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# The eukaryotic translation initiation factor eIF4E reprogrammes alternative splicing

Mehdi Ghram, Gavin Morris, Biljana Culjkovic-Kraljcic, Jean-Clément Mars, Patrick Gendron, Lucy Skrabanek, Maria Revuelta, Leandro Cerchietti, Monica L Guzman, and Katherine Borden
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## Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

## 1st Editorial Decision 22nd Dec 2021

Thank you for submitting your manuscript (EMBOJ-2021-110496) to The EMBO Journal. I have now read your study carefully and discussed the work with other members of the editorial team. However, I regret to inform you that we have decided not to pursue publication of this manuscript in The EMBO Journal.

We appreciate that you assess a potential involvement of eIF4E in splicing and find that increased levels lead to alternative splicing of specific targets and dysregulation of the splicing machinery. Specifically, you report that increased eIF4E leads to an increase in SF protein levels and that eIF4E interacts with the spliceosome. We recognize that this study extends beyond your previous work on eIF4E and now also implicates this factor in splicing. However, while we find that this link and the proposed model would be of interest, further experimental characterization and validation of the model would in our view be required. Here, to us it would for example be important to further define the molecular function of eIF4E is this process and to experimentally test the proposed model(s), as well as to further define and characterize eIF4E target mRNAs. Overall, we find that the mechanistic insight is not yet sufficient to provide the degree of conceptual advance that would be required to warrant further consideration for publication at EMBO Journal. However, as we appreciate the potential interest in the proposed role for eIF4E, we would be open to reconsider a revised version of the manuscript should you be able to address this in the future.

Referee #1 (Report for Author)

Ghram, Morris, Culjkovic-Krakjacic et al report results indicating that 1) over-expression of eIF4E in U2OS cells leads to increased expression of a variety of splicing factors, an effect also observed in AML NOMO-1 cells and that correlates with the status of eIF4E levels in AML specimens, 2) RIP and co-IP experiments +/- RNase treatment show an RNA-mediated association between eIF4E and these splicing factors, and association of eIF4E occurs both with precursors as well as mature mRNAs, 3) eiF4E overexpression in U2OS cells and higher levels of eIF4E in AML samples correlate with substantial -but not global- effects on alternative splicing, involving pathways with important cellular functions, and 4) the alternatively spliced regions most affected by eIF4E levels typically contain longer introns, have higher GC content and are enriched in potential binding sites for particular splicing factors and for ARE-binding proteins.

These are interesting results that can potentially link the clinically relevant increase in eIF4E levels in AML (supported by prognostic studies as well as by the clinical benefit of the eIF4E inhibitor Ribavirin) with downstream mechanisms of post-transcriptional gene regulation. In my opinion, however, to justify publication in EMBOJ the authors should dig deeper in at least one of these two lines of experiments:

1) Figure 1G clearly supports the notion that AML samples show much increased levels of a variety of splicing factors, along with increased expression of eIF4E. This in part recapitulated by overexpression of eIF4E in U2OS and NOMO-1 cells (other panels in Figure 1), arguing for a causal relationship. Results in Supplementary Fig 2 suggest that eIF4E overexpression enhances nucleo-cytoplasmic transport of mRNAs encoding splicing factors by about 2-fold. It is unclear at this point whether this rather modest increase can justify the large (may be 5-10 fold?) increase in splicing factor protein levels found in the more relevant context of AML samples (Figure 1G). This mechanistic link seems to me critical for building the authors' model, specially considering the various activities that eIF4E can exert on the gene expression pathway. I therefore encourage the authors to assess, whether eIF4E elevated levels are associated to/can cause changes in the mRNA levels, mRNA stability and/or mRNA engagement with polysomes of the mRNAs encoding the splicing factors under study (and others as controls). Ideally, this could be addressed by carrying out RIP-Seq (and eventually Ribo-Seq) to be able to correlate, transcriptome-wide, the levels of association with eIF4E and the impact on mRNA levels/translation.

2) The other important message of the paper is the ability of eIF4E to modulate alternative splicing, either through its effects on expression of a variety of splicing factors and/or through a more direct role, as suggested by the association of eIF4E with splicing factors on pre-mRNAs as well as in mRNAs. Possibly the main finding of the paper is that eIF4E can be associated, presumably through its binding to the CAP in competition with the CBC, with pre-mRNAs and this somehow influence alternative splice site choices. What is missing here is a more complete study of the extent to which eIF4E association with pre-mRNAs correlates with alternative splicing changes, something that -once again- could be achieved by combining RIP-Seq data with the RNA-seq datasets that the authors have already used to establish the impact of eIF4E overexpression on splicing. This could help to establish the basis for the selectivity of eIF4E association with a particular subset of pre-mRNAs in the nucleus, which at the basis of the authors' model. Another question is how association of eIF4E with pre-mRNAs impinges on splice site recognition, because the RNA-dependent association of eIF4E with splicing factors may just reflect their coexistence in the same pre-mRNA molecules. For example, is it conceivable that eIF4E can help to recruit ARE binding proteins?

