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eIF2A mediates translation of hepatitis C viral mRNA under stress conditions

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1st Editorial Decision 30 September 2010

Thank you for submitting your manuscript for consideration by The EMBO Journal. After some delay due to difficulties with the availability of suitable and willing referees during the past summer holiday period it has now been seen by three referees whose comments are shown below. As you will see while referee 2 is clearly more positive the other referees are not convinced that the conclusiveness and completeness of the experimental evidence provided is sufficient at this point to justify the conclusions drawn. One major issue is that the present study is in direct contradiction to a recent study that demonstrates that eIF2A and the Met-tRNAi binding activity of the "eIF2A" fraction can be separated from each other via a multi-step purification procedure (Dmitriev et al. 2010; PMID: 20566627). In the light of this publication, the evidence put forward here is not strong enough to be sufficiently convincing at this point. Clearly, these concerns affect key aspects of the experimental evidence provided, and an extensive amount of further experimentation, which includes crucial controls, would be required to address these issues. Furthermore, the outcome of such experiments cannot be predicted at this point. I am afraid to say that I therefore see little choice but to come to the conclusion that we cannot offer to publish the manuscript.

Please note that we receive many more submissions at The EMBO Journal than we can possibly publish, and that we can only accept those very few manuscripts, which are met with strong and enthusiastic support from at least a majority of reviewers upon initial review. As unfortunately this is not the case for the present submission, I am afraid to say our conclusion regarding its publication here cannot be a positive one.
Thank you in any case for the opportunity to consider this manuscript. I am sorry we cannot be more positive on this occasion, but we hope nevertheless that you will find our referees' comments helpful.

Yours sincerely,

Editor
The EMBO Journal

REFeree COMMENTS

Referee #1 (Remarks to the Author):

The eukaryotic PERK and PKR kinases are commonly activated during viral infection, leading to phosphorylation of eIF2-alpha, consequent sequestration of eIF2 and ultimately to a shut-off of translation of viral and cellular mRNAs. It has been known for almost a decade (e.g. Rivas-Estilla et al. (2002) J. Virol. 76: 10637) that initiation of translation on HCV and related viral IRESs is at least partially resistant to eIF2-alpha phosphorylation, implying the existence of alternative, eIF2-independent mechanisms of initiation. Three distinct eIF2-independent mechanisms of initiation have already been reported (see Specific comment 1), and in this manuscript, Kim et al. report that they have identified a fourth mechanism, in which the cellular protein eIF2A binds specifically to both the HCV IRES and apparently to initiator tRNA, and thus promotes eIF2-independent translation. After reporting experiments that recapitulate prior findings concerning the resistance of the HCV IRES to inhibition by eIF2-alpha phosphorylation (see Specific comment 3), Kim et al. determined that silencing eIF2A reduced HCV IRES-dependent translation under stress conditions, and that this impairment could, in cell-free extracts, be overcome by addition of recombinant eIF2A. This result directly contradicts the conclusion of a report from Dmitriev et al. (see Specific comment #1), who explicitly tested the activity of native eIF2A and found that it is unable to promote initiation on the HCV IRES: this report is not discussed or even cited by Kim et al. This is a particular concern because recent papers on eIF2D/Ligatin (Skabkjin et al., G&D, 2010; Dmitriev et al., JBC, 2010) strongly suggest that Ligatin was likely the active constituent of the protein fraction previously described "eIF2A".

In further experiments, Kim et al. identified interactions of eIF2A with domain IIIId of the HCV IRES and apparently with initiator tRNA (p16, line 13) that together suggest the basis for a model in which eIF2A binds to IRES/40S subunit complexes and recruits initiator tRNA to the P site. The robustness of this model is compromised by the well-established (but here, unacknowledged) importance of domain IIIid for stable IRES/40S subunit interaction (see Specific comment 4), and by the TOTAL ABSENCE of data to support the assertion that eIF2A binds initiator tRNA and to show that this interaction is specific. The latter point is important, because eIF2A has previously been reported to promote ribosomal binding of Phe-tRNA(Phe) to the ribosome, suggesting a lack of specificity (Merrick and Anderson, 1975). The translation experiments done using domain IIIId HCV IRES mutants lacks two important elements, namely assessment of the degree of eIF2 phosphorylation in each experiment, and analysis of the efficiency of translation of each mutant relative to wild-type in the absence of stress.

SPECIFIC COMMENTS

1. Three distinct eIF2-independent mechanisms of initiation on HCV and related IRESs have previously been reported, which involve (a) direct binding of the IRES to preformed 80S ribosomes, yielding elongation-competent ribosomal complexes (Lancaster et al. (2006) RNA 12: 894), (b) recruitment of initiator tRNA to IRES/40S subunit complexes by eIF5B (Pestova et al. (2008) EMBO J 27: 1060; Terenin et al. (2008) Nat Struct Mol Biol 15: 836) and (c) recruitment of initiator tRNA to IRES/40S subunit complexes by eIF2D/ligatin (Skabkjin et al., (2010) Genes Dev 24: 1787; Dmitriev et al. (2010) J Biol Chem 285: 26779). It is accordingly inappropriate for Kim et al. to make claims such as those on p3, lines 13-15 that "the molecular basis of continuous translation of specific mRNAs under stress conditions... is poorly understood", on p4, lines 4-6 that this process "remains an enigma", and p5, lines 18-20, that theirs is "the first report" that describes the molecular...
basis for translation of a specific mRNA under conditions when eIF2's activity is compromised. The claim (p5, lines 11-12) that the molecular basis for continuous translation of Sindbis mRNA under stress conditions has not been explored is incorrect: Skabkin et al. (op. cit.) characterized this eIF2-independent initiation mechanism in detail.

2. Remarkably, Kim et al. mention only one of the three currently known mechanisms for eIF2-independent initiation on HCV-class IRESs, and moreover in an appropriately dismissive manner (e.g. p4, lines 9-10 and p15, lines 16-21) that ignores the mechanistic insights described by Terenin et al. (2008) and Pestova et al. (2008). eIF5B is the eukaryotic homologue of the bacterial initiation factor IF2, which binds to the ribosomal 30S subunit before initiator tRNA and accelerates its recruitment to the 30S initiation complex by "by providing anchoring interactions or inducing a favourable ribosome conformation" (Milon et al., (2010) EMBO Rep. 11: 312). This bacterial mechanism is directly analogous to that proposed for eIF5B during initiation on HCV-class IRESs, as discussed extensively in the reports cited above: neither IF2 nor eIF5B is thought to act as a classical tRNA carrier such as eIF2, EF-Tu or eEF1alpha.

3. The response to different inducers of eIF2 phosphorylation and even the same inducer appears to be variable within and between experiments (Figs. 1b, 1c; Fig. 2a, 2b; Fig. 4C). It would have been appropriate if the authors had assessed the proportion of eIF2 that was phosphorylated under different conditions and in different experiments.

