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Nonsense-mediated mRNA Decay Involves Two Distinct Upf1-bound Complexes

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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 24th March 2018

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, the referees all recognise the value in providing a careful, quantitative analysis of Upf-interaction partners but they also all agree that further functional validation is needed before they can support publication of your manuscript here. I realise that several of the points raised are rather open-ended and that it is therefore hard to fully predict the outcome and conclusiveness of a revised manuscript at this point. However, I ran an additional round of cross-referee commenting - based on the reports - and here it became clear that all three referees are interested in seeing a revised version, as long as the functional assays for the roles for Ebs1 and Nmd4 (as well as a number of technical points and controls) are significantly extended.

More specifically, the referees provided the following, constructive input on the requirements for a revision (after seeing the reports)

Ref #3: In response to ref #1's report, ref #3 finds that studies on recombinant proteins, the Nmd4 PIN domain and phosphorylation-dependent Upf1 interaction are beyond the scope of the current manuscript. However, this person would need you to demonstrate that the tagged alleles have WT NMD function and that the putative NMD factors were shown to be bona fide NMD factors with straightforward northern assays.

Ref #1: Agrees with Reviewer 3 that the function of the Nmd4 PIN domain does not need to be analyzed if it is most likely inactive (point 5) but that this should be mentioned clearly in the manuscript. The same goes for the analysis of the interaction of Ebs1 with Upf1 (point 6). Regarding the helicase assays (point 4), the ref finds it relevant to include such an assay, especially when other functional data (i.e. NMD) are not available (ref #3 subsequently agreed to this point). The referee concludes: 'In summary, if clear results confirm the function of Ebs1 or Nmd4 as NMD

factors, I will be satisfied. Without such data I expect convincing alternative data about their molecular function'.

Ref #3: Suggests using known endogenous NMD targets to test for functionality and mentions the CYH2 pre-mRNA as a good option (since a transcript that is largely absent in cells WT for NMD function and considerably abundant when NMD is inactive). In addition, all three refs reflect on the level of effect one would have to see in order to conclude that something is a bona fide NMD factor. It is clear that one cannot draw a direct line for this but it will be important that you discuss the size of the effects seen in the manuscript.

Ref #2: Is less concerned about the overall size of the effects but agrees with the other two that additional functional assays will have to be performed for the revised manuscript.

Given the referees' overall interest and positive recommendations, I would therefore like to invite you to submit a revised version of the manuscript, addressing the comments of all three reviewers.

REFEREE REPORTS

Referee #1:

In the manuscript "Nonsense-mediated mRNA Decay Involves Distinct and Successive Upf1-bound Complexes" by Cosmin Saveanu the authors describe the mass-spectrometric analysis of different NMD complexes in yeast. Using Upf1 as bait protein, they find two distinct complexes associating with it, which they term the detector and the effector. While the detector complex contains the classical NMD factors Upf1, Upf2 and Upf3, the effector complex consists of Upf1 and different RNA-degrading enzymes. The authors also describe an interaction of the protein Ebs1 and Nmd4 with the helicase domain of Upf1. However, they only observe a mild NMD inhibition in yeast strains lacking Ebs1 or Nmd4 or both.
All in all, this interesting piece of work is written in a comprehensible manner (although the lack of line numbers may be criticized) and contains convincing data. However, a number of additional experiments have to be done before this work can be published.
One of the most important problems for me is that it is not quite clear to what extent the function of Ebs1 and Nmd3 in the effector complex is documented, because neither Nmd4 nor Ebs1 have proven degradation activity. Therefore, it is possible that these proteins belong to yet another Upf1-containing complex, which is not directly involved in the degradation of NMD substrates. In this case, the effects of the deletion of these protein on NMD activity would only be indirect and the "effector complex" incorrectly designated. One observation supporting this interpretation is that Ebs1 does not seem to interact with the decapping complex (see comment 1 below).
Additional comments
1) The RNase-independent interaction partner profiles of TAP-tagged Nmd4 and Ebs1 look different (Fig. 2d and 2e). Does this suggest that distinct effector subcomplexes exist? If decapping components are co-purified due to their interaction with Upf1 (based on Fig. 4b), why is this interaction lost in Ebs1 purification?
2) Nmd4 or Ebs1 deletion (or combined deletion) has only a mild effect on NMD. Did the authors test the function of Nmd4 or Ebs1 in a strain that lacks proteins of the decapping machinery? Or are Nmd4 or Ebs1 required to stimulate the decapping activity of Upf1-bound decapping proteins during NMD?
3) Do Nmd4 and Ebs1 affect the production of 3' RNA fragments that have been detected for Upf1 ATP hydrolysis mutants (Serdar et al., Nature Communications 2016, Volume 7, doi:10.1038/ncomms14021)? Could Ebs1 and Nmd4 be involved in the recycling of Upf1 or the unwinding of mRNPs by Upf1?
4) What is the role of Ebs1 in NMD? Does it impact on the helicase activity of Upf1? Does it bind directly to Upf1? Both could be tested in vitro using recombinant proteins.
5) The authors report that Nmd4 contains (or consists almost entirely of) a PIN domain. Has the potential endonuclease activity of Nmd4 been tested in vitro or in cells? Is it possible to identify and test putative catalytic residues required for endonucleolytic activity?
6) SMG5-7 interact with human UPF1 mainly via phosphorylated Serine and Threonine residues. Could the potential 14-3-3 domain of Ebs1 also confer binding to Upf1 in a phosphorylation-

