referee #1:

The authors have provided a revised and improved version of their previous manuscript that addresses and discusses many of the points raised by the three reviewers. This reviewer is still enthusiastic about the novelty of this study and convinced of its importance to the field of translational control by viruses. However, the responses to two of the comments are still unsatisfactory.

The first point concerns the experiments related to RNA-immunoprecipitation. Although the reviewer and the authors agree on the difficulty of this experiment and its inherent variability, this reviewer finds it very irritating that the authors refuse to adhere to the minimum standards of scientific accuracy and provide a minimum of three replicates before drawing any conclusions. Furthermore the histograms shown in figure EV4B do not show the individual points of the two experiments mentioned.

Second point. This reviewer recognizes the efforts and work done to consolidate the data obtained from polysome profiling (Figures 5, 6 and EV5) but does not share the same interpretation of the results for the reasons mentioned below.
It appears, however, that the question was not quite clear. To be able to compare the polysome results, translation efficiency must be calculated and additionally be shown for each replicate. The translation efficiency represents the proportion of RNA associated with polysome vs total ribosomes. In this case, the agarose gel analysis seems to show (but RNAs look particularly degraded) that fraction 1 corresponds to free RNAs (tRNAs), fraction 2 to RNA associated with the 40S subunit of the ribosomes, fraction 3 RNA associated with monosomes, fractions 4 to 11 with polysomes. This means that the the translation efficiency of the reporter RNAs and viral RNAs corresponds to the ratio of fractions 4-11 to total fractions. This must be calculated for each replicate and the average values shown, for example as a bar graph. This will further allow testing the statistical differences between the conditions and drawing appropriate conclusions. At this point, this reviewer cannot agree with the conclusions proposed for Figure 6C. None of the viral RNA shifts in the monosomal fractions. In addition analysis in cells in absence of DDX60 is not provided. This result is key to confirm the authors' claim of DDX60 specificity for type II IRESs.

Minor comments regarding

- the annotations of the RNA agarose gels: the labels are incorrect, the label "40S" should be replaced with "free RNA"; "60S" with "18S" and "80S" with "28S".
- Displaying mean +/- SD values for % RNA in each fraction would improve the readability of polysome graphs.

Referee #2:

In my opinion, all of the previous reviews have been adequately addressed.

Referee #3:

The revised manuscript by Sadic et al, is greatly improved. There are a few minor issues the authors might want to address. In particular Figures 1D and 2 are very nicely done!

Minor corrections for the authors at their discretion
1. Figure 1B the protein labels do not align with the bands.
2. Figure 1C in the results and figure it is not entirely clear how this experiment was carried out. The legend appears to be the clearest and the way this reviewer would expect with cells transfected with and RFP plasmid expressing IRF, Fluc or DDX60 and infected with a YFP HCV reporter. Some adjustments to the results and model in Figure 1C could be helpful.
3. Line 169 should the Schoggins 2011 reference be replaced with the original Ypet bicistronic HCV reporter paper Jones et al 2010 from Charlie Rice's lab?
4. Figure 1C can the authors clarify why in the figure legend the Ypet bicistronic reporter sounds like a reporter that would be transfected, yet in the cartoon it appears to be an infectious particle?
5. Lines 816 and 1040: ul should be µl; line 1078: ug should be µg.

Editorial Requests

1. Please change 'off' to 'of' in the title. DDX60 selectively reduces translation of viral type II internal ribosome entry sites

Thank you for this suggestion. As IRESs are themselves not translated, unfortunately "translation of viral type II IRESs" is not scientifically correct. After discussion with the Editor, we will leave the title as is.

2. We updated our journal's competing interests policy in January 2022 and request authors to consider both actual and perceived competing interests. Please review the policyhttps://www.embopress.org/competing-interests and update your competing interests if necessary. Please name this section 'Disclosure and Competing Interests Statement' and put it after the Acknowledgements section.

The DAS section has been included after the Acknowledgements section.