Other points:

a) Figures 1 and 2: the western blot results are generally convincing but would benefit from quantification. In panel 1B it is strange that the eIF4E signal (4th blot from the bottom) does not seem to increase upon overexpression of 2FLAG-eIf4E.
b) Figure 5D-E: the text and Figure legend indicate that these results correspond to RT-qPCR analyses of splicing events, however the y axis indicate "mRNA levels relative to Vector": are the authors quantifying mRNA levels or exon inclusion levels (PSI)?
c) The authors may want to discuss an earlier publication (https://pubmed.ncbi.nlm.nih.gov/21829374/) describing the impact of eIF4E in alternative splicing regulation during Drosophila sex determination. This work from Paul Schedl's lab argues that eIF4E plays a major role in splicing autoregulation of the master regulator Sex-lethal. Also work from the Sonnenberg's lab showing co-localization of eIF4E with splicing factors in speckles (https://pubmed.ncbi.nlm.nih.gov/10648556/).
d) Line 303 and elsewhere in the text: "observed 555 splicing events" should be "observed 555 splicing event changes". Line 313 "associated with repression or promotion of splicing": except for IR, for the rest of the alternative splicing categories (e.g. cassette exon inclusion/skipping) are not really associated with repression or promotion of splicing, but rather with different choices of splice sites. Line 501: PRP8 is a general core splicing factor involved in catalytic activation of the spliceosome and does not have a specific binding motif as most regulators (or core factors involving in splice site recognition like U2AF2) do.
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Referee #2 (Report for Author)

eIF4E is often focussed on as a core component of the eukaryotic translation initiation machinery and functioning in the cytoplasm to stimulate protein synthesis. However, eIF4E also exists in the nucleus, where it has been linked to regulating select nuclear mRNA export. Here the authors submit a manuscript linking eIF4E directly to the splicing machinery and regulating mRNA splicing at multiple levels. Overall, this is an interesting paper that could provide additional insights into eIF4E functionality, both in normal and cancer contexts.

Major points:

FLAG-eIF4E is mentioned to be around 3-fold higher levels than endogenous. However, the western blot appear as though the levels are significantly more than just 3-fold. Can the authors provide lower exposure westerns for eIF4E to put the FLAG-eIF4E signal more into the dynamic range?

One of the main conclusions of the manuscript is that eIF4E promotes the nuclear export of mRNAs coding for splicing factors. However, this would be significantly strengthened if the authors used reporters in U2OS cells with 5' and/or 3'UTRs of candidate targets that may have eIF4E-sensitivity elements that would be utilized for export. This would allow them to mutate putative elements on candidate mRNAs to demonstrate this is a direct effect of eIF4E rather than an indirect effect of eIF4E overexpression. In addition, the authors could deplete LRPPRC, which they had previously shown interacts with eIF4E in the nucleus and selectively effects eIF4E-dependent mRNA nuclear export.

Is it possible that the authors could generate an eIF4E mutant that cannot go nuclear? This would help to determine what aspects of the phenotypes they observe are dependent on eiF4E localization to the nucleus as opposed to just eiF4E overexpression.

Co-immunoprecipitation studies for NOMO-1 cells are mentioned to be in Figure 2D, but it's really Figure 2C. Regardless, for co-immunoprecipitations, it isn't mentioned whether these co-Ips are being carried out in the presence or absence of RNase. Actin control is missing from co-Ips carried out in U2OS cells. Also, it would be good to do a blot for eIF4G, which theoretically shouldn't come down from nuclear fractions, as well as for LRPPC, which should be a strong nuclear interactor with eIF4E. It should also be mentioned for the IPs, what level of input they are being compared to. Some of the input signals look a bit blown out and may be out of the dynamic range. Finally, a confirmation of these interactions by PLA would go a long way to strengthening the interactions between nuclear eIF4E and the splicing machinery.

For figure 6B, the authors show a western blot for eIF4E immunoprecipitation in the presence or absence of RNase. A minor thing: they mention TE in the figure but don't actually describe what this acronym stands for. In addition, they blot for HuR (Elav1) but do not mention this in the results section here...only later on. Is there specific relevance to this interaction? LRPPC should not be a RNA-dependent interaction with eIF4E...this would serve as a nice positive control for a RNA-independent eIF4E interactor. In addition, all blots of input nuclear lysate were individual cropped bands. Without inputs run on the same gel and a mention of what percentage of INPUT they represent, it is impossible to describe how robust these interactions truly are with eIF4E. Finally, the fact that RNase destroys these association because it breaks down mRNAs or also because it degrades the UsnRNAs present in the splicing machinery....this is unclear and highly relevant.

Taken together, it is still unclear from the data provided whether there truly is a physical interaction between eIF4E and the splicing machinery or merely a RNA-dependent association.

Thank you again for submitting a revised version of your manuscript EMBOJ-2022-1110496, which we had previously rejected editorially, as well as for sending the preliminary response to the two referee reports we received on the study (included again below).