dependent manner? How do mutations of the 14-3-3 domain affect the interaction between Ebs1 and Upf1?
7) NMD function is abolished in Upf2 or Upf3 deletion strains. However, the global interaction partner profile of Upf1 is unchanged (except for Upf2 and Upf3 of course). How does this fit with the detector/effector model? Should one not observe a loss of Upf1 interaction with Nmd4, Ebs1 and the decapping machinery upon depletion of the detector complex? Or is the Upf1-Nmd4-Ebs1-DCP complex the stable "end-point" NMD formation that needs to be remodeled/recycled by Upf2-Upf3 in order to engage a new RNA substrate?

Minor comments
8) Fig 2a-f; 3b-e; 4a-b; 6a-b and e: Please improve the labels of the x-axis, because they are rather difficult to read.
9) I do not think the Figure 2h contributes to understanding of the data. To me there seem to be a lot of arrows in all possible directions. In some cases, interactions vary in their RNAse-dependence, depending on the direction.

Referee #2:

This manuscript describes the results of a large quantitative mass spectrometry-based characterization of UPF1 complexes in yeast. Unlike previous mass spec-based identifications of protein interactors, Dehecq and colleagues employed a rigorous and quantitative experimental scheme, in which for each co-purified protein the enrichment score was determined, rather than just an abundance score. By this normalization of the IPs to the cellular abundance of a protein, typical contaminations of high abundant proteins could be eliminated and specific interactions of low abundant proteins that previously were lost in the noise, could now be reliably detected. Using this experimental approach, with and without RNase A treatments, for the TAP-tagged yeast NMD factors Upf1, Upf2 and Upf3, and additional Upf1-associated factors (Nmd4, Ebs1, Dcp1), the authors could identify two distinct Upf1-containing protein complexes, which they termed the detector (Upf1, Upf2, Upf3) and the effector (Upf1, Ebs1, Nmd4, decapping enzyme) complexes. Most interesting was the finding that interaction with Upf1 of Upf2 and Upf3 appeared to be mutually exclusive with the interaction of the effector complex constituents with Upf1. This contrast with current models, in particular with the widespread SURF-DECID model, which proposes that Upf2/3 would remain associated with Upf1 after recruitment of the downstream effector factors (SMG6-7 in metazoans).
Using Upf1 fragments, the authors demonstrated that Upf2/3 as well as the decapping complex and Hrr25 all interact with the CH domain of Upf1 in an RNase-resistant manner, whereas Ebs1 and Nmd4 interact with the helicase domain (HD) and the C-terminal part of Upf1 in an RNase-resistant manner.
While a previous study from the Lingner lab (Luke et al., 2007) already showed a function for Ebs1 in NMD, Nmd4 has been originally identified by the Jacobson lab in a screen for NMD factors but not further been followed up because they later couldn't find an effect on NMD in Δnmd4 strains. In this manuscript here, the authors also find only minor effects on NMD in Δnmd4 strains (Fig. 5). To see a greater effect, the authors needed to "weaken" NMD by expressing a truncated Upf1 version. Interestingly, the affected transcripts in Δnmd4 overlapped considerably with those detected in Δebs1, providing additional evidence that Nmd4 indeed is involved in NMD.
The last part of the manuscript addresses the question, if formation of the effector complex depends on previous formation of the detector complex. The authors chose a rather indirect way to address this hypothesized switch (Fig. 7), the results of which I do not find conclusive. The straight-forward way to address this would have been to test if in Δupf2 or Δupf3 strains, effector complex can still be detected to the same extent as in WT yeast. The authors state that they tried this but that it could not be done because the increased levels of NMD targets rendered enrichment calculations and comparisons to the WT situation unreliable. I don't understand this argument, since enrichment calculations involve the normalization to the input level, which should eliminate the problem. In any case, the authors should provide additional results, ideally from more direct assays, to better support their hypothesis of the switch from the detector to the effector complex. With the currently presented data, this part of the model remains speculative.

