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Dual function of UPF3B in early and late translation termination

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

1st Editorial Decision 04 May 2017

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees whose comments are shown below.

As you will see from the reports, our referees all highlight the importance and quality of your manuscript, although they also point to a number of technical points that need to be clarified before they can support publication in The EMBO Journal. This is particularly seen in the comments from ref #3 who is concerned that the reported role for Upf3b in ribosome release may be confounded by the experimental conditions of the in vitro translation assay. In our view, these are constructive comments that should be addressable and which will help strengthen the manuscript.

Given the referees' positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

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

REFEREE REPORTS

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Referee #1:

Despite three decades of research our understanding of the mechanism of NMD is still fragmented. By using an in vitro translation system fully reconstituted from purified components, Neu-Yilik and colleagues investigated whether UPF1 alone or together with UPF2 and/or UPF3B affected translation termination. This reconstituted translation system was originally developed in the Pestova lab (Alkalaeva et al.) and has been successfully used to investigate the transition from pre- to post-termination complexes. Here the assay was used by adding only limiting concentrations of eRF1 and eRF3a to mimic the situation of slowed translation termination, which is thought to trigger NMD according to current models. Interestingly, addition of UPF3B inhibited the eRF-dependent transition from the preTC to the postTC state, while UPF1 had no effect and UPF2 nullified the UPF3B effect when added together (Fig. 1). UPF3B's inhibitory effect on postTC formation was also unaffected by SMG1C (trimeric complex consisting of SMG1, SMG8 and SMG9). The authors then went on to show in vivo that UPF1 and UPF3B, but not UPF2, co-IP with eRF3a, both individually and together, suggesting that UPF3B might be part of the SURF complex (Fig. 2). Pulldown experiments using recombinant proteins confirmed that UPF3a interacts directly with eRF3a and eRF1 (Fig 3). In contrast to the prevailing view in the field that UPF3 and UPF1 only interact indirectly via UPF2, the pulldown experiments with the recombinant proteins indicate a hitherto undiscovered direct interaction between UPF1 and UPF3B in the absence of UPF2 (Fig. 4). The caveat is of course the high protein concentrations used for these pulldowns and it would therefore be interesting to determine the Kd values of this interaction to estimate how likely it is to occur in vivo. Using recombinant protein variants, the authors went on to characterize the interaction between UPF3B and eRF3a and could map this interaction to the N-terminal part of eRF3a and the middle domain of UPF3B (Fig. 5). This interaction was confirmed in vivo using co-IPs. Consistent with UPF3B's effect on translation termination, the authors document co-sedimentation of UPF3B and UPF1 with 80S ribosomes. By contrast UPF2 only co-sedimented with 80S ribosomes when incubated together with UPF3B but not alone. Finally, when the reconstituted translation termination assays were repeated with saturating amounts of eRFs, UPF3B no longer inhibited the preTC to postTC transition but instead now promoted the dissociation of postTCs (Fig. 6).
Based on their new findings, the authors postulate a modified NMD model in which UPF3B rather than UPF1 plays a central role in the mechanism of translation termination at PTCs. According to this model, which remains quite speculative over all, UPF3B first assists in the recruitment of the eRFs and later - after peptide release - contributes to dissociating of the ribosomal subunits. The model leaves open the question under which exact circumstances UPF3B would get involved in termination and when it is excluded. The authors should be more explicit on this point when describing their model.
Collectively, the findings presented here are novel and unexpected and hence represent an important advance towards elucidating the NMD mechanism. The data is of very high quality with each experiment being carefully controlled. The results and conclusions are therefore compelling and over-interpretations were avoided. Except for the few points below, the manuscript is clear and in fact was a pleasure to read. I am very much looking forward seeing this paper published.

Specific points to address:
- The statement on p. 2 that PTCs are contained in 5 - 15 % of normal transcripts needs a reference.
- Ottens & Gehring, 2016 (cited on p. 2) is missing in the bibliography
- P. 12: The statement "neither eRF1 nor eRF3a individually, nor the eRF1-eRF3a complex, detectably bound to UPF1" is not accurate, since Fig. 3B shows very weak yet clearly detectable bands for these two factors in the UPF1 pulldown. Similarly, a weak eRF1 band is also detectable in the UPF2L pulldown (Fig. 3C, lane 5). The same comment also applies for EV3 A and C.
- Fig. 3F, G; Fig. 4B: The size exclusion chromatography does not very well separate free UPF3B from its apparent complexes with eRFs. Did the authors try blue native gels? They might give a clearer answer.
- EV4B and p. 17: The text appears to refer to the wrong lane numbers. Please correct.

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Referee #2:

This is an excellent manuscript that uncovers a novel function for the NMD factor UPF3B on translation termination. Although NMD has been investigated for many years, the mechanism by which premature stop codons are recognized remains unclear. This manuscript investigates translation termination in an in vitro system supplemented with human NMD factors.

The authors observed that UPF3B delays translation termination and dissociates post-termination ribosomal complexes. Interestingly, UPF1 another key NMD factor does not directly participates in translation termination. The results presented in this manuscript are novel, unexpected and of great interest to the field of post-transcriptional mRNA regulation.

The manuscript is clearly written and the conclusions are well supported by experimental evidence.

The authors could comment more extensively on the possible role of UPF3A (for example how similar are UPF3A and UPF3B) and test whether UPF3A also interacts with eRF3a.

Minor comment

References in the text are in the wrong format.

Referee #3:

The manuscript by Neu-Yilik et al. analyses the effects of NMD factors in translation termination, as tested with an in vitro reconstituted termination system, previously established in the Pestova lab (Alkalaeva et al. 2006, Pisarev et al., 2007). Clear figures, clean results and a manuscript that contains a lot of information with many novel results. The main message of the manuscript is that UPF3B, one of the core NMD factors, is involved both in early and late stages of translation termination in human cells. These claims have alternative explanations in the framework of the very complex experimental system used, as explained in my questions for the authors. Thus, even if the manuscript brings a lot of new and important biochemical data on NMD factors, further experimental work is required before the main message becomes fully supported by the data.