4. A well-known characteristic of initiation on HCV-like IRESs that Kim et al. fail to mention is difficult to reconcile with their initiation model. The apical GGG element that they report as being required for binding of eIF2A to the HCV IRES has in numerous RNA protection, ribosome binding, inhibitor and translation studies on HCV and related IRESs been shown to bind directly to the 40S subunit and to be a key determinant of stable IRES/40S subunit interaction and of IRES function (Jubin et al. (2000) J Virol 74, 10430; Kolupaeva et al. (2000) J Virol 74: 6242; Kolupaeva et al. (2000) RNA 6: 1791; Kieft et al. (2001) RNA 7: 194; Lukavsky et al. (2000) Nat. Struct. Biol. 7:1105; Tallet-Lopez et al. (2003) Nucleic Acids Res. 31: 734; de Breyne et al. (2008) RNA 14: 367). How do Kim et al. reconcile its role as an element that interacts directly with the 40S subunit (and remains protected from cleavage/modification in ribosomal complexes) with the proposed role in recruitment of eIF2A?

5. Experimental questions.

a) Many experimental descriptions are inadequate. For example, details of plasmid construction (p17, lines 18-19) are unhelpful, because the cited reference (#10) simply refers to yet other papers, unpublished clones etc. The manuscript does not describe how the pQE31/His-eIF2A plasmid was made - or even the species from which the expressed eIF2A originates. The protocol for purification of His6-eIF2A ("Methods similar to those described elsewhere" - p18, line 17) is inadequate: previous reports by other groups have indicated a lack of success in expression of recombinant eIF2A (e.g. Dmitriev et al. 2010), and it is thus essential that the present authors describe their methods fully and accurately. The reference (#13) that is cited for descriptions of the method of preparation of the cell-free extract and of the translation conditions (p17, lines 13-14) again contains minimal information and instead refers back to another paper. The description of eIF2A add-back experiments (p27, lines 6-8) does not indicate how much eIF2A was added to what volume of translation reaction.

b) Figure 2A. Phosphorylation of eIF2 appears to be lower in lanes in which eIF2A had been silenced than in those subjected to mock-silencing (compare lane 1 vs. 3 and lanes 2 vs. 4). Is this reproducible?

Figure 2B. The western blot signal for eIF2A appears to be of comparable intensity in lanes 2 and 3, despite the supposed inclusion of recombinant eIF2A in lane 3. Why is this?

Figure 4d. The statement that "the persistence of wild-type and IIId-2 translation in the face of eIF2alpha translation disappeared..." (p11, lines 11-12) is an exaggeration: there is at least 30% residual translation. Similarly, the claim that "binding of eIF2A to the IIId domain of the HCV IRES is a prerequisite for translation ... under stress conditions" (p11, lines 16-17) is not consistent with the high residual activity of IRES mutants that fail to bind eIF2A.

6. Minor Comments
Referee #2 (Remarks to the Author):

In this manuscript, the authors provide evidence that eIF2A directs the binding of initiator tRNA to initiate translation of the HCV mRNA. Curiously however, this is observed only when concentrations of the ternary complex (eIF2•GTP•Met-tRNA) are reduced due to "stress". This is the second paper (Ventoso et al. being the first) to show a role of eIF2A allowing translation of any mRNA, but in these cases, viral mRNAs at a time when phosphorylation of eIF2alpha is extensive. The unusual finding with this report is that under normal circumstances, eIF2A appears to have a nuclear localization that becomes cytoplasmic with stress or infection. Although there are a series of comments below, this is very nicely done.

Comments

1. page 5 - "This higher Km for GTP hydrolysis in the eIF2A-dependent pathway relative to that of the eIF2-dependent pathway likely accounts for the predominant use of eIF2 in the translation of most mRNAs..." This is incorrect. Both pathways require eIF5B and the intracellular concentration of GTP is likely to be saturating for eIF5B (or any of the other GTP binding proteins in translation).

2. Page 7 - "...that eIF2A activity is specific for HCV mRNA." One assumes that there are likely cellular mRNAs that might also use eIF2A and not just HCV.

3. eIF2A has not been characterized as having a nuclear localization signal. The authors may want to point out several basic regions in the C-terminus that might represent this signal. Additionally, in Figure 6b, it would appear that the level of eIF2A in the cytoplasm is similar to that of what are apparently uninfected cells. Might this mean that there is some reasonable percentage of eIF2A in the cytoplasm of normal cells or is this just a difficulty this reviewer is having in viewing these images?

4. Page 16 - "...because eIF2A possess an intrinsic initiator tRNA binding property (data not shown)." It is not clear how this was determined even if the data were not shown. Previous published work had shown that eIF2A would bind either Met-tRNAi or E. coli Phe-tRNA to ribosomes and not other aminoacyl-tRNAs.

5. The authors seem to have missed the literature in yeast that describes eIF2A as a repressor of IRES-mediated translation (i.e. Komar et al., JBC 2005) in which it is suggested that eIF2A represses IRES-mediated translation by working synthetically to bind initiator tRNA but commit to being elongating ribosomes more slowly. More recent work identifies the IRES element of the URE2 gene (Reineke et al., 2009) and it is noted that the minimal IRES is similar in structure to the HCV IId element.

6. There has been a very recent report of a protein with similar properties to eIF2A called eIF2D (Dmitriev et al., JBC 2010). The authors may wish to consider its role as well in their Discussion.
Referee #3 (Remarks to the Author):

In this manuscript the authors show data that would strongly suggest that eIF2A is required by the HCV IRES for its translation under conditions of cell stress. In comparison it has little or no apparent effect on cap-dependent mRNAs under the same conditions. They show that this protein binds to domain IIId of the viral RNA and that mutants in this region are no longer sensitive to changes in the levels of this protein under stress conditions. Moreover, this protein is necessary to sustain viral infectivity. The authors conclude that lack of HCV translation during stress conditions in the absence of eIF2A is due to inhibition of the loading of the Met-tRNAi to the P site of the 40S ribosomal subunit. While the data are of high quality and this is an interesting topic I feel that additional work is essential to really prove the role of this protein as outlined below.

1. The experiments shown in 2 should be additionally carried out with some other viral (and possibly cellular) IRESs to show that the effect is specific for HCV. It would be of considerable interest if this protein were able to activate other IRESs. Some cellular IRESs have been shown to be relatively insensitive to the eIF2 alpha phosphorylation (e.g. PDGF).
2. To prove directly that HCV interacts with eIF2A (Figure 3) this protein should be immuno-precipitated from cells infected with the virus and RT-PCR performed to assess direct binding. In addition, filter binding studies should be carried out so that the dissociation constant can be measured.
3. The most important experiment to carry out is to generate 40S in vitro complexes and carry out a detailed series of experiments to show that in the absence of eIF2A there is no loading of Met-tRNAi. It will be possible to use sucrose density gradient analysis or else ribosome shift experiments for these studies.

Reviewers’ comments and replies to the comments:

Referee #1:

The eukaryotic PERK and PKR kinases are commonly activated during viral infection, leading to phosphorylation of eIF2-alpha, consequent sequestration of eIF2 and ultimately to a shut-off of translation of viral and cellular mRNAs. It has been known for almost a decade (e.g. Rivas-Estilla et al. (2002) J. Virol. 76: 10637) that initiation of translation on HCV and related viral IRESs is at least partially resistant to eIF2-alpha phosphorylation, implying the existence of alternative, eIF2-independent mechanisms of initiation. Three distinct eIF2-independent mechanisms of initiation have already been reported (see Specific comment 1), and in this manuscript, Kim et al. report that they have identified a fourth mechanism, in which the cellular protein eIF2A binds specifically to both the HCV IRES and apparently to initiator tRNA, and thus promotes eIF2-independent translation. After reporting experiments that recapitulate prior findings concerning the resistance of the HCV IRES to inhibition by eIF2-alpha phosphorylation (see Specific comment 3), Kim et al. determined that silencing eIF2A reduced HCV IRES-dependent translation under stress conditions, and that this impairment could, in cell-free extracts, be overcome by addition of recombinant eIF2A.