3. We now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section. Please use the free text box to provide more detailed descriptions. Thus, please remove the author contributions section from the manuscript text file. See also guide to authors: https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines

We removed the author contributions section from the manuscript.

4. The "Data Availability section" (DAS - placed after Materials and Methods) should list deposited primary datasets, accession numbers and links to the database. It seems no such datasets have created in this study and deposited? In that case, please state this in this section (e.g. 'No primary datasets have been generated and deposited'). Please also remove the paragraph 'New material availability statement'. Authors publishing in EMBO press journals always agree to make available reagents used in the study upon request.

Correct, no such datasets have been created in this study. Thus, we added "'No primary datasets have been generated and deposited." And removed the "'New material availability statement".

5. Please order the manuscript sections like this (using these names): Title page - Abstract - Keywords - Introduction - Results - Discussion - Materials & Methods - DAS - Acknowledgements - Disclosure and Competing Interests Statement - References - Figure legends - Expanded View Figure legends (please separate main and EV figure legends).

Done.

6. Please remove the point from all panel labels in the figures (it should be A, B, C ... not A., B. and C. ...).

All figures have been edited accordingly.

7. Please make sure that the number "n" for how many independent experiments were performed, their nature (biological versus technical replicates), the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values is indicated in the respective figure legends (main, EV and Appendix figures), and that statistical testing has been done where applicable. Please avoid phrases like 'independent experiment', but clearly state if these were biological or technical replicates. Please add complete statistical testing to all diagrams (main, EV and Appendix figures). Please also indicate (e.g. with

n.s.) if testing was performed, but the differences are not significant. In case n=2, please shoe the data as separate datapoints without error bars and statistics (see also the first point of referee #1 - please also apply this to the data in figures EV1 and EV3 where necessary).

Number “n”, their nature, bars and error bars etc have been added to all figure legends. The outcome of statistical testing has been added to all diagrams, including ns (non-significant), or nd (not determined, in case of n=2). Finally, in all cases n=2 data is now plotted as separate datapoints without error bars and statistics.

8. Please make sure that all the funding information is also entered into the online submission system and that it is complete and similar to the one in the acknowledgement section of the manuscript text file. Please include the funding information in the acknowledgements and remove the separate paragraph.

Checked and funding information moved to acknowledgements.

9. I wonder if the Appendix Figure could be included in one of the main or EV figures? Otherwise, we need a formal Appendix file. The Appendix should have page numbers and needs to include a table of content on the first page (with page numbers) and legends for all content. Please follow the nomenclature Appendix Figure Sx, Appendix Table Sx etc. throughout the text, and also label the figures and tables according to this nomenclature.

Thank you for suggesting this. The Appendix Figure has now been incorporated into main Figure 4.

10. Please upload the information provided in 'Appendix Table S1' as 'Reagents and Tools table'. I have attached templates for that in word or excel format. Please upload the filled in table to the manuscript tracking system as a 'Reagent Table' file. The example linked below shows how the table will display in the published article and includes examples of the type of information that should be provided for the different categories of reagents and tools. Please list your reagents/tools using the categories provided in the template and do not add additional subheadings to the table. Reagents/tools that do not fit in any of the specific categories can be listed under "Other":
https://www.embopress.org/pb%2Dassets/embo-site/msb_177951_sample_FINAL.pdf

We reformatted and renamed “Appendix Table S1” according to the provided “Reagent Table” file.

11. As they are significantly cropped, please provide the source data for the Western blots shown in the manuscript (including the EV figures). The source data will be published in separate source data files online along with the accepted manuscript and will be linked to the relevant figures. Please submit scans of entire gels or blots together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure (main and EV).

We prepared one PDF per figure for all figures containing cropped western blots.