In addition to the main message, important and original results are not mentioned in the abstract. An interaction between purified UPF3B and UPF1; thought previously to require UPF2 as a bridging molecule, as well as the dissection of the interaction between UPF3B fragments and eRF3, are important results. These interactions, as well as the lack of a direct link between UPF1 and eRF3 bring new and very interesting aspects to the mechanistic details of NMD.

In terms of impact, if the technical details around the translation termination system used can be solved, the manuscript challenges the current NMD models, brings interesting data on early aspects of NMD and suggests new links with translation termination. These results would reinforce and clarify previously suggested roles of NMD factors in ribosome dissociation from stop codons and their recycling as described using a yeast in vitro translation system (Amrani et al., 2004, Ghosh et al., 2010). Such results are timely even if they complicate the overall NMD picture, especially in light of the recent links between yeast UPF1 and ribosome dissociation at premature stop codons (Serdar et al., 2016). Moreover, this manuscript challenges current molecular mechanisms proposed for mammalian NMD, which can help future research in this area. Both for the biochemical data using recombinant proteins and co-immunoprecipitations and for the functional in vitro results, this manuscript will be of broad interest and could bring new light on NMD mechanism and translation termination mechanisms in eukaryotes.

<B>Questions for the authors and major issues:</B>

1. In the presence of limiting amounts of termination factors eRF1/eRF3a, UPF3B alone delayed the conformational change induced by the termination factors and GTP on a pre-termination complex. As explained in the text, pre-incubation with UPF3B led reproducibly to higher amounts of

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recovered pre-TC complexes, as seen by the amount of reverse transcription product on the reporter RNA (Fig. 1). If the amounts of pre-TC formed in the presence of UPF3B are larger than in its absence, the ratio between pre-TCs and eRF1/eRF3a changes. Since the termination factors are already limiting in the system used by the authors, an imbalance in the ratio between pre-TCs and termination factors could lead to a lower efficiency of termination that would reflect in the observed change in the pre-TC/post-TC toeprint levels. To be able to give an interpretation to the results, careful adjustment of eRF1/eRF3a levels in relation with the pre-TCs should be done and the results of such tests should be presented. In addition, it would be important to understand why the presence of UPF3B leads to higher amounts of pre-TCs. A sucrose gradient analysis, for example, of the complexes observed with or without UPF3B could bring important information about what happens in the system. Without clear data about how UPF3B can influence the translation termination system in indirect ways, no clear conclusion can be drawn from the experiments.

2. Figure 6 makes the case that UPF3B could affect ribosome dissociation from a post-termination complex. However, at 1 mM Mg²⁺, there is no obvious difference between the results obtained in the absence (lane 4, Fig 6C) and presence of UPF3B (lane 7, Fig 6C). The affirmation that "UPF3B also dissociates postTCs that have been generated in the absence of eRF3a (Fig 6B, lane 15)" does not take into account the fact that the primer extension profile in lane 15 is practically identical with the one seen in lane 7, in the absence of UPF3B. Thus, the conclusion of the last paragraph in the Results section that "UPF3B dissociates postTCs with flexible subunit association, possibly by accessing the ribosome subunit interface which is stabilized at higher Mg²⁺ concentrations", is not supported by the shown results and should not be presented this way in the revised version of the manuscript. It is thus important to either bring additional experimental evidence to be able to claim that UPF3B has an involvement in a late translation termination event or to present these data with a more moderate conclusion, in line with the results.

3. Many interesting protein-protein interactions are described in the manuscript. These data were obtained either with purified proteins or by co-immunoprecipitation of tagged proteins overexpressed in cultured cells. A surprising result, for example, is a direct interaction between UPF3B and UPF1. Since both these proteins can bind RNA non-specifically, and RNA traces can be present in purified fractions, it would be important to assess the RNA dependence of the presented interactions, or, at least of the ones that have not been previously published. The same RNA dependence should be tested for interactions that were tested by co-immunoprecipitation.

<B>Minor points.</B>

1. Since magnesium concentration or the order of addition of the different components in the reactions that test translation termination efficiency are critical, the exact conditions should be mentioned in the main text every time a result is presented.

2. The co-immunoprecipitation results are very interesting but some of the affirmations are not completely supported by the data presented in the figures. For example, the claim that "co-transfection of UPF2 strongly enhanced the ability of UPF1 to co-immunoprecipitate with eRF3a" is not entirely clear. It is hard to say that UPF2 increases the association of UPF1 with eRF3a because the level of UPF1 is higher in the input when UPF2 was co-expressed (Fig 2B). Such claims need further controls to be valid or should be discussed when presented.

3. Page 7, end of first section - the ATPase tests indicate that recombinant purified UPF1 is catalytically active and that the UPF1L variant has higher ATPase activity in the presence of both UPF2L and UPF3B. Thus the correct term would be "catalytically active" instead of "biologically active", since the term "biological activity" extends beyond a reconstituted enzymatic effect, specifically to the ability of a recombinant form to complement the absence of the corresponding gene in vivo. The "slight" enhancement of activity, presented in Fig. EV1E, does not need to be mentioned, as it will most likely confuse the reader.

4. The authors present a beautiful demonstration of the interaction between the middle domain of UPF3B and the N-terminus of eRF3, a region that is not required for translation termination. Strangely, the absence of the interaction did not affect the previously seen effects of UPF3B on toeprints in the in vitro translation system. This discrepancy needs to be discussed.

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5. Figure EV4C shows the independent binding of UPF1 and UPF3B to 80S ribosomes, and binding of UPF2 enhanced by UPF3B. This results is consistent with the idea that UPF3B could bind to RNA, maybe not specifically, and bring UPF2L along. This supplementary figure could be promoted to the main text as the result is important.