#1-1. This result directly contradicts the conclusion of a report from Dmitriev et al. (see Specific comment #1), who explicitly tested the activity of native eIF2A and found that it is unable to promote initiation on the HCV IRES: this report is not discussed or even cited by Kim et al. This is a particular concern because recent papers on eIF2D/Ligatin (Skabkjin et al., G&D, 2010; Dmitriev et al., JBC, 2010) strongly suggest that Ligatin was likely the active constituent of the protein fraction previously described “eIF2A”.

In the revised paper, we cited the papers pointed out by the reviewer. We showed that eIF2A, which was expressed in E. coli and purified in homogeneity, specifically interacts with
initiator $tRNA_{\text{Met}}$ but not with elongator $tRNAs$ ($tRNA_{\text{Met}}$ and $tRNA_{\text{Leu}}$) (Fig. S3). I would like to emphasize that nobody characterized eIF2A proteins purified in homogeneity in the previous studies. The eIF2D/Ligatin, described by Dmitriev et al. (2010), binds not only to the initiator $tRNA$ but also to elongator $tRNAs$. Therefore, eIF2A, which is thoroughly characterized in this study, has more plausible properties as a translational initiation factor than eIF2D. More importantly, none of the previous reports including Dmitriev et al.’s showed either the functionality (the delivery of the initiator $tRNA$ to ribosome) of the putative initiation factors using in vivo and/or in cellulo approaches or requirement of the proteins in HCV proliferation. On the other hand, we showed that eIF2A is required for translation of HCV mRNA in cells under stress conditions by using a siRNA against eIF2A (Fig. 2), and that eIF2A substitutes for eIF2 in delivering $\text{Met}-tRNA_{\text{Met}}$ to the 40S ribosomal subunit (Fig. 3). Notably, eIF2A is required for efficient proliferation of HCV in cells (Fig. 6b), indicating that eIF2A-dependent translation is important for translation of HCV mRNA during HCV infection which triggers eIF2A phosphorylation (Fig. 6a).

#1-2. In further experiments, Kim et al. identified interactions of eIF2A with domain IIId of the HCV IRES and apparently with initiator $tRNA$ (p16, line 13) that together suggest the basis for a model in which eIF2A binds to IRES/40S subunit complexes and recruits initiator $tRNA$ to the P site. The robustness of this model is compromised by the well-established (but here, unacknowledged) importance of domain IIId for stable IRES/40S subunit interaction (see Specific comment 4), and by the TOTAL ABSENCE of data to support the assertion that eIF2A binds initiator $tRNA$ and to show that this interaction is specific. The latter point is important, because eIF2A has previously been reported to promote ribosomal binding of Phe-$tRNA$(Phe) to the ribosome, suggesting a lack of specificity (Merrick and Anderson, 1973).

The effects of IIId mutations on translation and resistance to eIF2a phosphorylation were further analyzed and described in Supplementary Fig. 2. Through the analyses, we demonstrated that the resistance to eIF2a phosphorylation clearly depends on eIF2A-binding capability of the IIId domain not on translatability per se.

We showed that eIF2A, which was expressed in E. coli and purified in homogeneity, specifically interacts with initiator $tRNA_{\text{Met}}$ but not with elongator $tRNAs$ ($tRNA_{\text{Met}}$ and $tRNA_{\text{Leu}}$) (Fig. S3).

#1-3. The translation experiments done using domain IIId HCV IRES mutants lacks two important elements, namely assessment of the degree of eIF2 phosphorylation in each experiment, and analysis of the efficiency of translation of each mutant relative to wild-type in the absence of stress.

As suggested by the reviewer, we added Western blotting data showing phosphorylation levels of eIF2a and showed the efficiency of translation of each mutant relative to wild-type in the absence of stress in Fig. S2b and S2c, respectively.

SPECIFIC COMMENTS

1. Three distinct eIF2-independent mechanisms of initiation on HCV and related IRESs have previously been reported, which involve (a) direct binding of the IRES to preformed 80S ribosomes, yielding elongation-competent ribosomal complexes (Lancaster et al. (2006) RNA 12: 894), (b) recruitment of initiator $tRNA$ to IRES/40S subunit complexes by eIF5B (Pestova et al. (2008) EMBO J 27: 1060; Terenin et al. (2008) Nat Struct Mol Biol 15: 836) and (c) recruitment of initiator $tRNA$ to IRES/40S subunit complexes by eIF2D/Ligatin (Skabkin et al., (2010) Genes Dev 24: 1787; Dmitriev et al. (2010) J Biol Chem 285: 26779). It is accordingly inappropriate for Kim et al. to make claims such as those on p3, lines 13-15 that “the molecular basis of continuous translation of specific mRNAs under stress conditions ... is poorly understood”, on p4, lines 4-6 that this process "remains an enigma", and p5, lines 18-20, that theirs is "the first report" that describes the molecular basis for translation of a specific mRNA under conditions when eIF2's activity is compromised. The claim (p5, lines 11-12) that the molecular basis for continuous translation of Sindbis mRNA under stress conditions has not been explored is incorrect: Skabkin et al. (op. cit.) characterized this eIF2-independent initiation mechanism in detail.

As suggested by the reviewer, we described the reports related with this study. And we modified the manuscript accordingly (Page 4): Investigations into the mechanism of continuous
translation of HCV mRNA under stress conditions, in which eIF2 activity is compromised, have been conducted by several groups. Three distinct possible mechanisms have been reported: (a) HCV mRNA directs synthesis of peptides in the absence of any initiation factor (Lancaster et al., 2006). However, the peptide synthesis without initiation factors occurs at high Mg$^{2+}$ concentration but not at physiological concentration; (b) Recruitment of the initiator tRNA to a HCV IRES/ribosome complex is facilitated by eIF5B(Pestova et al., 2008; Terenin et al., 2008). The authors showed that eIF3 and eIF5B are sufficient for charging the initiator tRNA to a ribosome through reconstitution experiments with purified proteins and ribosomes. However, it remains unclear how eIF5B, which does not interact with Met-tRNA$_{Met}$(Roll-Mecak et al., 2000), charges Met-tRNA$_{Met}$ to the P site of a ribosome associated with a HCV mRNA; (c) Recruitment of the initiator tRNA to a HCV IRES/ribosome complex is facilitated by eIF2D/ligatin(Dmitriev et al., 2010; Skabkin et al., 2010). The authors showed that eIF2D, which binds to Met-tRNA$_{Met}^{i}$ and elongator tRNAs, stimulates charging the tRNAs to the P site of a 40S ribosome in a GTP-independent manner through toe-printing analyses with purified proteins and ribosomes. However, none of these reports showed the physiological role of the suggested proteins (eIF5B and eIF2D) in translation of HCV mRNA using an in vivo or an in cellulo system. Moreover, there was no report showing the importance of these proteins in HCV infection. Therefore, the function of these proteins in translation of HCV mRNA remains to be elucidated.

The sentences describing the Sindbis virus were changed to “eIF2A was recently proposed to participate in the translation of Sindbis viral 26S mRNA when functional eIF2 is limited by the stress of virus infection(Ventoso et al., 2006), but the molecular basis of the continuous translation of Sindbis mRNA by eIF2A was not explored.”