In summary, the proteomics approach is probably the most thorough that has so far been done with yeast NMD factors, the results are compelling and led to the identification of two distinct Upf1-containing complexes. The subsequent investigation of the functional relationship between these two complexes is much less thorough and the proposed model does conceptually not very much differ from previously proposed models, except that the model proposed by Dehecq and colleagues excludes Upf2 and Upf3 from complexes further downstream in the NMD pathway. With regards to Ebs1 and Nmd4, this study confirms a function for Ebs1 in NMD and for the first time also shows a function for Nmd4.

Specific points to address:
- Fig. 1: Is there an explanation why in the case of Upf1-TAP, co-IP of UpfF2 and Upf3 is RNase sensitive, while in the case of Upf2-TAP and Upf3-TAP, co-IP of the other two partners is RNase resistant?

- The methods section, although referred to it several times in the text, is missing from the manuscript.

- Fig. 3: Why was the Upf1-HD fragment not used to show that Nmd4 specifically binds to the helicase domain? This would have made the authors conclusion more compelling.

- The authors suggest that yeast Ebs1 represents a functional equivalent of the mammalian SMG5/SMG7 heterodimer, and Nmd4 a functional equivalent of SMG6. While it is an attractive hypothesis and the respective factors appear to contact similar regions on Upf1, it should however also be mentioned that the PIN domain of Nmd4 is unlikely to have endonuclease activity, because one of the three conserved glutamates in the catalytic center of SMG6-PIN is a lysine in Nmd4. In that sense, Nmd4 is more similar to SMG5 than to SMG6.

- The Discussion is to a large part a repetition of the result section. It should be shortened and emphasize on the comparison of the results with other studies rather than summarizing the results. Regarding the proposed "revised universal NMD model", it should be acknowledged that similar ideas can already be found in the literature. For example, that EJC-independent and EJC-enhanced NMD are mechanistically very similar has already been proposed by the Muhlemann lab ten years ago (Muhlemann et al., BBA 2008; Stalder and Muhlemann, TiCB 2008).

Minor points:
- Abstract: Modify sentence "...characterization of yeast NMD complexes in yeast"
- P. 4: Modify incomprehensible sentence "First, even if most of the RNA decay factors, and the key NMD proteins ..."

Referee #3:

General summary:
The authors are interested in the regulation of the NMD pathway in yeast and have used epitope-tagged alleles of all three UPF genes to search for co-purifying proteins that might expand our understanding of the factors involved in NMD. Tagged alleles of the genes encoding co-purifying proteins were also utilized to examine their respective interactors, and mass spec analyses were compared to protein abundances to establish relative enrichment scores for the proteins of interest. Ultimately, the respective candidate genes were tested for possible involvement in NMD regulation. The authors found the expected interactions between Upf1, Upf2, and Upf3, as well as expected interactions between Upf1 and components of the decapping complex. Additional interactors with Upf1 included Nmd4 (a potential NMD regulator identified previously in a Upf1 two-hybrid screen) and Ebs1, a protein previously identified as a potential NMD regulator by virtue of its homology to Smg5/7. The appearance of two possible "sets" of Upf1 interactors (namely Upf2 and Upf3 in one set, and Nmd4, Ebs1, Hrr25, and decapping factors in a second set) led the authors to propose the existence of two NMD complexes that they call the "detector" and "effector" complexes.
The experiments of the paper encompass a substantial amount of work and the results are potentially interesting. However, several substantial concerns, itemized below, limit the reliability of the conclusions, i.e., additional work needs to be done to provide suitable validation of the results.