As we discussed, both referees express an interest in the findings and acknowledge the additional insight into eIF4E function the work would provide. However, they also both find that further mechanistic clarity would be required and that additional experiments would be needed to further support the proposed models. Specifically, it will be important to address the question if the interaction of eIF4E with the splicing machinery is RNA-dependent or not (i.e. ref #2- point 4, 5, 6), for example through the m7G cap experiments you suggested in the preliminary response. In addition, the proposed link between eIF4E association and alternative splicing of target mRNA should be strengthened further, ideally by analyzing the genome-wide correlation between eIF4E binding and altered splicing. Further computational analyses of target (sub-)sets could also be helpful and should be added to the manuscript if they are conclusive. Moreover, additional experiments to differentiate effects from altered nuclear mRNA export, translation or splicing should be added. This point could for example be addressed using a type of separation-of-function mutant of eIF4E as referee #2 suggests and/or as you propose in the preliminary response. Finally, please also carefully consider all other referee comments and revise the manuscript and figures as appropriate, also keeping the broader readership of The EMBO Journal in mind. Please remember to provide a detailed response to each of the comments when submitting the revised version and that the manuscript must then fulfill all formatting guidelines (please also see below).

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**Detailed response to reviewers.**
**Reviewer 1.**
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**Point 1.** *This mechanistic link seems to me critical for building the authors' model, specially considering the various activities that eIF4E can exert on the gene expression pathway. I therefore encourage the authors to assess, whether eIF4E elevated levels are associated to/ can cause changes in the mRNA levels, mRNA stability and/or mRNA engagement with polysomes of the mRNAs encoding the splicing factors under study (and others as controls).*

We agree that it is important to further characterize the impact of eIF4E on production of splice factors (SFs). We have added data to address each of the above points. With regard to RNA levels and RNA stability, our RT-qPCR data show that eIF4E did not alter steady state levels of the SF-encoding RNAs examined (Figure 1H). Additionally, RNA-Seq assessment of transcript levels in 2FLAG-eIF4E and vector U2OS cells showed that ~400 transcripts had altered RNA levels (equally divided between elevated and reduced) (Culjkovic-Kraljacic et al, PNAS, 2020). Here, we further analyzed these data and observed that **no** SF-encoding RNAs changed. The same observation applies to the analysis of the RNA-Seq data from AML specimens used in this study i.e. not change in transcript levels of SF-encoding RNAs; we now comment on this in the text. We also note that eIF4E did not alter the levels of the *U*snRNAs themselves (Figure 1&3). Thus, transcript-wide, eIF4E does not modulate the steady-state levels of SF-encoding RNAs or *U*snRNAs. In this way, they are neither transcription nor stability targets. We also note that eIF4E can modulate the splicing of 5 SFs in U2OS (Supplemental Table 2) and ~20 SFs in AML (Supplemental Table 6) suggesting that splicing reprogramming also contributes to modified SF landscape. We now discuss this in the discussion.

We now also include data with a separation-of-function mutant, S53A eIF4E. This mutant separates the nuclear export and translation functions of eIF4E. Specifically, the S53A mutant is unable bind to RNAs in the nucleus or to increase their nuclear export (Culjkovic-Kraljacic et al, 2012). However, in the cytoplasm, the S53A mutant can both bind RNAs and increase their translation efficiency (Culjkovic-Kraljaic et al, 2012}(Kaufman *et al*, 1993; Zhang *et al*, 1995). Importantly, this mutant no longer oncogenically transform cells (Culjkovic-Kraljaic et al, 2012; Lazaris-Karatzas et al 1990). Here, we assessed the capacity of the S53A mutant and wildtype eIF4E to increase SF production relative to vector controls using western blots (Figure 2). We used 3 stable clones for each cell line. As expected for control RNAs which are established eIF4E RNA export targets such as *CCND1*, the S53A mutant could not stimulate production of Cyclin D1 protein (yielding levels similar to vector controls), while levels were elevated in wildtype eIF4E-overexpressing cells as anticipated. Analysis of a subset of SFs revealed a

similar pattern for SNRNP200, PRPF6, PRPF31, and U2AF1 whereby the levels of SFs were roughly equivalent between S53A and vector controls indicating that the RNA export function was the primary means by which to enhance levels of these proteins. For some SFs such as PRPF8, SF3B1 and U2AF2 there was an intermediary impact of S53A mutant, elevating protein relative to vector cells but not to the same extent as wildtype eIF4E. This suggests that there was a contribution from both nuclear RNA export and on translation in these cases. The negative controls β-actin and HSP90 were unchanged by mutant or wildtype protein, as expected. These data are included in Figure 2A.