2. Remarkably, Kim et al. mention only one of the three currently known mechanisms for eIF2-independent initiation on HCV-class IRESs, and moreover in an inappropriately dismissive manner (e.g. p4, lines 9-10 and p15, lines 16-21) that ignores the mechanistic insights described by Terenin et al. (2008) and Pestova et al. (2008). eIF5B is the eukaryotic homologue of the bacterial initiation factor IF2, which binds to the ribosomal 30S subunit before initiator tRNA and accelerates its recruitment to the 30S initiation complex by “by providing anchoring interactions or inducing a favorable ribosome conformation” (Milon et al., 2010 EMBO Rep. 11: 312). This bacterial mechanism is directly analogous to that proposed for eIF5B during initiation on HCV-class IRESs, as discussed extensively in the reports cited above: neither IF2 nor eIF5B is thought to act as a classical tRNA carrier such as eIF2, EF-Tu or eEF1alpha.

As suggested by the reviewer, we described the reports related with this issue in the introduction and discussion sections.

3. The response to different inducers of eIF2 phosphorylation and even the same inducer appears to be variable within and between experiments (Figs. 1b, 1c; Fig. 2a, 2b; Fig. 4C). It would have been appropriate if the authors had assessed the proportion of eIF2 that was phosphorylated under different conditions and in different experiments.

Indeed, phosphorylation level of eIF2a varies depending on the reagents inducing the phosphorylation. Therefore, it was very difficult to get the same level of eIF2a phosphorylation in experiments requiring different stress conditions. The important point is that the levels of eIF2a phosphorylation correlate with the levels of translational repression in various experiments.

4. A well-known characteristic of initiation on HCV-like IRESs that Kim et al. fail to mention is difficult to reconcile with their initiation model. The apical GGG element that they report as being required for binding of eIF2A to the HCV IRES has in numerous RNA protection, ribosome binding, inhibitor and translation studies on HCV and related IRESs been shown to bind directly to the 40S subunit and to be a key determinant of stable IRES/40S subunit interaction and of IRES function (Jubin et al. (2000) J Virol 74, 10430; Kolupaeva et al. (2000) J Virol. 74: 6242; Kolupaeva et al. (2000) RNA 6: 1791; Kieft et al. (2001) RNA 7: 194; Lukavsky et al. (2000) Nat. Struct. Biol. 7:1105; Tallet-Lopez et al. (2003) Nucleic Acids Res. 31: 734; de Breyn et al. (2008) RNA 14: 367). How does Kim et al. reconcile its role as an element that interacts directly with the 40S subunit (and remains protected from cleavage/modification in ribosomal complexes) with the proposed role in recruitment of eIF2A?
We showed that the 40S ribosomal subunit, HCV IRES, and Met-tRNA_{iMet} make a complex in an eIF2A-dependent manner (Fig. 3). This demonstrates that the model shown in Fig. 8 is a plausible one. Moreover, we demonstrated that the resistance to eIF2a phosphorylation clearly depends on eIFA-binding capability of the IIIId domain not on translatability per se (Supplementary Fig. 2).

5. Experimental questions.

a) Many experimental descriptions are inadequate. For example, details of plasmid construction (p17, lines 18-19) are unhelpful, because the cited reference (#10) simply refers to yet other papers, unpublished clones etc. The manuscript does not describe how the pQE31/His-eIF2A plasmid was made - or even the species from which the expressed eIF2A originates. The protocol for purification of His6-eIF2A ("Methods similar to those described elsewhere" - p18, line 17) is inadequate: previous reports by other groups have indicated a lack of success in expression of recombinant eIF2A (e.g. Dmitriev et al. 2010), and it is thus essential that the present authors describe their methods fully and accurately. The reference (#13) that is cited for descriptions of the method of preparation of the cell-free extract and of the translation conditions (p17, lines 13-14) again contains minimal information and instead refers back to another paper. The description of eIF2A add-back experiments (p27, lines 6-8) does not indicate how much eIF2A was added to what volume of translation reaction.

As suggested by the reviewer, we described the method to clone the human eIF2A gene in a bacterial expression vector and the procedure to purify eIF2A protein in detail (pages 19 and 21). In fact, purification of eIF2A was very difficult and inefficient. High skill and patience was demanded for purification of the protein.

b) Figure 2A. Phosphorylation of eIF2 appears to be lower in lanes in which eIF2A had been silenced than in those subjected to mock-silencing (compare lane 1 vs. 3 and lanes 2 vs. 4). Is this reproducible?

The phosphorylation levels of eIF2a are similar in both control siRNA- and eIF2A siRNA-treated samples. The slight change in the phosphorylation level in Fig. 2a is attributed to experimental error.

Figure 2B. The western blot signal for eIF2A appears to be of comparable intensity in lanes 2 and 3, despite the supposed inclusion of recombinant eIF2A in lane 3. Why is this?

We overexposed the film in Western blotting of eIF2A to visualize the eIF2A band from the eIF2A siRNA-treated cell extracts. The difference in eIF2A level on lanes 2 and 3 was observed in shorter exposure of the Western blotting (data not shown).

Figure 4d. The statement that "the persistence of wild-type and IIIId-2 translation in the face of eIF2alpha translation disappeared..." (p11, lines 11-12) is an exaggeration: there is at least 30% residual translation. Similarly, the claim that "binding of eIF2A to the IIIid domain of the HCV IRES is a prerequisite for translation ... under stress conditions" (p11, lines 16-17) is not consistent with the high residual activity of IRES mutants that fail to bind eIF2A.

We change the manuscript as follows: The translation of wild-type and IIIId-2 mutant HCV mRNAs was minimally reduced (~20%) under stress conditions. In contrast, translation of ΔIIId and IIId-1 mutant mRNAs was reduced by about 60% under the same stress conditions, a decrease in translation efficiency that approached that of control capped mRNA (up ~80%) under stress conditions (page 11).

...binding of eIF2A to the IIId domain of the HCV IRES plays an important role in translation of HCV mRNA under stress conditions (page 12).

6. Minor Comments

a) Citations the text and in the reference list are formatted for a journal other than EMBO Journal.

b) Many experimental details are included in the Figure legends rather than in Methods.

c) p2, line 3; p4, lines 8, 10: The assertion that eIF2 or eIF5B charge initiator tRNA to the P -
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site uses inappropriate terminology: in the context of tRNA, charging is always understood to mean aminoacylation.

d) p4, lines 17-18. The cited paper (Ref. 14) concerns elongation factor 2 rather than initiation factor 2A.
e) p16, lines 10-11. Contrary to what is stated, the GTPase activity of eIF5B is not required for subunit joining, but is required for subsequent release of eIF5B from the assembled 80S ribosome.
f) p5, lines 2-3. I was unable to identify primary data in either Ref. 15 or 16 that supports this claim. It would be better to cite the primary literature.

For a) to f), we changed the manuscript as suggested by the reviewer.

g) p27. Line 1. "Indicated antibodies" - specific for which subunit of eIF3?

We described in the manuscript that we used eIF3b antibody.

h) Figure 5b. The panel is not labeled to indicate silencing vs. mock-silencing of cells.

We changed the figure as suggested by the reviewer.