Major concerns:
1. The experiments of this manuscript utilize numerous TAP-tagged alleles in various formats, e.g., 5' or 3' tagging, expression from plasmids or chromosomal loci, and different degrees of expression level. None of these alleles has been shown to leave NMD intact, and this is unacceptable. For example, overexpression of a particular tagged allele could have a dominant-negative effect on NMD and thus lead to the recruitment of interacting proteins that are not relevant. Hence, it must be shown that all tagged alleles used in the study do not affect NMD, i.e., they do not elevate the levels of known NMD substrates. (more on that test in item #2, below).
2. While most of the protein co-purification experiments are convincing, the experiments showing that certain factors, e.g., Nmd4, have a role in NMD are very weak. One would expect that deletion of an NMD regulator would yield substantial increases in the levels of known NMD substrates. This is the type of experiment that was done previously in multiple labs to identify all three Upfs, and to demonstrate roles for Dcp1, Dcp2, and Xrn1 in NMD. Here, the authors have used RNA-Seq to address this point and their results largely point to very modest effects (e.g., Fig. 5b). The use of IGV browser "shots" as validation (e.g., Fig. 5e) is simply a reuse of the same RNA-Seq data in a different format. Fortunately for the authors, decades of prior studies in other labs have provided the necessary tests, i.e., northern blotting assessments of the levels of known NMD substrates in cells that are WT or mutant for the putative factor. The authors need to use these definitive (and very reliable) tests on cells that are WT or mutant for NMD4, EBS1, etc. in order to prove that these are bona fide NMD factors.
3. Recent studies (Feng et al, Mol Cell 67: 239, 2017; Kuroha et al, JBC 288: 28630, 2013) point to a role of Upf1 in proteasome-mediated degradation. Hence, some of the variations in polypeptide recoveries seen here may reflect protein decay roles, not RNA decay roles, and may also account for some of the differential recoveries of specific proteins. The authors must provide some indication that they have considered this potential complication and ruled it out.
4. The manuscript has a considerable amount of unprofessional boasting that needs to be toned down substantially. Use of unnecessary terms like "first extensive characterization," and the frequent ignoring of previously published work which makes similar points is not appropriate for EMBO J. In the same vein, the NMD field already has a surfeit of names for various complexes (surveillance complex, SURF complex, DECID complex) and all of them are unnecessary and may simply reflect the frequently discussed transition of the Upf proteins from recognition of a substrate to recruitment of decay enzymes, and perhaps even to recruitment of the proteasome. I strongly recommend that the authors abandon their use of "detector" and "effector" and adopt the more conventional thinking about a "pathway."
5. It would be very helpful if the authors could provide relative abundances of the different proteins that comprise the different complexes. The demonstration of stoichiometric relationships would certainly support their arguments.
6. The authors state that the C-terminus of Upf1 is able to complement a upf1 deletion and restore NMD. This needs to be validated because the results in the literature are inconsistent.

Minor concerns:
7. The authors' interpretation of their polysome data appears to be incorrect. They state that most of the Nmd4 signal was in monosomes in the Upf WT strain (and therefore state that it is NMD substrate-associated, by their reference to Heyer, et al), but this is not the case because 20% was in the polysome fraction, and 9% was in the 80S. They also claim that the upf2 deletion had similar effects as the upf1 deletion on Nmd4 polysome association. While polysome/80S association was down in the upf2 deletion, there was still twice as much Nmd4 associated with polysomes/80S as there was in upf1 deletion. This indicates that Nmd4 requires Upf1 to bind to the ribosome, and that this interaction is stabilized by Upf2. They also state that Upf1 and Nmd4 are part of their "effector" complex, but there is no evidence that Upf1/Nmd4 interaction occurs in the same complex as Upf1/Dcp1/2. They also don't reconcile this with Fig. 6a/b in which they showed that Upf1 interaction with Nmd4 is unaffected by deletion of Upf2 or Upf3. This suggests that the Upf1/Nmd4 interaction that they observe in their IP is probably occurring off of the ribosome.
8. Fig 2G: It would be helpful if the authors included a positive control for a protein whose interaction with Upf1 is sensitive to RNase, e.g., Pat1.
9. Fig. 1E arrows are pointing at the wrong bars.