Also, to further dissect the nuclear versus cytoplasmic functions of eIF4E, we investigated whether eIF4E impacted the translational efficiency of SF-encoding RNAs. We monitored polysomal loading as a function of eIF4E overexpression relative to vector controls to investigate this (Figure 2B). Consistent with the S53A mutant studies above, we observed that *PRPF8, SF3B1* and *U2AF2* had increased translational efficiency upon eIF4E overexpression whereas other examined targets were not changed *e.g.*, *PRPF6*, *PRPF31, SNRNP200 and U2AF1.* In all, eIF4E's nuclear export function is important for the eIF4E-mediated elevation of these SFs and in a few cases, these RNAs are also sensitive to eIF4E at the translation efficiency.

*Results in Supplementary Fig 2 suggest that eIF4E overexpression enhances nucleo-cytoplasmic transport of mRNAs encoding splicing factors by about 2-fold. It is unclear at this point whether this rather modest increase can justify the large (may be 5-10 fold?) increase in splicing factor protein levels found in the more relevant context of AML samples (Figure 1G).*

Our quantification of the 2FLAG-eIF4E blots from all three clonal cell lines (versus 3 vector cell lines) indicate that the change is 2.7-fold change. In this case, we sum the eIF4E from the endogenous and 2FLAG-eIF4E in the 2FLAG-eIF4E cells and compare to the endogenous eIF4E in the vector controls (Figure 2A). The splicing factors are elevated ranging from ~2-3 fold depending on the factor by wildtype eIF4E, when averaged across the cell lines. We now include quantification for the blots in Figure 2A. The data are positioned here because we re-ran gels with S53A, wildtype eIF4E and vector for quantification purposes and thus are all given in together in Figure 2A where the separation-of-function mutant is described.

It is notable that even if the elevation of eIF4E is 2.7-fold, that it is acting in the export of many mRNAs, and thus it is not likely to produce a precisely linear output given its activity is spread across multiple transcripts (*SF3B1*, *U2AF2* etc). Also, see above for our dissection of the nuclear export and translation contributions of eIF4E to SF protein production.

*Ideally, this could be addressed by carrying out RIP-Seq (and eventually Ribo-Seq) to be able to correlate, transcriptome-wide, the levels of association with eIF4E and the impact on mRNA levels/translation.*

This is an excellent idea. We have now analyzed our nuclear LY1 eIF4E RIP-Seq data to determined which SF-encoding RNAs were bound to eIF4E. We found that 109 RNAs encoded proteins involved in splicing. Of these, 53 RNAs encoded components of the spliceosome including SFs validated here *e.g. SNRNP200, PRPF6, PRPF8* in U2Os and AML cells (Figure 1A&E). We found SF-encoding RNAs that are components of each the 5 major *U*snRNPs; STRING analysis revealed that enrichment for each *U*snRNP was statistically significant with FDRs ranging from .0016 to $2.19 \times 10^{-49}$. This is presented in Figure 1A.

We thank the reviewer for their suggestions which we feel that has substantially deepened the insights the mechanisms by which eIF4E controls production of SFs.

**Point 2.**

*The other important message of the paper is the ability of eIF4E to modulate alternative splicing, either through its effects on expression of a variety of splicing factors and/or through a more direct role, as suggested by the association of eIF4E with splicing factors on pre-mRNAs as well as in mRNAs. Possibly the main finding of the paper is that eIF4E can be associated, presumably through its binding to the CAP in competition with the CBC, with pre-mRNAs and this somehow influences alternative splice-site choices. What is missing here is a more complete study of the extent to which eIF4E association with pre-mRNAs correlates with alternative splicing changes, something that -once again- could be achieved by combining RIP-Seq data with the RNA-seq datasets that the authors have already used to establish the impact of eIF4E overexpression on splicing.*

We completely agree and now include this analysis. We inspected a previously collected endogenous nuclear eIF4E RIP-seq dataset derived from an aggressive B-cell lymphoma LY1 cell line where nuclear eIF4E associated with ~3000 transcripts (Culjkovic-Kraljacic et al, 2016). The overall transcriptomes were most similar between the two hematological malignancy-derived datasets i.e. AML and lymphoma. LY1 and AML share ~9000 transcripts (with >10 TPM) as observed from the RNA-Seq data. We found 1326 transcripts in the eIF4E LY1 RIP were also identified in the ~4600 eIF4E-dependent splicing targets in AML from the rMATs analysis. This constitutes ~50% of the RNAs in the nuclear eIF4E RIP (1326/2800) and ~30% (1326/4600) of the RNAs that are alternatively spliced in AML. Moreover, we also identify within this RNA subset validated splicing targets expressed in both LY1 and AML and enriched in the RIP-Seq data e.g. *MAPK3IP8*. Among the 1446 targets in the eIF4E-RIP-Seq that were not eIF4E splicing targets, only 327 (~20%) were not expressed in AML. In this way, ~1100 RNAs that were in the nuclear eIF4E RIPs were not AS targets and are likely targets of other nuclear eIF4E activities such as capping, CPA and/or export.