1st Revision - authors' response 27 June 2017

Point-by-point Response to the Referees' Comments

Referee #1:
Despite three decades of research our understanding of the mechanism of NMD is still fragmented. By using an in vitro translation system fully reconstituted from purified components, Neu-Yilik and colleagues investigated whether UPF1 alone or together with UPF2 and/or UPF3B affected translation termination. This reconstituted translation system was originally developed in the Pestova lab (Alkalaeva et al.) and has been successfully used to investigate the transition from pre- to post-termination complexes. Here the assay was used by adding only limiting concentrations of eRF1 and eRF3a to mimic the situation of slowed translation termination, which is thought to trigger NMD according to current models. Interestingly, addition of UPF3B inhibited the eRF-dependent transition from the preTC to the postTC state, while UPF1 had no effect and UPF2 nullified the UPF3B effect when added together (Fig. 1). UPF3B's inhibitory effect on postTC formation was also unaffected by SMG1C (trimeric complex consisting of SMG1, SMG8 and SMG9). The authors then went on to show in vivo that UPF1 and UPF3B, but not UPF2, co-IP with eRF3a, both individually and together, suggesting that UPF3B might be part of the SURF complex (Fig. 2). Pulldown experiments using recombinant proteins confirmed that UPF3a interacts directly with eRF3a and eRF1 (Fig 3). In contrast to the prevailing view in the field that UPF3 and UPF1 only interact indirectly via UPF2, the pulldown experiments with the recombinant proteins indicate a hitherto undiscovered direct interaction between UPF1 and UPF3B in the absence of UPF2 (Fig. 4). The caveat is of course the high protein concentrations used for these pulldowns and it would therefore be interesting to determine the Kd values of this interaction to estimate how likely it is to occur in vivo. Using recombinant protein variants, the authors went on to characterize the interaction between UPF3B and eRF3a and could map this interaction to the N-terminal part of eRF3a and the middle domain of UPF3B (Fig. 5). This interaction was confirmed in vivo using co-IPs. Consistent with UPF3B's effect on translation termination, the authors document co-sedimentation of UPF3B and UPF1 with 80S ribosomes. By contrast UPF2 only co-sedimented with 80S ribosomes when incubated together with UPF3B but not alone. Finally, when the reconstituted translation termination assays were repeated with saturating amounts of eRFs, UPF3B no longer inhibited the preTC to postTC transition but instead now promoted the dissociation of postTCs (Fig. 6).
Based on their new findings, the authors postulate a modified NMD model in which UPF3B rather than UPF1 plays a central role in the mechanism of translation termination at PTCs. According to this model, which remains quite speculative over all, UPF3B first assists in the recruitment of the eRFs and later - after peptide release - contributes to dissociating of the ribosomal subunits. The model leaves open the question under which exact circumstances UPF3B would get involved in termination and when it is excluded. The authors should be more explicit on this point when describing their model.
Collectively, the findings presented here are novel and unexpected and hence represent an important advance towards elucidating the NMD mechanism. The data is of very high quality with each experiment being carefully controlled. The results and conclusions are therefore compelling and over-interpretations were avoided. Except for the few points below, the manuscript is clear and in fact was a pleasure to read. I am very much looking forward seeing this paper published.

We thank the referee for his/her very positive assessment of our work and the very helpful suggestions to improve the manuscript.

The referee suggests to define more explicitly under which circumstances UPF3B gets involved in termination. In fact, in the previous version of the manuscript we have elaborated on this point in the paragraph before we described our suggested model in detail. In order to include our vision as to when and how UPF3B gets involved in termination in vivo we have now integrated the 2nd

paragraph on p. 23 with the third. The new paragraph now explains the model of when UPF3B does or does not interact with the ribosome. We thus include our suggestion that UPF3B may bind to the terminating ribosome in the absence of PABPC1 in the description of our model.

The paragraph now reads:
Because UPF3B interferes with translation termination we assign a central role to UPF3B in a modified model for NMD. Mechanistically, we suggest that at a PTC in the absence of PABPC1 either EJC-bound or free UPF3B binds to the terminating ribosome, interacts with the release factors and then delays termination by sterically impeding stop codon recognition and peptide release by eRF1. This hypothesis also provides a mechanistic rationale for the NMD-enhancing effect of EJCs, which may increase the local concentration of UPF3B at the premature termination site.

Specific points to address:
- The statement on p. 2 that PTCs are contained in 5 - 15 % of normal transcripts needs a reference.

We have now added several references to this statement on p. 2:
PTCs can be introduced into mRNAs by mutations, transcriptional errors, and aberrant splicing, but are also contained in 5 -15 % of normal transcripts (Karousis et al, 2016, Mendell et al, 2004, Nguyen et al, 2014).

- Ottens & Gehring, 2016 (cited on p. 2) is missing in the bibliography

This reference is now included in the bibliography.

- P. 12: The statement "neither eRF1 nor eRF3a individually, nor the eRF1-eRF3a complex, detectably bound to UPF1" is not accurate, since Fig. 3B shows very weak yet clearly detectable bands for these two factors in the UPF1 pulldown. Similarly, a weak eRF1 band is also detectable in the UPF2L pulldown (Fig. 3C, lane 5). The same comment also applies for EV3 A and C.

We thank the referee for pointing out that we need to clarify the description of the experiment and our conclusions. In all cases mentioned by the referee the apparent binding of UPF1 or UPF2L to the release factors is not above background binding of the untagged proteins to the affinity matrix. For instance, please compare Fig 3B, lanes 1, 2, and 4 where a low binding of eRF1 and eRF3a to the Ni-NTA resin occurs also in the absence of His-UPF1. To facilitate the interpretation of the respective experiments we have now changed the text on p. 12/13 in the revised manuscript as follows:

We found that neither eRF1 nor eRF3a individually, nor the eRF1-eRF3a complex, bound to UPF1 (Fig 3B, lanes 5-7), or to UPF2L (Fig 3C, lanes 5-7) above background (Fig 3B, C, lanes 1, 2, 4, Fig EV3A, C lanes 3).

In addition, we now explain this fact in the legends to Figure 3 on p. 34:
B-D: Bands in lanes 1, 2, and 4 (eluate panels) represent background binding of the untagged eRFs to the Ni-NTA resin.

and to Fig EV3 on p. 39:
Bands in lanes 1, 2, and 5 of A-C (eluate panels) represent background binding of untagged proteins to the Ni-NTA resin.