Referee #2:

In this manuscript, the authors provide evidence that eIF2A directs the binding of initiator tRNA to initiate translation of the HCV mRNA. Curiously however, this is observed only when concentrations of the ternary complex (eIF2/GTP/Met-tRNA) are reduced due to “stress”. This is the second paper (Ventoso et al. being the first) to show a role of eIF2A allowing translation of any mRNA, but in these cases, viral mRNAs at a time when phosphorylation of eIF2alpha is extensive. The unusual finding with this report is that under normal circumstances, eIF2A appears to have a nuclear localization that becomes cytoplasmic with stress or infection. Although there are a series of comments below, this is very nicely done.

Comments
1. page 5 - "This higher Km for GTP hydrolysis in the eIF2A-dependent pathway relative to that of the eIF2-dependent pathway likely accounts for the predominant use of eIF2 in the translation of most mRNAs..." This is incorrect. Both pathways require eIF5B and the intracellular concentration of GTP is likely to be saturating for eIF5B (or any of the other GTP binding proteins in translation).

We agree with the reviewer. Therefore, we deleted this sentence.

2. Page 7 - "...that eIF2A activity is specific for HCV mRNA." One assumes that there are likely cellular mRNAs that might also use eIF2A and not just HCV.

This sentence could mislead the readers. Therefore we changed this sentence to “eIF2A activity is required for translation of specific mRNAs including HCV mRNA under stress conditions.”

3. eIF2A has not been characterized as having a nuclear localization signal. The authors may want to point out several basic regions in the C-terminus that might represent this signal. Additionally, in Figure 6b, it would appear that the level of eIF2A in the cytoplasm is similar to that of what are apparently uninfected cells. Might this mean that there is some reasonable percentage of eIF2A in the cytoplasm of normal cells or is this just a difficulty this reviewer is having in viewing these images?

There seems to be equilibrium between the cytoplasmic and nuclear eIF2A. According to the immunecytochemistry data, the equilibrium shifts toward the nuclear localization under normal conditions, and the equilibrium shifts toward the cytoplasmic localization under stress conditions. In other words, some eIF2A proteins reside in the cytoplasm even under normal conditions, and some eIF2A proteins may localize in the nucleus under stress conditions.
4. Page 16 - "...because eIF2A possess an intrinsic initiator tRNA binding property (data not shown)." It is not clear how this was determined even if the data were not shown. Previous published work had shown that eIF2A would bind either Met-tRNAi or E. coli Phe-tRNA to ribosomes and not other aminoacyl-tRNAs.

In the revised manuscript, we showed that eIF2A, which was expressed in E. coli and purified in homogeneity, specifically interacts with initiator tRNA\textsubscript{Met} but not with elongator tRNAs (tRNA\textsubscript{Met} and tRNA\textsubscript{Leu})(Fig. S3).

5. The authors seem to have missed the literature in yeast that describes eIF2A as a repressor of IRES-mediated translation (i.e. Komar et al., JBC 2005) in which it is suggested that eIF2A represses IRES-mediated translation by working synthetically to bind initiator tRNA but commit to being elongating ribosomes more slowly. More recent work identifies the IRES element of the URE2 gene (Reineke et al., 2009) and it is noted that the minimal IRES is similar in structure to the HCV IIId element.

As suggested by the reviewer, we included the references in the revised manuscript.

6. There has been a very recent report of a protein with similar properties to eIF2A called eIF2D (Dmitriev et al., JBC 2010). The authors may wish to consider its role as well in their Discussion.

As suggested by the reviewer, we described Dmitriev et al.’s work in the introduction and discussion sections.

Referee #3:

In this manuscript the authors show data that would strongly suggest that eIF2A is required by the HCV IRES for its translation under conditions of cell stress. In comparison it has little or no apparent effect on cap-dependent mRNAs under the same conditions. They show that this protein binds to domain IIId of the viral RNA and that mutants in this region are no longer sensitive to changes in the levels of this protein under stress conditions. Moreover, this protein is necessary to sustain viral infectivity. The authors conclude that lack of HCV translation during stress conditions in the absence of eIF2A is due to inhibition of the loading of the Met-tRNA\textsubscript{i} to the P site of the 40S ribosomal subunit. While the data are of high quality and this is an interesting topic I feel that additional work is essential to really prove the role of this protein as outlined below.

1. The experiments shown in 2 should be additionally carried out with some other viral (and possibly cellular) IRESs to show that the effect is specific for HCV. It would be of considerable interest if this protein were able to activate other IRESs. Some cellular IRESs have been shown to be relatively insensitive to the eIF2 alpha phosphorylation (e.g. PDGF).

   We tested the effects of eIF2A on translation of EMCV and polioviral mRNAs containing IRES elements under stress conditions (Fig. S1). Knockdown of eIF2A did not show any effect on translation of these viral mRNAs under normal or stress conditions (Fig. S1). We also tested the effect of eIF2A knockdown on translation of a cellular mRNA e-Src containing a stress-insensitive IRES element. Knockdown of eIF2A did not show any effect on translation of this cellular mRNA under normal or stress conditions (data not shown).

2. To prove directly that HCV interacts with eIF2A (Figure 3) this protein should be immuno-precipitated from cells infected with the virus and RT-PCR performed to assess direct binding. In addition, filter binding studies should be carried out so that the dissociation constant can be measured.

   We tried to perform the experiments suggested by the reviewer several times. However, we could not obtain eIF2A-specific specific signal from RT-PCR reactions, because the HCV RNA (or RNA protein complex) bound to the agarose resin nonspecifically. The non-specific binding of HCV RNA to the resin may be attributed to cellular protein(s) associated with the viral RNA. The direct binding of eIF2A with HCV RNA was shown in Fig. 4b by observing the RNA-protein
interaction with purified eIF2A proteins and purified RNAs corresponding to nt 229-280 of HCV RNA (Fig. 4b).

3. The most important experiment to carry out is to generate 40S in vitro complexes and carry out a detailed series of experiments to show that in the absence of eIF2A there is no loading of Met-tRNAi. It will be possible to use sucrose density gradient analysis or else ribosome shift experiments for these studies.

We performed filter-binding assays to monitor the delivery of Met-tRNAiMet to 40S ribosome in a HCV IRES- and eIF2A-dependent manner (Fig. 3). The data indicated that both eIF2A and HCV IRES RNA are required for the delivery of Met-tRNAiMet to 40S ribosome.

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2nd Editorial Decision 09 February 2011

Thank you for submitting your revised manuscript as a new submission. In the meantime, I have sent it back to our original referees 2 and 3 (now referees 1 and 2, respectively) as well as to a new referee, referee 3. You will see that all three referees now support publication here after appropriate revision. I should add that referee 1 mentioned to the editor that it would be highly advisable to tone down your conclusions about the efficiency of eIF2A-mediated translation initiation in the discussion section of your manuscript. All in all, we should be able to consider a revised version of the manuscript that addresses the remaining points raised by the referees in an adequate manner. Furthermore, I would like to urge you to take another careful look through the text and correct and improve the spelling and grammar throughout. I would suggest having a native English speaker read the final draft before returning it to our office.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When preparing your letter of response to the referees’ comments, please bear in mind that this will form part of the Peer Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Yours sincerely,

Editor
The EMBO Journal

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REFEREE COMMENTS

Referee #1 (Remarks to the Author):

This is a much improved manuscript although there are still several concerns that should be addressed. The positive feature is the indication of an alternate pathway for the recruitment of the initiator tRNA under conditions of stress. In this regard, this paper is similar to that of Ventoso et al. that found that alphavirus also appears to use eIF2A to direct met-tRNA binding under conditions of stress and limiting ternary complex (eIF2•GTP•met-tRNA).