Using this data enables us to propose a model whereby all ~4600 AML eIF4E-dependent AS targets undergo differential splice-site selection based on the altered splicing landscape induced by eIF4E-mediated changes to SF protein production. In addition, ~1300/4600 would also have altered splice site selection stemming from the physical interactions with nuclear eIF4E which suggests that eIF4E chaperones these RNAs through splicing and modifies their splice-site selection through its simultaneous interactions with substrate RNAs and spliceosome components. Consistent with this model is our observations that eIF4E can bind both splicing substrate and product RNAs (Figure 7).

*This could help to establish the basis for the selectivity of eIF4E association with a particular subset of pre-mRNAs in the nucleus, which at the basis of the authors' model.*

Our original analysis into transcript features that could imbue eIF4E-dependency included all ~4600 transcripts as AS targets. Now, we carried out analysis of those AS targets that were found in the eIF4E RIPs (~1300 targets) and compared that to those that were AS targets but not found in the eIF4E RIPs (~3000 targets). We found no difference in the profiles between these groups or when compared to the original group analysis. Thus, there was no change in the event-type profile or AU rich content in these targets. Clearly there will be a USER code potentially a structure based one such as the 4ESE for export. Studies to identify these are important but we feel are beyond the scope of the current work.

We also now include data to show eIF4E-dependent AS targets have more exons than average transcripts (Supplemental Figure 4). This indicates that eIF4E-dependent AS is linked to transcripts which are more complex from the splicing perspective.

*Another question is how association of eIF4E with pre-mRNAs impinges on splice site recognition, because the RNA-dependent association of eIF4E with splicing factors may just reflect their coexistence in the same pre-mRNA molecules.*

To address this, we investigated whether the interactions of eIF4E with SFs were solely reliant on the presence of the mRNA. Originally, we only used RNAses to examine this dependency, but as reviewer 2 pointed out, the RNAse will target both the *U*snRNAs and mRNAs. Thus, we added new data where we investigated whether excess $m^7G$ cap analogues competed for eIF4E interactions with the SFs identified. We conducted eIF4E IPs as a function of $m^7$GpppG or the negative control GpppG (using nuclear lysates) and monitored SF association by western blot (Figure 4D). We found that the interactions with HuR/ELAVL1 were substantially reduced by $m^7$GpppG but not GpppG treatment; while interaction with eIF4E-binding protein 1 (4EBP1) was not affected by $m^7$GpppG treatment consistent with the direct protein-protein interactions between eIF4E and 4EBP1. We observed that the examined SFs such as PRPF6 and U2AF2, had a reduced association (~2-5 fold) upon $m^7$GpppG treatment but not the GpppG control. Thus, the interaction between eIF4E and some SFs is partially mediated and/or stabilized by capped RNA.

*Is it conceivable that eIF4E can help to recruit ARE binding proteins?*

We agree with the reviewer that one way that eIF4E could impinge on splice-site selection is through the recruitment of ARE-binding proteins. This is particularly relevant given our finding that many of the targeted introns contain A or U rich regions (Supplemental Figure 7) which prompted us to examine ELAVL1/HuR which is the major A/U rich binding protein. We show that ELAVL1/HuR, binds to eIF4E in the nucleus and that this interaction is disrupted by RNAse or $m^7$GpppG cap treatment but not by the controls (Figure 4). This sensitivity to $m^7$GpppG indicated that HuR/ELAVL1 relied on capped-RNAs to bind to eIF4E strongly suggesting that HuR/ELAV1 could be involved in recruitment via AU rich regions. We now discuss this in the text.

**Other points.**

*a) Figures 1 and 2: the western blot results are generally convincing but would benefit from quantification. In panel 1B it is strange that the eIF4E signal (4th blot from the bottom) does not seem to increase upon overexpression of 2FLAG-eIf4E.*

We note that the 2FLAG-eIF4E is substantially increased versus the endogenous eIF4E (2.7-fold) and not present in the vector control. In this case, we calculated the intensity of 2FLAG-eIF4E plus endogenous eIF4E in the 2FLAG cells relative to the endogenous eIF4E in the vector cells from western blots. We detected it using an antibody which recognizes both 2FLAG-eIF4E and endogenous eIF4E. Because of the tag, 2FLAG-eIF4E migrates at a higher molecular weight than endogenous eIF4E. It appears that the reviewer may have been looking at the endogenous eIF4E band and not noticed the 2Flag-eIF4E band at the top of the blot shown. We now label these 2FLAG-eIF4E and endog-4E to avoid confusion.

*b) Figure 5D-E: the text and Figure legend indicate that these results correspond to RT-qPCR analyses of splicing events, however the y axis indicate "mRNA levels relative to Vector": are the authors quantifying mRNA levels or exon inclusion levels (PSI)?*

We agree with the reviewer and have changed the text to note exon inclusion levels as suggested throughout.

*c) The authors may want to discuss an earlier publication (https://pubmed.ncbi.nlm.nih.gov/21829374/) describing the impact of eIF4E in alternative splicing regulation during Drosophila sex determination. This work from Paul Schedl's lab argues that eIF4E plays a major role in splicing autoregulation of the master regulator Sex-lethal. Also work from the Sonnenberg's lab showing co-localization of eIF4E with splicing factors in speckles (https://pubmed.ncbi.nlm.nih.gov/10648556/).*

We now describe these findings and discuss their relevance to our model in the discussion section (page 32).