- Fig. 3F, G; Fig. 4B: The size exclusion chromatography does not very well separate free UPF3B from its apparent complexes with eRFs. Did the authors try blue native gels? They might give a clearer answer.

We tried native gels and blue native gels but these efforts did not result in a clear and interpretable separation of the eRF-UPF3B complexes versus free UPF3B and eRF1 or eRF3a.

- EV4B and p. 17: The text appears to refer to the wrong lane numbers. Please correct.

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We thank the referee for pointing this out. Following the suggestion of referee #3, we have promoted Fig EV4 to the main text. The previous Fig EV4 therefore is now Fig 6 in the revised manuscript. We have changed the description of Fig 6B (previous Fig EV4B) on p. 17 to better explain that only lanes 3-12 in Fig 6B directly correspond to Fig 6C. The text now reads:

To explore the ability of UPF3B, UPF1, and UPF2L to interact with ribosomes we performed co-sedimentation assays (Fig 6C). Centrifugation without ribosomes served as controls (Fig 6B, lanes 3-12).

We have also re-aligned the lane numbers under Fig 6B (previous Fig EV4B) and changed the legend to Fig 6B (p. 36) in the following way:

B Sucrose cushion co-sedimentation analysis of either ribosomes (lanes 1, 2) or of UPF1, UPF2L, or UPF3B or of combinations as indicated. After ultracentrifugation, the supernatant (S) and pellet (P) fractions were analysed by SDS-PAGE.

Referee #2:
This is an excellent manuscript that uncovers a novel function for the NMD factor UPF3B on translation termination. Although NMD has been investigated for many years, the mechanism by which premature stop codons are recognized remains unclear. This manuscript investigates translation termination in an in vitro system supplemented with human NMD factors.
The authors observed that UPF3B delays translation termination and dissociates post-termination ribosomal complexes. Interestingly, UPF1 another key NMD factor does not directly participates in translation termination. The results presented in this manuscript are novel, unexpected and of great interest to the field of post-transcriptional mRNA regulation.
The manuscript is clearly written and the conclusions are well supported by experimental evidence.

We thank this referee for his/her very positive evaluation of our manuscript and the constructive suggestions.

The authors could comment more extensively on the possible role of UPF3A (for example how similar are UPF3A and UPF3B) and test whether UPF3A also interacts with eRF3a.

As requested, we have now added information regarding the similarity of and differences between UPF3A and UPF3B on p. 25 of the revised manuscript:

This fundamental functional difference appears to root in the impairment of UPF3A's EJC binding domain (EBD). Swapping of UPF3A's weak EBD with UPF3B's strong EBD converts UPF3A into an NMD activator and UPF3B into an NMD suppressor (Shum et al., 2016). However, the EBD plays no role in the dual function of UPF3B described here. Interestingly, UPF3A and UPF3B not only differ in their NMD activity but also in their ability to stimulate translation (Kunz et al., 2006), a function that may be related to translation termination and ribosome dissociation at a PTC.

We agree with this referee that a detailed analysis of UPF3A would be an interesting addition to our data. However, to conform with the context of our present study this would require time-consuming synthesis of the protein and multiple downstream experiments including testing UPF3A's interaction with the other UPF proteins and the eRFs and investigating its role in the elaborate in vitro translation termination assays. Therefore, this addition is beyond the scope of this review and will be subject of subsequent study.

Minor comment

References in the text are in the wrong format.

The reference format has now been corrected.

Referee #3:
The manuscript by Neu-Yilik et al. analyses the effects of NMD factors in translation termination, as tested with an in vitro reconstituted termination system, previously established in the Pestova lab

(Alkalaeva et al. 2006, Pisarev et al., 2007). Clear figures, clean results and a manuscript that contains a lot of information with many novel results. The main message of the manuscript is that UPF3B, one of the core NMD factors, is involved both in early and late stages of translation termination in human cells. These claims have alternative explanations in the framework of the very complex experimental system used, as explained in my questions for the authors. Thus, even if the manuscript brings a lot of new and important biochemical data on NMD factors, further experimental work is required before the main message becomes fully supported by the data.

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In terms of impact, if the technical details around the translation termination system used can be solved, the manuscript challenges the current NMD models, brings interesting data on early aspects of NMD and suggests new links with translation termination. These results would reinforce and clarify previously suggested roles of NMD factors in ribosome dissociation from stop codons and their recycling as described using a yeast in vitro translation system (Amrani et al., 2004, Ghosh et al., 2010). Such results are timely even if they complicate the overall NMD picture, especially in light of the recent links between yeast UPF1 and ribosome dissociation at premature stop codons (Serdar et al., 2016). Moreover, this manuscript challenges current molecular mechanisms proposed for mammalian NMD, which can help future research in this area. Both for the biochemical data using recombinant proteins and co-immunoprecipitations and for the functional in vitro results, this manuscript will be of broad interest and could bring new light on NMD mechanism and translation termination mechanisms in eukaryotes.

We very much appreciate this referee's overall positive evaluation of our work and the helpful suggestions. We have addressed all major and minor issues and questions in the revised version of our manuscript, leading to an improved manuscript.

As suggested, we have now included the information that UPF3B interacts with release factors, with UPF1 and with ribosomes in a revised version of the abstract which now reads:

Nonsense-mediated mRNA decay (NMD) is a cellular surveillance pathway that recognizes and degrades mRNAs with premature termination codons (PTCs). The mechanisms underlying translation termination are key to the understanding of RNA surveillance mechanisms such as NMD and crucial for the development of therapeutic strategies for NMD-related diseases. Here, we have used a fully reconstituted in vitro translation system to probe the NMD proteins for interaction with the termination apparatus. We discovered that UPF3B (1) interacts with the release factors, (2) delays translation termination, and (3) dissociates post-termination ribosomal complexes that are devoid of the nascent peptide. Furthermore, we identified UPF1 and ribosomes as new interaction partners of UPF3B. These previously unknown functions of UPF3B during the early and late phases of translation termination suggest that UPF3B is involved in the crosstalk between the NMD machinery and the PTC-bound ribosome, a central mechanistic step of RNA surveillance.