Manuscript: “Nonsense-mediated mRNA decay involves distinct and successive Upf1-bound complexes” by M. Dehecq *et al*.

Answers to the editor’s questions

Question: ...I ran an additional round of cross-referee commenting - based on the reports - and here it became clear that all three referees are interested in seeing a revised version, as long as the functional assays for the roles for Ebs1 and Nmd4 (as well as a number of technical points and controls) are significantly extended.

> **Answer 1.** To investigate the involvement of Nmd4 and Ebs1 in NMD we used the "gold standard" of RNA degradation studies, the half-life measurement for a reporter RNA whose synthesis can be switched off using a repressible promoter. The obtained results are now part of **Figure 5**, as panels **F**, **G** and **H**. We used an RNA that codes for a HA-tagged protein to show that : 1) more reporter protein accumulates in cells without *NMD4*, *EBS1* or in a double mutant with deletion of both *NMD4* and *EBS1* (**Fig. 5G**) and 2) in light of the cumulative effect of the double mutant on protein production from the NMD-sensitive reporter, we tested the RNA half-life in this strain compared with a wild-type. As shown in **Fig. 5H**, the absence of *NMD4* and *EBS1* led to a delay in the degradation of the reporter RNA. After 5 minutes of transcriptional repression, the cellular levels of the reporter diminished to 60% of the original level, while, at the same time, the level of the reporter remained unchanged in the strain lacking both *EBS1* and *NMD4*. We conclude that Nmd4 and Ebs1 are required for the degradation of this specific NMD RNA reporter. Together with the highly specific physical association of these proteins with Upf1 complexes and with the global, and correlated effect of the *NMD4* and *EBS1* gene deletion on the levels of NMD substrates in yeast, we now provide three distinct and convergent experimental results that strongly suggest that Ebs1 and Nmd4 are NMD factors.

Question: More specifically, the referees provided the following, constructive input on the requirements for a revision (after seeing the reports)

Ref #3: In response to ref #1's report, ref #3 finds that studies on recombinant proteins, the Nmd4 PIN domain and phosphorylation-dependent Upf1 interaction are beyond the scope of the current manuscript. However, this person would need you to demonstrate that the tagged alleles have WT NMD function and that the putative NMD factors were shown to be bona fide NMD factors with straightforward northern assays.

> **Answer 2.** We are sorry that we did not include in the initial version of the manuscript the control experiments for the different tagged strains used. The original version of the manuscript showed only the ability of the overexpressed N-terminal tagged Upf1 to complement NMD (**Fig. 5E** in the revised version). The strains expressing C-terminal tagged proteins from the original chromosomal locus (Upf1, Upf2, Upf3, Nmd4, Ebs1, Dcp1 and Hrr25) were tested for NMD by looking at the relative levels of the RPL28 (CYH2) unspliced precursor both by RT-qPCR and Northern blots. Except for Upf2-TAP, which showed a 1.5 fold increase in the levels of the tested NMD substrate (compared with a much larger 5.5 fold increase in an NMD-deficient strain), all the other strains were NMD competent **(Fig. EV1 A** and **B**). Thus, our results are based on tagged proteins in yeast strains that are functional for NMD.

Answer 3. To answer the observation about Northern assays, we now present in **Appendix Fig. S4**, the equivalent of the Q-PCR results shown in **Fig. 5E**, obtained by Northern blot with probes specific for the pre-RPL28 intron-containing NMD substrate and the NMD-insensitive mature RPL28 mature form. These results mirror the RT-qPCR data.

Question: **Ref #1**: Agrees with Reviewer 3 that the function of the Nmd4 PIN domain does not need to be analyzed if it is most likely inactive (point 5) but that this should be mentioned clearly in the manuscript. The same goes for the analysis of the interaction of Ebs1 with Upf1 (point 6). Regarding the helicase assays (point 4), the ref finds it relevant to include such an assay, especially when other functional data (i.e. NMD) are not available (ref #3 subsequently agreed to this point). The referee concludes: 'In summary, if clear results confirm the function of Ebs1 or Nmd4 as NMD factors, I will be satisfied. Without such data I expect convincing alternative data about their molecular function'.