12. You indicate in the author checklist that your study could fall under dual use research restriction. Please detail this in your point-by-point response and in a dedicated paragraph termed 'biosafety' in the methods section (see below). Dual Use Research of Concern (DURC) is life sciences research that, based on current understanding, can be reasonably anticipated to provide knowledge, information, products, or technologies that could be directly misapplied to pose a significant threat with broad potential consequences to public health and safety, agricultural crops and other plants, animals,

Referee #1:

Major points:

1. The first point concerns the experiments related to RNA-immunoprecipitation. Although the reviewer and the authors agree on the difficulty of this experiment and its inherent variability, this reviewer finds it very irritating that the authors refuse to adhere to the minimum standards of scientific accuracy and provide a minimum of three replicates before drawing any conclusions. Furthermore the histograms shown in figure EV4B do not show the individual points of the two experiments mentioned.

We are very sorry that our rebuttal has caused irritation. We value the input given by this reviewer, and generally agree that experiments should be done in three replicates. However in this case we feel that a third replicate of each complimentary approach would not change the overall conclusion. In accordance with EMBO guidelines, we now show separate datapoints without error bars and statistics in Figure EV4B.

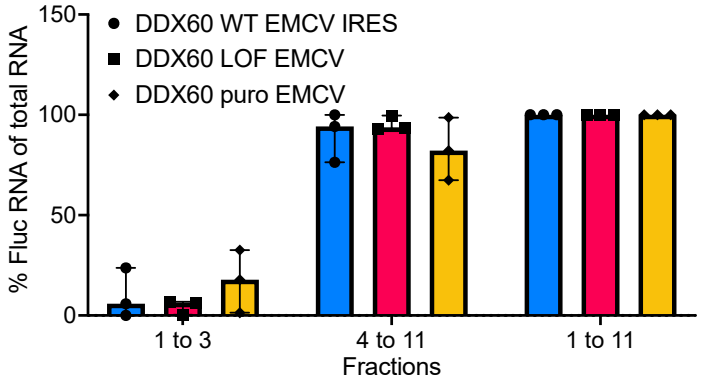
2. Second point. This reviewer recognizes the efforts and work done to consolidate the data obtained from polysome profiling (Figures 5, 6 and EV5) but does not share the same interpretation of the results for the reasons mentioned below. It appears, however,

that the question was not quite clear. To be able to compare the polysome results, translation efficiency must be calculated and additionally be shown for each replicate. The translation efficiency represents the proportion of RNA associated with polysome vs total ribosomes. In this case, the agarose gel analysis seems to show (but RNAs look particularly degraded) that fraction 1 corresponds to free RNAs (tRNAs), fraction 2 to RNA associated with the 40S subunit of the ribosomes, fraction 3 RNA associated with monosomes, fractions 4 to 11 with polysomes. This means that the translation efficiency of the reporter RNAs and viral RNAs corresponds to the ratio of fractions 4-11 to total fractions. This must be calculated for each replicate and the average values shown, for example as a bar graph. This will further allow testing the statistical differences between the conditions and drawing appropriate conclusions. At this point, this reviewer cannot agree with the conclusions proposed for Figure 6C. None of the viral RNA shifts in the monosomal fractions. In addition analysis in cells in absence of DDX60 is not provided. This result is key to confirm the authors' claim of DDX60 specificity for type II IRESs.

This valuable reviewer comment has prompted us to revisit the presentation of our polysome datasets, as well as language of our conclusions.