Questions for the authors and major issues:

1. In the presence of limiting amounts of termination factors eRF1/eRF3a, UPF3B alone delayed the conformational change induced by the termination factors and GTP on a pre-termination complex. As explained in the text, pre-incubation with UPF3B led reproducibly to higher amounts of recovered pre-TC complexes, as seen by the amount of reverse transcription product on the reporter RNA (Fig. 1). If the amounts of pre-TC formed in the presence of UPF3B are larger than in its absence, the ratio between pre-TCs and eRF1/eRF3a changes. Since the termination factors are already limiting in the system used by the authors, an imbalance in the ratio between pre-TCs and termination factors could lead to a lower efficiency of termination that would reflect in the observed change in the pre-TC/post-TC toeprint levels. To be able to give an interpretation to the results, careful adjustment of eRF1/eRF3a levels in relation with the pre-TCs should be done and the results of such tests should be presented. In addition, it would be important to understand why the presence of UPF3B leads to higher amounts of pre-TCs. A sucrose gradient analysis, for example, of the

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complexes observed with or without UPF3B could bring important information about what happens in the system. Without clear data about how UPF3B can influence the translation termination system in indirect ways, no clear conclusion can be drawn from the experiments.

We thank the referee for this comment. To clarify this important point, we have reformulated the methodological description of the toeprinting experiments, pointing out that (1) preTCs were not made during the termination reaction but preformed on the MVHC-STOP mRNA and subsequently purified using sucrose density gradients. (2) Identical amounts of purified preTCs were added to each translation termination reaction. Importantly, the final concentration of pre-TCs and eRFs included into the termination reactions are identical in all reactions composing a figure regardless, whether they contain UPF3B or not. The addition of UPF3B (or any of the other tested proteins) can therefore not change the ratio between preTCs and eRFs or the concentration of these factors in the assays.
In order to avoid this technical ambiguity, we have changed the text on p. 8 in the revised version of the manuscript which now reads:

To test if UPF proteins affect the efficiency of translation termination, equal amounts of preTCs that had been assembled on MVHC-STOP mRNA and purified by sucrose density-gradient centrifugation were incubated with UPF1, UPF2L, UPF3B or combinations of these proteins as indicated (Fig 1B, lanes 3-9).

And on p. 9 of the revised manuscript:

We found that here, too, all toeprint signals were stronger than in the presence of UPF3B (Fig 1B, lanes 1 and 10), which is likely caused by more efficient recovery of ribosomal complexes and RNA in the reverse transcription reaction and the subsequent purification steps following the in vitro translation termination reaction.

We have also changed a sentence in the section in Supplementary Materials and Methods describing the in vitro termination assay which now reads:

Briefly, 0.1 pmol of preTCs that had been assembled on MVHC-STOP mRNA and purified by sucrose density gradient-centrifugation as described under "Pre-termination complex assembly and purification" were incubated for 10 minutes at 37°C with 3 pmol of UPF proteins or BSA in a total volume of 35 µL translation buffer E (10 mM Tris pH 7.5, 100 mM KCl, 1 mM free MgCl2, 2 mM DTT, 0.25 mM spermidine) supplemented with 0.5 mM GTP and 1 mM ATP or AMPPNP as indicated.

2. Figure 6 makes the case that UPF3B could affect ribosome dissociation from a post-termination complex. However, at 1 mM Mg^{2+}, there is no obvious difference between the results obtained in the absence (lane 4, Fig 6C) and presence of UPF3B (lane 7, Fig 6C). The affirmation that "UPF3B also dissociates postTCs that have been generated in the absence of eRF3a (Fig 6B, lane 15)" does not take into account the fact that the primer extension profile in lane 15 is practically identical with the one seen in lane 7, in the absence of UPF3B. Thus, the conclusion of the last paragraph in the Results section that "UPF3B dissociates postTCs with flexible subunit association, possibly by accessing the ribosome subunit interface which is stabilized at higher Mg^{2+} concentrations", is not supported by the shown results and should not be presented this way in the revised version of the manuscript. It is thus important to either bring additional experimental evidence to be able to claim that UPF3B has an involvement in a late translation termination event or to present these data with a more moderate conclusion, in line with the results.

Figure 7C in the revised manuscript (previous Fig 6C) illustrates that in the presence of higher Mg^{2+} concentrations UPF3B cannot dissociate postTCs. Therefore, we have to compare pairwise (lanes 7/10, lanes 8/11, lanes 9/12) the signals generated in the presence of UPF3B and either in the presence or absence of eRFs at the various Mg^{2+} concentrations. Moreover, we have to take the signal-enhancing effect of UPF3B into consideration. To avoid the misleading impression that there is no major difference between lanes 4 and 7, we have now reassembled Fig 7C by using for lanes 1-6 a two-times longer exposure than for lanes 7-12. This leads to a more even overall signal intensity between all lanes. With this "normalization", the difference in the primer extension profile

of the postTCs between lane 4 and lane 7 becomes more evident. We have changed the text on p. 20 of the revised manuscript accordingly:
Importantly, we found that at 2.5 or 5 mM Mg^{2+} no postTC dissociation occurred in the presence of equimolar amounts of the eRFs and UPF3B (compare lanes 7/10, 8/11, and 9/12).

Furthermore, we have adjusted the legend to Fig 7C (previously Fig 6C) on p. 36, indicating the longer exposure of lanes 1-6.

More subtle differences between the primer extension profiles are sometimes hard to evaluate in the small scale scans and reproductions of the autoradiographs of large sequencing-type gels. To better support our statement that "UPF3B also dissociates postTCs that have been generated in the absence of eRF3a (Fig 7B, lane 15)", we have now added enlargements of the pre-/postTC profiles of the critical lanes (lanes 6-8 and lanes 14-16) to Figure 7B (previous Fig 6B). We are confident that these enlarged inserts of the new panel 7B visualize more clearly that the postTC signals in lane 7 (generated without UPF3B) are decidedly stronger than those in lane 15 (generated in the presence of UPF3B). We interpret the loss of postTCs that have been generated in the absence of eRF3a but in the presence of UPF3B to be due to the postTC-dissociating activity of UPF3B.