Answer 4. We agree that the situation of the Nmd4 PIN domain needed clarification in the manuscript. The short discussion about the conservation of 3 out of 4 aminoacids that are required for the enzymatic activity of the PIN domain in human SMG6 and other PIN domain endonucleases is illustrated now by local multiple alignments and the accompanying annotated structure of the SMG6 PIN domain (**Appendix Fig. S6**).

Answer 5. Concerning the helicase activity of Upf1 in the presence or absence of Ebs1, we could not obtain significant amounts of soluble purified Ebs1, required for such an assay. Previous attempts to produce recombinant Ebs1 were unsuccessful in one of our collaborators laboratory (personal communication M. Graille, Ecole Polytechnique, Saclay, France) and a different strategy of protein production will need to be set up for this assay. However, the transcriptome-wide effect of EBS1 deletion (**Fig. 5B**, **D**), its specific effect in the degradation of an NMD reporter (**Fig. 5F**, **G**, **H**), the impact of Ebs1 on NMD elicited with the helicase domain of Upf1 (**Fig 3E** and **Appendix Fig. S4**) and the specific interactions with Nmd4 and Upf1 (**Fig. 2**), are strong indications that the main function of Ebs1 is to assist Upf1 during yeast NMD.

Answer 6. To replace the helicase assay, that was not technically possible in our hands (as explained above), with a different type of functional data, we include now an *in vitro* RNA binding assay that shows a role for Nmd4 in stabilizing the binding of Upf1 to RNA (**Fig. 4E**). This result strongly suggest that even if the PIN domain of Nmd4 is not enzymatically active, Nmd4 binding to Upf1 could play a role in the residence time of the helicase on RNA. Since mutants that affect binding of Upf1 to RNA are no longer able to trigger the degradation of NMD substrates (Weng, Czaplinski & Peltz, RNA, 1998, PMID 9570320), our findings, together with the other biochemical and functional results on Nmd4, indicate that it could affect the affinity or residence of Upf1 on NMD substrates. By extension, these results suggest that the PIN domain of the human NMD co-factors SMG6 and SMG5 could play similar roles, that were, until now never tested. An effect of Nmd4 on Upf1 binding to RNA could also explain its accessory role *in vivo*, where the affinity of Upf1 for RNA could only become limiting under specific conditions. An experimental "stress" condition that we identified in the current manuscript is the absence of the CH domain, when Nmd4 and Ebs1 were required for the destabilization of an NMD substrate *via* Upf1.

Questions. **Ref #3**: Suggests using known endogenous NMD targets to test for functionality and mentions the CYH2 pre-mRNA as a good option (since a transcript that is largely absent in cells WT for NMD function and considerably abundant when NMD is inactive). In addition, all three refs

reflect on the level of effect one would have to see in order to conclude that something is a bona fide NMD factor. It is clear that one cannot draw a direct line for this but it will be important that you discuss the size of the effects seen in the manuscript.

Answer 7. We have used the CYH2 mRNA precursor in the manuscript as a classical readout of NMD activity, albeit we used the current name of the gene, which is RPL28. The magnitude of the effects seen in our transcriptome data is indeed low and stimulated our search for a more reliable phenotype associated with the absence of *NMD4* or *EBS1*. The requirement for these genes in the destabilization of NMD substrates by a truncated form of Upf1 is now reinforced by a different type of experiment, in which we looked at the stabilization of an artificial NMD reporter (see **Answer 1** for details). Moreover, we further extended our investigation of the mechanism by which the Upf1 HD-Cter fragment affects RNA levels, by showing that the observed effects depend on decapping, like *bona fide* yeast NMD (**Fig. EV4B**).

Question. **Ref #2**: Is less concerned about the overall size of the effects but agrees with the other two that additional functional assays will have to be performed for the revised manuscript.

Answer 8: please see our **Answer 1** (page 1, line 8) and **Answer 6** (page 2, line 26), explaining the additional functional data on Ebs1 and Nmd4 in NMD, that are now part of the manuscript.

Answers to referee comments and questions:

Referee #1:

In the manuscript "Nonsense-mediated mRNA Decay Involves Distinct and Successive Upf1-bound Complexes" by Cosmin Saveanu the authors describe the mass-spectrometric analysis of different NMD complexes in yeast. Using Upf1 as bait protein, they find two distinct complexes associating with it, which they term the detector and the effector. While the detector complex contains the classical NMD factors Upf1, Upf2 and Upf3, the effector complex consists of Upf1 and different RNA-degrading enzymes. The authors also describe an interaction of the protein Ebs1 and Nmd4 with the helicase domain of Upf1. However, they only observe a mild NMD inhibition in yeast strains lacking Ebs1 or Nmd4 or both.