*d) Line 303 and elsewhere in the text: "observed 555 splicing events" should be "observed 555 splicing event changes". Line 313 "associated with repression or promotion of splicing": except for IR, for the rest of the alternative splicing categories (e.g. cassette exon inclusion/skipping) are not really associated with repression or promotion of splicing, but rather with different choices of splice sites. Line 501: PRP8 is a general core splicing factor involved in catalytic activation of the spliceosome and does not have a specific binding motif as most regulators (or core factors involving in splice site recognition like U2AF2) do.*

We agree and have added the requested corrections and re-worded the text.

Interestingly, recent eCLIP studies identifies a PRPF8-binding site which is used by the Atlas of UTR regulatory activity (AURA) database. Certainly, we agree that PRPF8 is involved in catalytic activation, but these recent studies and its inclusion in AURA underlie our discussion in the text. Please refer to: http://aura.science.unitn.it/

*e) The manuscript contains a significant number of spelling mistakes, incomplete sentences and duplicated verbs. Panel 2D is mentioned in the text (p 19) but not shown in Figure 2.*
We apologize and have improved the writing throughout.

**Reviewer 2.**
**Major points.**
"*Flag-eIF4E is mentioned to be 3 fold*…."

We agree and now quantify as requested. Our quantification showed this was indeed 2.7 fold of the 2FLAG-4E versus endogenous eIF4E in vector cells. In this case, we calculated the intensity of 2FLAG-eIF4E plus endogenous eIF4E in the 2FLAG cells relative to the endogenous eIF4E in the vector cells from western blots 3 different clones in two replicates each. The mean and standard deviation are given in Figure 2A. We re-ran gels with S53A, wildtype eIF4E and vector for quantification purposes and thus are all given in together in Figure 2A where the separation-of-function mutant is described.

"*eIF4E promotes the nuclear export of mRNAs controlling splice factors…this point would be strengthened if the authors used reporter and used the 5' or 3'UTRs of candidate targets*.. " &"*is it possible to generate a nuclear mutant that cannot go nuclear?....the phenotypes they observe are dependent on the nuclear localization of eIF4E and not due to eIF4E overexpression*"

We thank the reviewer for the idea of using separation-of-function mutants as it contributes significantly to the mechanism. At heart the reviewer would like a dissection of impacts of the nuclear and cytoplasmic contributions of eIF4E to its effects on the production of SFs. Mechanistically this is an important point in our understanding of how eIF4E influences splicing. We have added new data to address these important considerations including an entirely new

Figure 2. Along the lines of the mutant requested, we have a separation-of-function mutant, S53A eIF4E. This mutant separates the RNA export and translation functions of eIF4E. Specifically, the S53A mutant is unable bind to RNAs in the nucleus or to increase their nuclear export (Culjkovic-Kraljacic et al, 2012). However, in the cytoplasm, the S53A mutant can both bind RNAs and increase their translation efficiency (Culjkovic-Kraljacic et al, 2012; Kaufman *et al*, 1993; Zhang *et al*, 1995). Importantly, this mutant no longer oncogenically transform cells (Culjkovic-Kraljacic et al, 2012; Lazaris-Karatzas et al 1990). Here, we assessed the capacity of the S53A mutant and wildtype eIF4E to increase SF production relative to vector controls using western blots (Figure 2A). We used 3 stable clones for each cell line. As expected for control RNAs which are established eIF4E RNA export targets such as *CCND1*, the S53A mutant could not stimulate production of Cyclin D1 protein (yielding levels similar to vector controls), while levels were elevated in wildtype eIF4E-overexpressing cells as anticipated. Analysis of a subset of SFs revealed a similar pattern for SNRNP200, PRPF6, PRPF31, U2AF1, whereby the levels of SFs were roughly equivalent between S53A and vector controls indicating that the RNA export function was the primary means by which to enhance levels of these proteins. For some SFs such as SF3B1, PRPF8 and U2AF2 there was an intermediary impact of S53A mutant, elevating protein relative to vector cells but not to the same extent as wildtype eIF4E. This suggests that there was a contribution from both nuclear RNA export and on translation. The negative controls Actin and HSP90 were unchanged by mutant or wildtype protein, as expected. This data is included in Figure 2A.

Also, to further dissect the nuclear versus cytoplasmic functions of eIF4E, we investigated whether eIF4E impacted the translational efficiency of SF-encoding RNAs. We monitored polysomal loading as a function of eIF4E overexpression relative to vector controls to investigate this (Figure 2B). Consistent with the S53A mutant studies above, we observed that *PRPF8, SF3B1* and *U2AF2* had increased translational efficiency upon eIF4E overexpression whereas other examined targets did not e.g. *SNRNP200, U2AF1*, *PRPF6* and *PRPF31.* In all, eIF4E's nuclear export function is important for the eIF4E-mediated elevation of these SFs and in a few cases, these RNAs are also sensitive to eIF4E at the translation efficiency.