We have changed the text describing Fig 7B (previous Fig 6B) on p. 20 as follows:

Notably, UPF3B was unable to dissociate preTCs in the absence of eRFs (Fig 7B, lane 9) or residual preTCs in termination reactions that were incubated with eRF1 or puromycin alone (lanes 15, 16 in main panel and enlargement). These data indicate that UPF3B dissociates postTCs after both GTP and peptidyl-tRNA hydrolysis, but not preTCs or postTCs before peptide hydrolysis. UPF3B also dissociates postTCs that have been generated in the absence of eRF3a (Fig 7B, compare lanes 7 and 15 in main panel and enlargement). Therefore, the eRF3a-UPF3B interaction is not required for the function of UPF3B in ribosome dissociation.

Likewise, we have changed the legend to Fig 7B on p. 36 as follows:

B Toeprinting analysis of ribosomal complexes obtained by incubating preTCs as in (A) with UPF3B or BSA and combinations of eRF1, eRF1AGQ, eRF3a, and puromycin in the presence of GTP or GMPPNP. Pre/postTC profiles of lanes 6-8 and 11-14 are enlarged to allow a better assessment. The gel on the left was exposed 2x longer than gel on the right. Note that puromycin-treated preTCs are relatively unstable at the low Mg^{2+} concentrations used (Skabkin et al., 2013).

3. Many interesting protein-protein interactions are described in the manuscript. These data were obtained either with purified proteins or by co-immunoprecipitation of tagged proteins overexpressed in cultured cells. A surprising result, for example, is a direct interaction between UPF3B and UPF1. Since both these proteins can bind RNA non-specifically, and RNA traces can be present in purified fractions, it would be important to assess the RNA dependence of the presented interactions, or, at least of the ones that have not been previously published. The same RNA dependence should be tested for interactions that were tested by co-immunoprecipitation.

We thank the referee for this valid point.

The co-immunprecipitations shown in Figures 2 and 4 have been performed after digestion of the cell lysates with 30-40 µg/ml RNase A. The shown interactions thus are independent of RNA. We have modified the respective sentence on p. 11/12 in the following way to more clearly express this fact:

Immunoprecipitations on FLAG-antibody beads were performed in the presence of RNase A to ensure that interactions between the eRFs and the UPF proteins were not mediated by mRNA.

And on p. 16:

To examine if the interaction between UPF3B and N-terminally truncated eRF3a was also impaired in vivo, we transiently co-transfected HeLa cells with plasmids encoding a FLAG-tagged version of eRF3a lacking the first 199 aa (FLAG-eRF3aD199) and with V5-eRF1 or V5- UPF1, -UPF2, or -

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UPF3B, either individually or simultaneously (Fig 5F) and immunoprecipitated on FLAG-antibody beads after digestion of the lysates with RNase A

All our preparations of recombinant proteins were examined for contaminations of nucleic acids. Only RNA-free batches as juged by their OD260nm/280nm ratio of ~0.5 were admitted to the pulldown experiments presented in this study. We have now added this information to the Supplementary Materials and Methods section as follows:

As judged by a 260/280 ratio of ~ 0.5 all recombinant proteins were free of nucleic acid contaminations.

In addition we have specified this fact for UPF1 and UPF3B on p.15:

Both protein preparations were virtually RNA-free as indicated by their OD260nm/280nm ratios of ~0.5 (Raynal et al, 2014). Therefore, the interaction between UPF1 and UPF3B is not mediated by RNA contaminations of the recombinant proteins.

Moreover, as suggested by the referee, we have investigated whether the interaction of UPF1 and UPF3B is affected by the presence of RNA by incubating UPF1 with UPF3B and a 24 nt RNA oligomer and analysing complex formation by Size Exclusion Chromatography (SEC) (new Figure EV4). Since both UPF1 and UPF3B can bind RNA, we have added the RNA oligomer in threefold excess.

We show that UPF1 and UPF3B can form a complex in the presence of RNA. SEC is a non-equilibrium method, and we observe only a small signal corresponding to RNA in the UPF1-UPF3B peak fraction (Figure EV4B). The strong tailing of this peak suggests that the RNA oligomer dissociates from the UPF1-3 complex during the experiment. We have included this information in the main text on p. 15 of the revised manuscript as follows:

To investigate whether RNA interferes with the formation of the UPF1-UPF3B complex, we incubated UPF3B, or UPF1 and UPF3B with a threefold excess of a 24 nt RNA oligomer which is too small for a concomitant binding of both proteins (Fig EV4). In SEC analysis, the UPF3B-UPF1 complex eluted earlier than UPF1 or UPF3B from the SEC column (1.18 mL versus 1.31 or 1.3 mL, respectively) indicating that the oligomer did not compete with UPF1 for UPF3B binding or vice versa (Fig EV4A). The OD260nm/280nm ratio of 0.76 in the UPF1-UPF3B complex peak and the tailing of the peak (Fig EV4B, 3rd panel) suggest that a UPF1-UPF3B-RNA complex could transiently form since the complex partly dissociates during the SEC experiment.
The legend to the new Figure EV4 reads:

Figure EV4 UPF1-UPF3B complex formation is not prevented by RNA
A SEC elution profile of UPF3B (green), UPF3B and RNA (orange) or UPF3B, UPF1 and three-fold excess of RNA (blue). Below: SDS-PAGE analysis of eluate fractions.
B Analysis of SEC peak fractions. Peaks representing UPF3B (green) and UPF1 (purple) elute with an OD 260nm/280nm ratio of 0.54 and 0.51, respectively, whereas the RNA oligomer (red) elutes with an OD 260nm/280nm ratio of 2.0. The peak containing the UPF3B-UPF1 complex after incubation of UPF1, UPF3B, and RNA (blue) has a higher OD 260nm/280nm ratio of 0.76 due to the presence of RNA in this peak.