Specific concerns:
Page 4 - The use of eIF5B as a factor to direct the binding of initiator tRNA was initially shown by Choi et al. (1998) and this indicated a more direct interaction between eIF5B and met-tRNA.

Page 5 - It would appear that the references are off on this page, from 17 to 19. The authors should recheck this. Also the Ventoso et al. virus was alphavirus, not Sindbis virus.
Page 8 - The elongator species is indicated as met-tRNAm, not met-tRNAe. At the bottom of the page, the word "charging" should be changed to "binding" as charging is lab jargon for the attachment of the amino acid to a tRNA.

Figure 3 vs. Figure S3. Figure 3 appears to show that the binding of met-tRNA to eIF2A requires both HCV RNA and 40S subunits. Figure S3 appears to show that eIF2A can bind directly to the initiator tRNA in the absence of either HCV RNA or 40S subunits and that the binding is independent of the attachment of the amino acid methionine. Although the methodology is slightly different between these assays, these results are essentially contradictory. The authors need to resolve this contradiction, especially in light of the results of Dmitriev et al. 2010.

There continue to be several typos that should be corrected.

Referee #2 (Remarks to the Author):

The authors have addressed my concerns from the first review. This is an interesting story which adds to the knowledge of the field.

Referee #3 (Remarks to the Author):

This paper provides some interesting evidence in support of the model that polypeptide chain initiation factor eIF2A supports the translation of hepatitis C virus RNA in infected cells. This is proposed to occur under conditions where the stress of viral infection causes phosphorylation (and hence inactivation) of the initiation factor eIF2, the factor normally used for Met-tRNA-dependent initiation of protein synthesis. Several pieces of evidence are presented that are consistent with the mechanism suggested.

As well as being of interest in the field of HCV biology, this work has wider implications in that it suggests that mammalian cells may possess an eIF2A-mediated mechanism for the translation of at least some cellular mRNAs under stress conditions, in an eIF2-independent manner.

The authors are requested to address the following specific points in revision of their manuscript:

1. Page 8, line 20 and Fig. S3a. The dissociation constant of 16.5 nM refers to uncharged tRNAi, whereas the text says Met-tRNAi. More importantly, the authors need to make more emphasis of the point that eIF2A binds to the uncharged tRNAi equally as well as to Met-tRNAi. To my knowledge this is not the case for eIF2.

2. Page 12 and Fig. 6. Panel (a) of Fig. 6 should show a blot for total eIF2α as well as the phosphorylated protein to establish that virus infection really has increased the level of phosphorylation here. More importantly, it is not safe automatically to equate luciferase activity of a virally-encoded reporter with HCV proliferation or infectivity. At the very least the authors should look at the synthesis of one or more viral proteins in this type of experiment.

3. Discussion and Fig. 8. Have the authors examined directly (by a filter binding or pull-down assay) whether eIF2A can bind simultaneously to tRNAi and the IIIId domain of the HCV IRES?

Minor points

4. The manuscript needs to be checked for linguistic/grammatical errors, and preferably be proof-read by a native English speaker.

5. Page 4. In several places the authors use the word "charging" in relation to the process of bringing the initiator tRNA to the ribosome. This term is normally reserved for the process of attaching the amino acid to the tRNA, catalysed by aminoacyl-tRNA synthetases, and a better word would be...
"recruiting", "binding" or "loading" of the Met-tRNAi on to the ribosome.

6. Page 6, line 17 - delete "and HCV IRES-dependent".

7. Page 8, line 5 - delete "not"

8. Page 8, paragraph 2 - in several places "ribosome" or "subunit" should be in the plural.

9. Legend to Fig. 3 - this should only describe what was done in the experiment and conclusions should not be part of the text here.

10. Fig. 4 legend, last line - anti-eIF3b antibodies

Referee #1 (Remarks to the Author):

This is a much improved manuscript although there are still several concerns that should be addressed. The positive feature is the indication of an alternate pathway for the recruitment of the initiator tRNA under conditions of stress. In this regard, this paper is similar to that of Ventoso et al. that found that alphavirus also appears to use eIF2A to direct met-tRNA binding under conditions of stress and limiting ternary complex (eIF2-GTP-met-tRNA).

Specific concerns:

Page 4 - The use of eIF5B as a factor to direct the binding of initiator tRNA was initially shown by Choi et al. (1998) and this indicated a more direct interaction between eIF5B and met-tRNA.

We included this information on page 4:
The relationship between eIF5B and the initiator tRNA was also demonstrated by a partial suppression of severe slow growth phenotype of fun12Δ yeast strain with a mutation in the eIF5B gene through overexpression of the gene encoding tRNAiMet (Choi et al, 1998). However, it remains unclear how eIF5B, which does not make stable complex with Met-tRNAiMet contrast to IF2 (Roll-Mecak et al, 2000), delivers Met-tRNAiMet to the P site of a ribosome associated with a HCV mRNA;

Page 5 - It would appear that the references are off on this page, from 17 to 19. The authors should recheck this. Also the Ventoso et al. virus was alphavirus, not Sindbis virus.

As suggested by the reviewer, we rechecked the references and changed ‘Sindbis’ to ‘alphavirus’.

Page 8 - The elongator species is indicated as met-tRNAam, not met-tRNAe. At the bottom of the page, the word "charging" should be changed to "binding" as charging is lab jargon for the attachment of the amino acid to a tRNA.

As suggested by the reviewer, we changed ‘met-tRNAe’ and tRNAeLeu to ‘met-tRNA’ and tRNALeu, respectively. ‘Met-tRNAam’ is usually termed for prokaryotic elongator tRNA. ‘met-tRNA’ is usually used for eukaryotic elongator tRNA.

We also changed the word ‘charging’ to ‘binding’, ‘loading’ or ‘recruiting’ depending on the situations as suggested by referee #3.

Figure 3 vs. Figure S3. Figure 3 appears to show that the binding of met-tRNA to eIF2A requires both HCV RNA and 40S subunits. Figure S3 appears to show that eIF2A can bind directly to the initiator tRNA in the absence of either HCV RNA or 40S subunits and that the binding is independent of the attachment of the amino acid methionine. Although the methodology is slightly different between these assays, these results are essentially contradictory. The authors need to resolve this contradiction, especially in light of the results of Dmitriev et al. 2010.
Figure 3 depicts a filter binding assay (Ni-NTA resin was not used in this experiment). His-eIF2A-Met-tRNA_{i}^{Met} complex passes through the filter if it is not associated with the 40S ribosome since the size of this complex is smaller than the pore size. However, this complex (His-eIF2A-Met-tRNA_{i}^{Met}) cannot pass through filter if it is associated with the 40S ribosome. In other words, only the ribosome associated complexes show signals.

In contrast, Figure S3 is a resin binding assay. In this assay, His-eIF2A is bound to Ni-NTA resin that cannot pass through the filter. Therefore, \(^{35}\text{S}\)Met-tRNA associated with eIF2A cannot pass through the filter.

In conclusion, there is no contradiction between the results in Figure 3 and in Figure S3.

There continue to be several typos that should be corrected.

We corrected typos.

Referee #2 (Remarks to the Author):

The authors have addressed my concerns from the first review. This is an interesting story which adds to the knowledge of the field.