As to the nuclear export assays, we demonstrated that eIF4E IPs with SF-encoding RNAs and promoted their nuclear export (Figure 1&3). We agree with the reviewer that dissection of the UTRs would enable us to identify the elements required for export. However, creating these constructs requires us to delineate the full-length UTRs. which are not well curated in databases. This issue presents two problems; first one must define the full-length UTR for each RNA using methods such as RACE and second, one must then clone these out from human cells and then make the reporter constructs. Moreover, reporter assays would only define the element for one RNA at a time and it would be best to isolate USER codes from multiple RNAs to understand the conserved features of these elements. In all, this is a non-trivial task and could not be completed within the time frame allotted. It is a wonderful future direction. However, we feel that the other suggestions the reviewers made such as the separation-of-function S53A mutant which allows monitoring multiple SFs, and further direct measurement of translation via polysomes, have allowed us to dissect these possibilities and robustly demonstrate that the nuclear export function plays a major role in controlling SF protein production.

*the authors could deplete LRPPRC, which they had previously shown interacts with eIF4E in the nucleus and selectively effects eIF4E-dependent mRNA nuclear export*.

We feel that the S53A separation-of-function mutant, the analysis of the nuclear eIF4E RIPs, the accompanying RNA export assays, transcript level analysis and studies into the polysomal

loading address the question about the relevance of the nuclear export functions of eIF4E to its capacity to modulate production of SFs. The S53A mutant is clearly impaired in eIF4E-dependent elevation of all the splice factors examined, consistent with the central role of eIF4E-dependent nuclear RNA export. We agree that depletion of LRPPRC would be interesting but feel that the reviewers' other suggestions have allowed us to address the overarching question: the relevance of nuclear eIF4E to its capacity to elevate SF protein production.

*Co-immunoprecipitation studies for NOMO-1 cells are mentioned to be in Figure 2D, but it's really Figure 2C. Regardless, for co-immunoprecipitations, it isn't mentioned whether these co-Ips are being carried out in the presence or absence of RNase. Actin control is missing from co-Ips carried out in U2OS cells.*

We apologize and have corrected the Figure numbering. We apologize for our lack of clarity. Unless stated otherwise, the IPs were carried out using formaldehyde cross-linked nuclei to prevent reassortment followed by IP. Except when explicitly stated as in Figure 4C-D, there were no RNAse or $m^7G$ cap treatments. We note that we have now included $m^7G$ cap treatments to avoid issues of RNAse affecting *U*snRNAs (as brought up by the reviewer). Further, there was no crosslinking step for IPs monitoring $m^7G$ cap or RNA dependence. We note that the crosslinked and not-crosslinked IPs both demonstrated binding of eIF4E to the SFs examined. For example, buffer treated IPs in the RNAse experiment had the same complement of eIF4E-SF interactions as did the cross-linked RNAs supporting the robust nature of these interactions (Figure 4A-B versus 4C-D).

We have now added the β-actin control for U2OS cells in figure 4A. We apologize for the oversight.

*"A confirmation of these interactions by PLA would strengthen*…."

In principle this is a nice idea. However, it is difficult because SFs are typically spread diffusely throughout the nucleus and identification of specific interactions by microscopy will be difficult as these could be coincidental. Indeed, earlier studies did report that eIF4E co-localized with *U*snRNAs and Sc35 (Dostie et al, JCB 2000), but this was difficult to interpret since the *U*snRNAs were found diffusely throughout the nucleus and thus it is uncertain whether this colocalization indicated physical association or was spurious *i.e.* a happenstance of two proteins being nearby coincidentally. We now include a discussion of this paper. It is for this reason we opted to use immunoprecipitation with formaldehyde cross-linking rather than addressing this by microscopy.

We note that we cross-link nuclei with paraformaldehyde immediately after isolating nuclei and then carry out IP; in this way, we obviate the concern that interactions are a result of reassortment during preparation of nuclear lysates. Thus, we preserve physiologically relevant interactions using a cross-linking strategy similar to that in PLA. Obviously, we do not use the crosslink for the cap-competition or RNAse experiments, and we observe the same eIF4E-SF interactions in controls in those experiments as from the cross-link IPs supporting that the IPs work with or without crosslinking (Figure 4A-B versus 4C-D). We have highlighted these issues in the text.