Minor points.

1. Since magnesium concentration or the order of addition of the different components in the reactions that test translation termination efficiency are critical, the exact conditions should be mentioned in the main text every time a result is presented.

To respect the length restrictions of the main text, we have now added the magnesium concentrations and order of addition of components to each legend of figures depicting translation termination experiments.

2. The co-immunoprecipitation results are very interesting but some of the affirmations are not completely supported by the data presented in the figures. For example, the claim that "co-

transfection of UPF2 strongly enhanced the ability of UPF1 to co-immunoprecipitate with eRF3a" is not entirely clear. It is hard to say that UPF2 increases the association of UPF1 with eRF3a because the level of UPF1 is higher in the input when UPF2 was co-expressed (Fig 2B). Such claims need further controls to be valid or should be discussed when presented.

We thank the referee for pointing this out. We have changed the part of the description of Fig 2 on p. 12 that deals with UPF2 in the eRF3a-coIP experiment which now reads:

Surprisingly, UPF2 could not be detected in these complexes as well as in complexes containing eRF3a and UPF3B (lanes 6, 8 and 9). We conclude that UPF2 does not partake in complexes containing eRF3a together with UPF1, UPF3B, or both.

Moreover we have deleted the following sentence in the Discussion on p. 23 of the previous version:

As our eRF3a co-IP experiments indicate (Fig 2B), UPF2 could assist with this interaction, but is not itself part of a complex that contains, both, UPF1 and the terminating ribosome.

3. Page 7, end of first section - the ATPase tests indicate that recombinant purified UPF1 is catalytically active and that the UPF1L variant has higher ATPase activity in the presence of both UPF2L and UPF3B. Thus the correct term would be "catalytically active" instead of "biologically active", since the term "biological activity" extends beyond a reconstituted enzymatic effect, specifically to the ability of a recombinant form to complement the absence of the corresponding gene in vivo. The "slight" enhancement of activity, presented in Fig. EV1E, does not need to be mentioned, as it will most likely confuse the reader.

As suggested by the referee, we have changed the terms "biological activity" and "biologically active" to "catalytical activity" and catalytically active", respectively, on p. 7 of the revised manuscript. We have also deleted the sentence referring to a "slight enhancement of activity" in the legend to Fig EV1E in the revised version of the manuscript.

4. The authors present a beautiful demonstration of the interaction between the middle domain of UPF3B and the N-terminus of eRF3, a region that is not required for translation termination. Strangely, the absence of the interaction did not affect the previously seen effects of UPF3B on toeprints in the in vitro translation system. This discrepancy needs to be discussed.

We thank the referee for this comment and have tried to better explain why we believe that the in vitro termination system is unable to mirror a possible role of UPF3B in the recruitment of eRF3a to the termination site and why we therefore also do not see a difference in the toeprints with eRF3a vs eRF3aDN. We have modified the text on p. 17 accordingly:

We reasoned that the eRF3a(DN)-eRF1 interaction is much stronger than the eRF3a-UPF3B interaction. UPF3B's potential role in eRF3a recruitment or its inability to recruit eRF3aDN could thus be bypassed by eRF1 and therefore cannot be mirrored by the in vitro translation system. Moreover, the effect of UPF3B in delaying translation termination may also involve direct binding to the ribosome, thus interfering with efficient stop codon recognition by the release factors.

5. Figure EV4C shows the independent binding of UPF1 and UPF3B to 80S ribosomes, and binding of UPF2 enhanced by UPF3B. This result is consistent with the idea that UPF3B could bind to RNA, maybe not specifically, and bring UPF2L along. This supplementary figure could be promoted to the main text as the result is important

As suggested by the referee, we have now moved this Figure as Fig 6 to the main text in the revised version of the manuscript. Consequently, the previous Fig 6, and Fig 7 have been re-designated Fig 7 and Fig 8, respectively.

2nd Editorial Decision 14 July 2017

Thank you for submitting a revised version of your manuscript to The EMBO Journal. It has now been seen by two of the original referees whose comments are shown below. As you will see they

both find that all criticisms have been sufficiently addressed and recommend the manuscript for publication. However, before we can go on to officially accept the manuscript there are a few editorial issues concerning text and figures that I need you to address in a final revision:

-> We generally require that all information relevant to the main experiments in the manuscript should be included in Materials and Methods. Since most of that information is currently present in the Appendix File I would encourage you to move as much as possible into the main manuscript (or at least the following sections: Plasmids, Protein production and purification, Pulldown assays to probe protein-protein interaction using purified proteins, In vitro translation and toe-printing analysis of pre- and post-termination complexes, Peptide release assays). We do not have a fixed character limit in The EMBO Journal so you do not have to shorten any other sections of the manuscript to fit in this additional M&M content.

-> In case you decide to leave some of this information in the Appendix I would ask you to include a brief Table of Content overview on the first page of the Appendix file and to also add the word 'appendix' in front of the sections: Supplementary Materials and Methods and Supplementary references.

-> Please make sure that any discontinuity in the gels depicted is clearly marked with a line/frame (ie when running two different toe-print assays with a shared sequencing lanes in EV figs 2 and 5).

-> In addition, we generally encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.
For this point it would be helpful if you could also upload the source data files you have already sent to us, since they need to be provided as one pdf file per full figure.

-> Based on our email discussion about the Upf3b lanes in fig 3F and 4B, I would like to remind you to clarify to the reader how these experiments were done and why the same Upf3b lane is used in both figures.

-> Papers published in The EMBO Journal include a 'Synopsis' to further enhance discoverability. Synopses are displayed on the html version of the paper and are freely accessible to all readers. The synopsis includes a short standfirst - written by the handling editor - as well as 2-5 one sentence bullet points that summarise the paper and are provided by the authors. I would therefore ask you to include your suggestions for bullet points.

-> In addition, I would encourage you to provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels.

Thank you again for giving us the chance to consider your manuscript for The EMBO Journal, I look forward to receiving your final revision.