Referee #3 (Remarks to the Author):

This paper provides some interesting evidence in support of the model that polypeptide chain initiation factor eIF2A supports the translation of hepatitis C virus RNA in infected cells. This is proposed to occur under conditions where the stress of viral infection causes phosphorylation (and hence inactivation) of the initiation factor eIF2, the factor normally used for Met-tRNA-dependent initiation of protein synthesis. Several pieces of evidence are presented that are consistent with the mechanism suggested.

As well as being of interest in the field of HCV biology, this work has wider implications in that it suggests that mammalian cells may possess an eIF2A-mediated mechanism for the translation of at least some cellular mRNAs under stress conditions, in an eIF2-independent manner.

The authors are requested to address the following specific points in revision of their manuscript:

1. Page 8, line 20 and Fig. S3a. The dissociation constant of 16.5 nM refers to uncharged tRNA_{i}, whereas the text says Met-tRNA_{i}. More importantly, the authors need to make more emphasis of the point that eIF2A binds to the uncharged tRNA_{i} equally as well as to Met-tRNA_{i}. To my knowledge this is not the case for eIF2.

As suggested by the reviewer, we included the information in the manuscript on page 8: eIF2A strongly bound to the initiator tRNA_{i}^{Met} but only weakly to elongator tRNAs (Supplementary Figure S3A). Both charged Met-tRNA_{i}^{Met} (Kd = 12.4 nM) and uncharged tRNA_{i}^{Met} (Kd = 16.5 nM) bound to eIF2A strongly (Figure S3).

2. Page 12 and Fig. 6. Panel (a) of Fig. 6 should show a blot for total eIF2a; as well as the phosphorylated protein to establish that virus infection really has increased the level of phosphorylation here. More importantly, it is not safe automatically to equate luciferase activity of a virally-encoded reporter with HCV proliferation or infectivity. At the very least the authors should look at the synthesis of one or more viral proteins in this type of experiment.

As suggested by the reviewer, we measured the level of eIF2a as well as the level of phosphorylated eIF2a by Western blotting (Figure 6A). Moreover, we measured the infectivity of HCV by monitoring the level of HCV core (Figure 6B).

3. Discussion and Fig. 8. Have the authors examined directly (by a filter binding or pull-down assay) whether eIF2A can bind simultaneously to tRNA_{i} and the IIId domain of the HCV IRES?
We monitored the simultaneous binding of tRNAi and HCV IRES to eIF2A (see referee only supplementary data). We could detect the Met-tRNAi/eIF2A/HCV IRES ternary complex.

Referee only figure 1

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<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>HCV IRES (ΔIIId)</td>
</tr>
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</table>
| His-eIF2A   | - | - | + | - | + | [35S]Met-tRNAi
| Met         |

Purified His-eIF2A proteins and [35S]-labeled Met-tRNAis were incubated with biotinylated RNAs corresponding to the HCV IRES (WT or ΔIIId), and then RNA-protein complexes were precipitated with streptavidin-agarose beads. Radioactivity in the precipitants was measured with a liquid scintillation counter. The data indicate that eIF2A can interact with Met-tRNAi and the wild type HCV IRES simultaneously.

Minor points

4. The manuscript needs to be checked for linguistic/grammatical errors, and preferably be proof-read by a native English speaker.

We corrected the manuscript.

5. Page 4. In several places the authors use the word "charging" in relation to the process of bringing the initiator tRNA to the ribosome. This term is normally reserved for the process of attaching the amino acid to the tRNA, catalysed by aminoacyl-tRNA synthetases, and a better word would be "recruiting", "binding" or "loading" of the Met-tRNAi on to the ribosome.

As suggested by the reviewer, we changed the word ‘charging’ to ‘binding’, ‘loading’ or ‘recruiting’.

6. Page 6, line 17 - delete "and HCV IRES-dependent".

We changed the manuscript as suggested.

7. Page 8, line 5 - delete "not"

We changed the manuscript as suggested.

8. Page 8, paragraph 2 - in several places "ribosome" or "subunit" should be in the plural.

We changed the manuscript as suggested.

9. Legend to Fig. 3 - this should only describe what was done in the experiment and conclusions
should not be part of the text here.

The conclusion part in the figure legend was deleted.

10. Fig. 4 legend, last line - anti-eIF3b antibodies

We changed the manuscript as suggested.

Thank you for sending us your (re)revised manuscript. In the meantime, referees 1 and 3 have seen it again. While both referees are positive overall, referee 1 still raises concerns regarding the data in figure 3 versus figure S3. There appears to be a disagreement on the nature/basis of and the conclusions that can be drawn from your filter binding assay. This issue therefore still needs to be sorted out beyond any doubt.

Furthermore, there are a number of editorial issues that need further attention. First, I need to ask you to include the statistical details for figure 3 and the figure for the referee into the figure legends. Furthermore, a scale bar should be included into figure 7 (and the legend). Finally, I would like to ask you to include an authors' contribution section into the manuscript text according to our updated guidelines.

Thank you very much for your cooperation.

Yours sincerely,
Editor
The EMBO Journal

REFeree COMMENTS

Referee #1 (Remarks to the Author):

This revised manuscript is nearly complete. There is only one major scientific concern. In their response, the authors state that "His-eIF2A-Met-tRNA complex passes through the filter if it is not associated with the 40S ribosome since the size of this complex is smaller that the pore size." This is NOT how the Millipore filter binding assay works. All proteins are retained by the filter (and hence its use also for Westerns, etc.) while RNA and nucleotides are not. Anything that is bound to the protein is also retained. In experiments with eIF2, the retention of GTP and Met-tRNA can be demonstrated in the absence of ribosomes. The key feature is the Kd. Ligands for which the Kd is about 10^{-6} (micromolar) are retained poorly, mostly as the off rate is too high. Ligands for which the Kd is 10^{-7} (0.1 micromolar) are retained well. Given the value obtained in Figure S3, with a Kd of roughly 0.015 micromolar, there is no reason why such a complex would not be detected by the Millipore filter assay. Thus, Figure 3 and Figure S3 continue to be a contradiction.

On page 17, line 23, the impression is given that "eIF5B may assist in the translation of HCV mRNA by mediating the release of eIF2-GDP from the 40S ribosomal subunit." Generally it is believed that the hydrolysis of GTP is sufficient to trigger the release of eIF2 from the 40S subunit. This sentence needs to be redone.

With respect to grammar and spelling, the authors have still not generated a polished manuscript. Given below are the lines and pages where corrections need to be made:

1. page 3, line 13 insert "an" between containing and IRES
2. page 4, line 6 - insert Mg++ after physiological
3. page 4, line 14 should be "form a stable complex with Met-tRNAiMet, in contrast to IF2
4. page 4, line 21 - in vivo and in cellulo should be in italics
5. page 5, line 6 - remain should be remains
6. page 5, line 8 - this is the wrong reference
7. page 6, line 5 "where" should be "while"
8. page 7, line 9 - Merrick and Anderson, 1975 would be a better reference
9. page 8, line 2 - use italics (in vitro)
10. page 8, line 4 - use italics (in vitro)
11. page 12, line 23 - 36 hours should be 36 h
12. page 17, line 5 - should be However, the fact that ...
13. page 17, line 7 - participates should be participate
14. page 21, line 3 - The same column purification step ...