*Also, it would be good to do a blot for eIF4G, which theoretically shouldn't come down from nuclear fractions, as well as for LRPPC, which should be a strong nuclear interactor with eIF4E. It should also be mentioned for the IPs, what level of input they are being compared to. Some of the input signals look a bit blown out and may be out of the dynamic range. & LRPPC should*

*not be a RNA-dependent interaction with eIF4E...this would serve as a nice positive control for a RNA-independent eIF4E interactor.*

Interestingly, eIF4G is also found in the nucleus (McKendrick et al, MCB, 2001), as is the eIF4E binding protein (4EBP1) (Rong et al, 2008, RNA). The molecular weights of LRPPRC and eIF4G overlap with many of the SFs of interest and so to avoid cycles of stripping and re-probing the blots, we opted for the low molecular weight 4EBP1 as ideal for our purposes. It is now included in the RNAse and cap competition experiments (Figure 4) since it directly binds to eIF4E through protein-protein mediated interactions and serves as a negative control for RNAse and $m^7G$ cap treatment. We use actin, lamin and H2B as negative controls for IPs. The levels of input are now provided (Figure 4).

*A minor thing: they mention TE in the figure but don't actually describe what this acronym stands for.*

We apologize and improved the labels.

*In addition, they blot for HuR (Elav1) but do not mention this in the results section here...only later on. Is there specific relevance to this interaction?*

We apologize for our lack of clarity on HuR/ELAVL1. Our sequence analysis had shown that many of eIF4E-dependent splicing targets were A/U-rich and contained HuR/ELAVL1 binding sites (Supplemental Figure 7). Given HuR/ELAVL1 is the major AU-rich binding protein and plays a role in splicing of some RNAs, we investigated whether it physically associated with eIF4E. Indeed, we observed a physical interaction between eIF4E and HuR/ELAVL1 and that this is RNAse and $m^7G$-cap dependent (Figure 4). We now explain this in the results section in the place that matches its figure position (Figure 4).

*In addition, all blots of input nuclear lysate were individual cropped bands. Without inputs run on the same gel and a mention of what percentage of INPUT they represent, it is impossible to describe how robust these interactions truly are with eIF4E.*

We note that all input samples were run on the same gel as the IPs. While there was no cropping for the IPs in the absence of RNAse (Figure 4A and B), intervening lanes on the same gel with samples not related to this project were removed for presentation purposes for the RNAse experiment in the original version. Where necessary, we ran new gels without the need for cropping. We now mention percentage of inputs and provide gels without these unrelated samples to avoid cropping.

"*Taken together it is unclear whether there is truly a physical interaction between eIF4E and the splicing machinery or a RNA-dependent association… the fact that RNase destroys these association because it breaks down mRNAs or also because it degrades the UsnRNAs present in the splicing machinery....this is unclear and highly relevant.*

To address this, we investigated whether the interactions with SF factors were solely reliant on the presence of the mRNA. We completely agree that RNAse will degrade both types of RNA and unfortunately there are no RNAses specific to mRNAs or *U*snRNAs. Thus, we opted to carry out cap-competition studies to determine which physical interactions were $m^7G$ cap-dependent, and thus mRNA dependent. To this end, we investigated whether excess $m^7G$ cap analogues competed for eIF4E interactions with SFs. We conducted nuclear eIF4E IPs in the presence of $m^7GpppG$ or the negative control GpppG and monitored SF association by western blot (Figure 4D). We found that the interaction with HuR/ELAVL1 was substantially reduced by

$m^7$GpppG relative to GpppG; while interaction with eIF4E-binding protein 1 (4EBP1) was not affected by $m^7$GpppG treatment consistent with the direct protein-protein interaction between eIF4E and 4EBP1. We similarly observed that SFs had a decrease in their association with eIF4E indicating that $m^7$G-capped RNAs mediated and/or stabilized the interactions (Figure 4D).

It is incredibly exciting, and unexpected, to observe that eIF4E plays any role in splicing. From our global analysis, it appears that most splicing target RNAs do not physically associate with eIF4E, and thus their altered splice-site selection is a function of the reprogrammed SF landscape induced by eIF4E. For those that do physically associate with eIF4E, which includes intron-containing RNAs and resultant spliced product RNAs, their splice-site selection (or intron removal) is impacted not only by the altered landscape but also by their interactions with eIF4E, co-factors such as ELAVL1/HuR, and the spliceosome. We have now provided a new discussion and updated summary figure (Figure 7E) to better reflect this updated model.

We thank the reviewers for their efforts on this manuscript which have substantially improved it.

Thank you for submitting your revised manuscript. We have now received comments from both initial referees (please see below) and I am happy to say they now support publication. Referee #2 notes an issue regarding statistics and I would ask you to please provide this information for figure 2 in the final revised version. In addition, I would ask you to please address and answer the queries our data editors have raised (please see below for more detail). Please use the data edited document for any and all changes.

Referee #1:

The authors have adequately addressed the issues raised in my previous report and I am happy to recommend the paper for publication in EMBO Journal. I believe it will have a significant impact in the field.

Referee #2:

The authors have gone a long way to providing new data to respond to my previous comments/concerns. Aside from needing to provide statistical analysis (t-test) for data in Figure 2A , I feel that the manuscript is now acceptable for publication in EMBO J.

All editorial and formatting issues were resolved by the authors.

Thank you for sending the final revised version of your manuscript and the production files. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal.

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| If publicly available data were reused, provide the respective **data citations in the reference list**. | Yes | References, Results, and Materials and Methods |