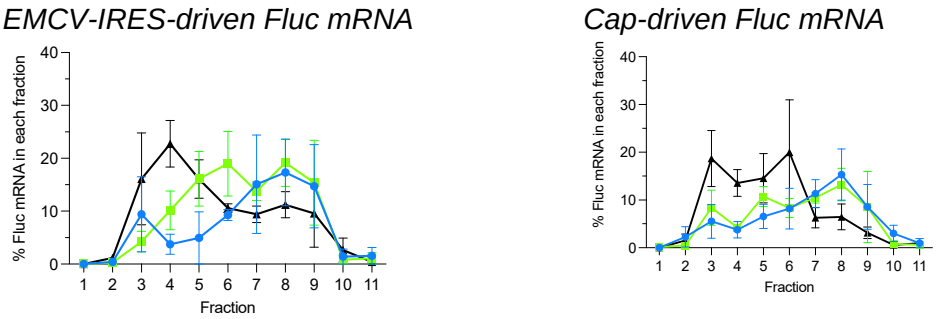
First, we apologize for the mislabeling of the RNA gel bands. Further, we completely agree that the RNA gels were not publication quality – we re-analyzed the same samples by electrophoresis on and now provide the according Bioanalyzer images in Figure EV5 and Figure 6. Fraction 1 corresponds to free RNAs (tRNAs), fraction 2 to RNA associated with the 40S subunit of the ribosomes, fraction 3 RNA associated with monosomes, fractions 4 to 11 with polysomes; we added this interpretation to the main text.

Second, we plotted the “translation efficiency” as suggested, collapsing the individual fractions into “polysomes”, presumably translated, (fractions 4-11) and “non-polysomes” (fractions 1-3). Shown below is the example of cells expressing DDX60 wild type or loss-of-function mutant, detecting the EMCV-IRES-driven Fluc reporter mRNA by RT-qPCR:



From this graph it is apparent that the data does not reflect a decrease in overall translation, in contrast to our results from luciferase assays, where we found a significant reduction of Fluc protein translated off EMCV IRES in the presence of DDX60 wild type. This discrepancy may be explained by the fact that the “collapsed view” of polysome data will only show differences if the inhibition uniquely occurs at the translation initiation step and / or prompts ribosomes to dissociate from the mRNA, both of which would lead to a net reduction of RNA bound to ribosomes. This view would not capture if the elongation process is disturbed, which may lead to an increase of RNA in some fractions due to a “traffic jam” scenario. This phenomenon may cancel out any net reduction in ribosome-bound RNA. We do not know at which step of translation DDX60 acts – it may be one or all of them. With all these considerations, we made the following changes to the manuscript:

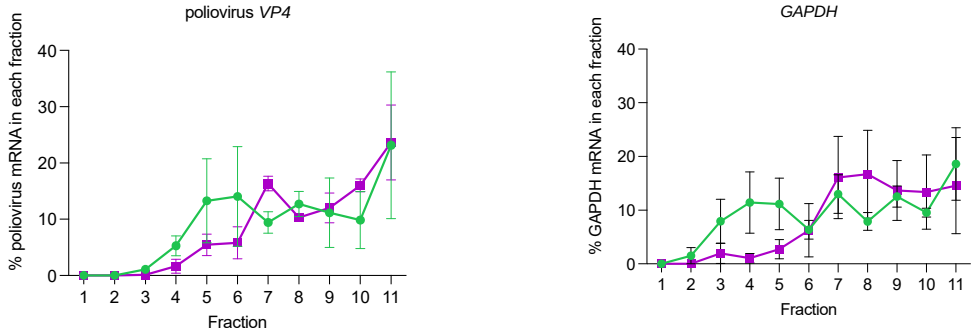
- We chose to not include the collapsed view of polysome profiling due to the loss of resolution. We are confident that our results from the luciferase assays (Figure 2) allow for interpretation that DDX60 selectively and significantly inhibits translation off type II IRESs.
- We now explain the abovementioned theoretical outcomes of initiation/dissociation/stalling on shifting of fractions in the polysome profile data in the results section.
- As suggested by this reviewer, we now show the data as mean +/- SEM. Figure 5:



black: DDX60 LOF + puromycin green: DDX60 LOF (reference) blue: DDX60 WT

The lower RNA amount in DDX60 WT-expressing cells in fractions 4-6 compared to DDX60 LOF is evident, yet not statistically significant. We stated this in the text. DDX60 WT and LOF follow the same trend for cap-driven Fluc mRNA.