Materials and Methods and Figure Legends

Abbreviations

a. minutes should be min
b. hours should be h
c. OAc should be (CH3CO2)
d. [35S]Met-tRNAi, not [35S] Met-tRNAiMet
e. Met-tRNAm is also the term used for eukaryotic elongator tRNA
f. 175 µl, not 175µl (i.e. numbers should have a space before the unit)

Referee #3 (Remarks to the Author):

This manuscript has been revised in response to the previous comments I made and, in my view, is now acceptable for publication. The only further change I would suggest is an amendment to the legend of Figure 6B to describe the blot for the HCV core which has been added in the revised version.

Replies to the comments:

Editor:

Furthermore, there are a number of editorial issues that need further attention. First, I need to ask you to include the statistical details for figure 3 and the figure for the referee into the figure legends. Furthermore, a scale bar should be included into figure 7 (and the legend). Finally, I would like to ask you to include an authors' contribution section into the manuscript text according to our updated guidelines.

We included the statistical details for figure 3 and referee only figure 1 into the figure legends.
We included a scale bar into figure 7 (and the legend).
We included an authors’ contribution section into the manuscript.

Referee #1 (Remarks to the Author):

This revised manuscript is nearly complete. There is only one major scientific concern. In their response, the authors state that "His-eIF2A-Met-tRNA complex passes through the filter if it is not associated with the 40S ribosome since the size of this complex is smaller than the pore size." This is NOT how the Millipore filter binding assay works. All proteins are retained by the filter (and hence its use also for Westerns, etc.) while RNA and nucleotides are not. Anything that is bound to the protein is also retained. In experiments with eIF2, the retention of GTP and Met-tRNA can be demonstrated in the absence of ribosomes. The key feature is the Kd. Ligands for which the Kd is about 10-6 (micromolar) are retained poorly, mostly as the off rate is too high. Ligands for which the Kd is 10-7 (0.1 micromolar) are retained well. Given the value obtained in Figure S3, with a Kd
of roughly 0.015 micromolar, there is no reason why such a complex would not be detected by the Millipore filter assay. Thus, Figure 3 and Figure S3 continue to be a contradiction.

We used a Millipore membrane (CAT MAHVN45), which has low binding affinity to proteins and nucleic acids. Most of eIF2A proteins passed through the filter during the washing step. On the other hand, ribosomes remained on the filter even after the washing step (please refer to the referee only figure 2). We owe the reviewer a big apology since we mislead the reviewer by describing ‘nitrocellulose filter (Millipore type HA, 0.45 μm) where proteins stick’ in the previous manuscript. In fact, we used Millipore membrane (CAT MAHVN45) in our experiments. I hope this explanation would be a proper answer to the contradiction problem. We changed ‘Millipore type HA, 0.45 μm’ to ‘Millipore membrane (CAT MAHVN45)’ in the revised manuscript.

Referee only figure 2

The followings are the websites where one can find the characters of the Millipore filters.

http://www.appliedcytometry.com/Technotes/Sample_Preparation/Technote_50/Millipore_MultiScreen_Assay_System.pdf

http://www.millipore.com/catalogue/module/c7631#0

On page 17, line 23, the impression is given that "eIF5B may assist in the translation of HCV mRNA by mediating the release of eIF2-GDP from the 40S ribosomal subunit.” Generally it is believed that the hydrolysis of GTP is sufficient to trigger the release of eIF2 from the 40S subunit. This sentence needs to be redone.

Different scientists have different opinions on the role of eIF5B. We deleted the sentence in the revised manuscript since this study is related with translation under stress conditions. Therefore, the sentence is not essential for the paper.

With respect to grammar and spelling, the authors have still not generated a polished manuscript. Given below are the lines and pages where corrections need to be made:
1. page 3, line 13 insert “an” between containing and IRES
2. page 4, line 6 - insert Mg++ after physiological
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This manuscript has been revised in response to the previous comments I made and, in my view, is now acceptable for publication. The only further change I would suggest is an amendment to the legend of Figure 6B to describe the blot for the HCV core which has been added in the revised version.

As suggested by the reviewer, we added the information in the figure legend.

Thank you for providing us with further clarifications regarding the filter-binding assay. I have now had a chance to consult with referee 1 once more. He/she now supports publication, but still feels that further clarifications in the manuscript text will be required (please see below). I would therefore suggest following this final suggestion. Please send us an amended version of the manuscript text via e-mail. We will upload it to the system for you, and I will then formally accept the manuscript.

I am looking forward to receiving the final version of the manuscript.

Yours sincerely,

Isabel Arnold, PhD
Editor
The EMBO Journal

Referee 1

This manuscript is now acceptable for publication and will add a new and interesting role for eIF2A in IRES-mediated translation. That said, the authors should clarify the point about the Millipore filter used. Using the URL provided by the authors, the following Millipore filters were listed: group 1, low protein binding - VVLP, GVWP, HVLP, DVPP, and SVLP; group two, higher protein binding - VVHP, GVHP and HVHP. None of these is the filter listed by the authors as MAHVN45. Therefore, it is not clear which filter was used. Second, from this same URL, the smallest pore size is listed as 0.45 micrometers. The rough size of the ribosome is 20-30 nm or 0.020-0.030 micrometers, about one tenth the size of the pore. Thus retention due to pore
restriction is unlikely (although the "referee only" figure 2 does show retention of ribosomes). Anything that would help clarify here would be helpful. It is noted that the ribosome-dependent binding of met-tRNA is likely of the "old" nitrocellulose filter type (Figure 3) and is distinct from the binding observed in Supplementary Figure 3 which includes Ni-nitritriacetic acid-agarose beads in the assay.

Thank you very much for your generous offer. I am sending you the replies to the questions of the reviewer and a revised manuscript.

Replies to the questions of the reviewer:

1. We have provided two URLs previously. One (http://www.millipore.com/catalogue/module/c7781#1) has description of HVPP that we have used (also see below for the description of HVPP). HVPP membrane has low binding affinity to proteins and nucleic acids. The other URL introduces supporting materials.

   As suggested by the reviewer, we included the description of HVPP in the modified manuscript as follows (page 20, lines 6-8): “the sample was filtered through a membrane (HVPP membrane, Millipore MAHV45, 0.45 µm), which has low binding affinity to proteins and nucleic acids, under negative pressure.” In fact, the HVPP membrane has the same characteristics (low protein binding affinity) as HVLP membrane (described in the second URL). HVPP is a multiscreen plate version of HVLP membrane.

2. The second issue was pore size. As pointed by the reviewer, the average pore size of HVPP is 0.45 µm. However, many pores are smaller than 0.45 µm, and the shapes of the pores are not circular. Therefore, many ribosomes, which are smaller than 0.45 µm were trapped in the pores and retained within the wells as indicated by the blue color in the the "referee only figure 2". By this property, we could detect tRNAs associated with ribosomes with this filter.
# MultiScreen Ordering Information

This section lists ordering information for low protein and nucleic acid binding plates, high protein or nucleic acid binding plates, and special filter papers.

**NOTE:** New plates (including black plates) are continually introduced for new assay techniques. Call your nearest Millipore Technical Service Department for more information.

## Low Protein and Nucleic Acid Binding Plates

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<th>Membrane</th>
<th>Pore Size</th>
<th>Plate</th>
<th>Pretreat</th>
<th>Description</th>
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<th>Catalogue No.</th>
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<td>Aqueous solution</td>
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