REFEREE REPORTS

Referee #1:

The authors have addressed my initial comments - which were few - satisfactorily in the revised version of the manuscript. I think this is going to be an important paper for the NMD field and I am looking forward seeing it published.

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Referee #3:

The revised version of the manuscript clarifies the points that were of concern. In view of the very interesting set of experiments and of the exciting conclusions about the links between Upf3, NMD and release factors, translation termination and NMD, I wholeheartedly recommend the publication of this paper in the EMBO Journal.

2nd Revision - authors' response 07 August 2017

Point-by-Point response to editor's requests

1. We generally require that all information relevant to the main experiments in the manuscript should be included in Materials and Methods. Since most of that information is currently present in the Appendix File I would encourage you to move as much as possible into the main manuscript (or at least the following sections: Plasmids, Protein production and purification, Pulldown assays to probe protein-protein interaction using purified proteins, In vitro translation and toe-printing analysis of pre- and post-termination complexes, Peptide release assays). We do not have a fixed character limit in The EMBO Journal so you do not have to shorten any other sections of the manuscript to fit in this additional M&M content.

We have now moved all information on materials and methods as well as the Supplementary References to the Materials & Methods section of the main manuscript.

2. In case you decide to leave some of this information in the Appendix I would ask you to include a brief Table of Content overview on the first page of the Appendix file and to also add the word 'appendix' in front of the sections: Supplementary Materials and Methods and Supplementary references.

We have deleted the Appendix file.

3. Please make sure that any discontinuity in the gels depicted is clearly marked with a line/frame (ie when running two different toe-print assays with a shared sequencing lanes in EV figs 2 and 5).

We have now framed all discontinuties in gel pictures (Figures 7, EV2, and EV5)
We have deleted one discontinuity in Figure 2B (anti-FLAG panel of the input gels). This discontinuity did not exist in the source scan (compare source data for Figure 2).

4. In addition, we generally encourage the publication of source data, particularly for electrophoretic gels and blots, with the aim of making primary data more accessible and transparent to the reader. We would need 1 file per figure (which can be a composite of source data from several panels) in jpg, gif or PDF format, uploaded as "Source data files". The gels should be labelled with the appropriate figure/panel number, and should have molecular weight markers; further annotation would clearly be useful but is not essential. These files will be published online with the article as a supplementary "Source Data". Please let me know if you have any questions about this policy.
For this point it would be helpful if you could also upload the source data files you have already sent to us, since they need to be provided as one pdf file per full figure.

We have created source data-files for all Figures and EV-Figures.

5. Based on our email discussion about the Upf3b lanes in fig 3F and 4B, I would like to remind you to clarify to the reader how these experiments were done and why the same Upf3b lane is used in both figures.

To state this fact we have changed the legend to Figure 4B which now reads (p 41):
B Left: SEC elution profile of UPF1 (purple), UPF3B (green) or both (orange). Right: SDS-PAGE analysis of eluate fractions. Representative of 2 independent experiments. Since the experiments described in Figures 3F and 4B were performed in parallel, the same UPF3B SEC elution profile

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(green) and the corresponding SDS-PAGE analysis served as control for both experiments. More UPF3B SEC experiments are depicted in Figures 3G, 6B and EV3E.

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In addition, I would encourage you to provide an image for the synopsis. This image should provide a rapid overview of the question addressed in the study but still needs to be kept fairly modest since the image size cannot exceed 550x400 pixels.

A suggestion for both synopsis figure and bullet points are provided together with the final version of the manuscript.

By comparison with the original scans, we have noticed that in Figure 1B and in Figure EV4 the MW-markers were not at the correct positions. We have now corrected these mistakes in the final version of these figures. Moreover, we have adjusted a few slight mislocations of lane numbers or arrows in the final versions of several figures.

3rd Editorial Decision 10 August 2017

Thank you for submitting the final version of your manuscript, I am pleased to inform you that your study has now been accepted for publication in The EMBO Journal.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

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Andreas E. Kulozik
EMBO Journal
EMBOJ-2017-97079

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
- graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
- if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
- Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions

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

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
  - common tests, such as t-test (please specify whether paired vs. unpaired), simple $\chi 2$ tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
  - are tests one-sided or two-sided?
  - are there adjustments for multiple comparisons?
  - exact statistical test results, e.g., P values = x but not P values < x;
  - definition of 'center values' as median or average;
  - definition of error bars as s.d. or s.e.m.

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

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

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

B- Statistics and general methods

Please fill out these boxes ↓ (Do not worry if you cannot see all your text once you press return)

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Experiments were performed 2 - 5 times as specified in the Figure Legends
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	NA
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Samples that were technically abortive (like failed gel runs) were excluded
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	Different batches of proteins and pre-termination complexes were used when repeating the experiments
For animal studies, include a statement about randomization even if no randomization was used.	NA
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	No investigator blinding was applied
4.b. For animal studies, include a statement about blinding even if no blinding was done	NA
5. For every figure, are statistical tests justified as appropriate?	Fig 1C: the test is described in the figure legend
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
Is there an estimate of variation within each group of data?	NA
Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

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http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tun

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

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6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	Antibody catalog numbers are provided in Supplementary Materials and Methods
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	The cells used for the co-IP experiments were a Hela cell strain that is used in our lab for more than 20 years.

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D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	NA
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	NA
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	NA

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	NA
17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	does not apply

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	does not apply. Protein and RNA constructs used are described in Supplementary Material and Methods.
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	NA
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	NA
21. As far as possible, primary and referenced data should be formally cited in a Data Availability section. Please state whether you have included this section. Examples: **Primary Data** Wetmore KM, Deutschbauer AM, Price MN, Arkin AP (2012). Comparison of gene expression and mutant fitness in Shewanella oneidensis MR-1. Gene Expression Omnibus GSE39462 **Referenced Data** Huang J, Brown AF, Lei M (2012). Crystal structure of the TRBD domain of TERT and the CR4/5 of TR. Protein Data Bank 4O26 AP-MS analysis of human histone deacetylase interactions in CEM-T cells (2013). PRIDE PXD000208	NA
22. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	NA

G- Dual use research of concern

23. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	No