- Figure 6 is now also shown as mean +/- SEM:



green: parental poliovirus-infected purple: EMCV-IRES-poliovirus-infected

- We expanded the paragraph in the discussion about possible mechanisms of translation inhibition by DDX60.
  We rewrote the paragraphs interpreting the above graphs. In brief, we are arguing that DDX60 may act on type II IRES both by perturbing translation initiation (see fractions 4-6) and by ribosome stalling (see fraction 7). Together with our data showing significant reduction of EMCV-IRES-driven Fluc protein production in the presence of DDX60 (Fig 2B) and reduced replication of EMCV-IRES-PV (Fig 3E), this suggests that DDX60 reduces viral type II IRES-driven protein synthesis by modulating ribosome occupancy on type II IRES-driven mRNA.

<u>Minor points:</u>

3. the annotations of the RNA agarose gels: the labels are incorrect, the label "40S" should be replaced with "free RNA"; "60S" with "18S" and "80S" with "28S".

Thank you much for catching this, it has now been corrected and also mentioned in the text to clarify fraction interpretation.

4. Displaying mean +/- SD values for % RNA in each fraction would improve the readability of polysome graphs.

We agree and now show data as mean +/- SEM.

Referee #2:

In my opinion, all of the previous reviews have been adequately addressed.

Thank you much!

Referee #3:

The revised manuscript by Sadic et al, is greatly improved. There are a few minor issues the authors might want to address. In particular Figures 1D and 2 are very nicely done!

Minor points:

1. Figure 1B the protein labels do not align with the bands.

Thank you for catching this, it has been fixed.

2. Figure 1C in the results and figure it is not entirely clear how this experiment was carried out. The legend appears to be the clearest and the way this reviewer would expect with cells transfected with and RFP plasmid expressing IRF, Fluc or DDX60 and infected with a YFP HCV reporter. Some adjustments to the results and model in Figure 1C could be helpful.

We made some adjustments in the text to more clearly state that the cells are first transfected to express the indicated transgenes, then infected with the YFP HCV reporter.

3. Line 169 should the Schoggins 2011 reference be replaced with the original Ypet bicistronic HCV reporter paper Jones et al 2010 from Charlie Rice's lab?

Both are cited – the bicistronic reporter HCV citation is indeed Jones et al 2010, whereas the screening for ISG effectors is from Schoggins 2011.

4. Figure 1C can the authors clarify why in the figure legend the Ypet bicistronic reporter sounds like a reporter that would be transfected, yet in the cartoon it appears to be an infectious particle?

It is indeed an infectious particle – we clarified this in the legend and throughout the text.

5. Lines 816 and 1040: ul should be µl; line 1078: ug should be µg.

Thank you, this has been corrected.

Dr. Meike Dittmann
University Grossman School of Medicine, New York
United States

Dear Dr. Dittmann,

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.

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Yours sincerely,

Achim Breiling
Senior Editor
EMBO Reports

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New_manu_number: EMBOR-2022-55218V3

Corr_author: Dittmann
Title: DDX60 selectively reduces translation off viral type II internal ribosome entry sites

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Reporting Checklist for Life Science Articles (updated January 2022)

This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your manuscript.

Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data Presentation.

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- 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 vs. unpaired), simple χ2 tests, Wilcoxon 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?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Yes	Materials and Methods

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Materials and Methods; Tools and Reagents Table

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Materials and Methods; Tools and Reagents Table

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Yes	Materials and Methods
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Not Applicable	
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Materials and Methods

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Not Applicable	
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Not Applicable	

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Not Applicable	

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Not Applicable	
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Not Applicable	
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Not Applicable	

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figure legends
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure legends

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: Include a statement confirming that **informed consent** was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Not Applicable	
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	No DURC research was performed as per USDA, ARS policy P&P 621.0 v.3 approved on June 19, 2020.

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
For **tumor marker prognostic studies,** we recommend that you follow the **REMARK** reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	Not Applicable	
For **phase II and III randomized controlled trials**, please refer to the **CONSORT** flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Not Applicable	
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	