All in all, this interesting piece of work is written in a comprehensible manner (although the lack of line numbers may be criticized) and contains convincing data. However, a number of additional experiments have to be done before this work can be published.

One of the most important problems for me is that it is not quite clear to what extent the function of Ebs1 and Nmd4 in the effector complex is documented, because neither Nmd4 nor Ebs1 have proven degradation activity. Therefore, it is possible that these proteins belong to yet another Upf1-containing complex, which is not directly involved in the degradation of NMD substrates. In this case, the effects of the deletion of these protein on NMD activity would only be indirect and the "effector complex" incorrectly designated. One observation supporting this interpretation is that Ebs1 does not seem to interact with the decapping complex (see comment 1 below).

Some concerns raised by the reviewer are now part of the **Answer 1** (page 1, line 8). We used the name 'Effector' for the complex associated with Nmd4 and Upf1 as a result of the presence of the decapping enzyme in the purified fractions. It would be certainly very interesting to test if the decapping enzyme that is enriched in a Nmd4 or Ebs1-TAP purification is enzymatically active or not. However, such an experiment is technically challenging, as illustrated by the lack of equivalent results in the published literature on NMD. For example, even if the physical association of Upf1 with the decapping factors has been known for more than 10 years (Lykke-Andersen, 2002 Mol Cell Biol PMID 12417715, in mammalian cells, Ford et al., 2006 Eukaryot. Cell PMID 16467471 co-immunoprecipitation of yeast Dcp1 and Upf1, Tarassov et al., 2008 Science PMID 18467557, large scale PCA assay in yeast, Swisher & Parker 2011 PLoS One PMID 22065998, yeast two-hybrid), the direct decapping activity of Upf1-associated complexes *in vitro* was, to our knowledge, not yet demonstrated. Setting up a new experimental system to answer this very interesting question might take a long time and, we believe, is beyond the scope of the current manuscript. However, our data provide an essential tool for this type of experiment, because it identifies Nmd4 as an ideal tagged protein for the purification of the fraction of yeast Upf1 not engaged in an interaction with Upf2 and Upf3, but interacting strongly with the decapping factors.

While the association of Nmd4 with the decapping complex was robust and resisted even a harsh RNase treatment, we agree with the reviewer that the situation of Ebs1 is more complex. Both in the Ebs1-TAP purification and in the Dcp1-TAP purification, the interactions between Ebs1 and decapping factors were sensitive to an RNase treatment. A similar result was previously obtained by immunoblot of myc-Dcp1 co-purified with HA-Ebs1 (Ford et al., 2006).

In the same article, the authors identified a strong synthetic lethal phenotype in a double mutant affecting *DCP1* and *EBS1*. The interpretation of our results in the context on the previously published data is that Ebs1 interacts with the decapping complex via the C-terminal domain of Upf1, but that this interaction is stabilized by the presence of RNA. Unlike Nmd4, for which we could identify a role in increasing the stability of Upf1 binding to RNA (**Fig. 4E**), we do not have yet data about what molecular function Ebs1 could play. We hope that the current manuscript will stimulate studies to elucidate this question in the future.

Additional comments

1) The RNase-independent interaction partner profiles of TAP-tagged Nmd4 and Ebs1 look different (Fig. 2d and 2e). Does this suggest that distinct effector subcomplexes exist? If decapping components are co-purified due to their interaction with Upf1 (based on Fig. 4b), why is this interaction lost in Ebs1 purification?

Our best current explanation concerning the differences between the results obtained with Nmd4 and Ebs1 is, as stated above, that Ebs1 has a lower affinity for Upf1 than Nmd4, and that this affinity is also modified as a function of RNA. We do not have enough data to speculate over the possibility that there are several Effector subcomplexes. However, we distinguish now between two types of Effector complexes, one bound to RNA and another that is RNA-free, by adding in the text of the manuscript, after mentioning the Detector/Effector model (**Fig. 7F, G**), the sentence: "This model includes two forms of Effector: one that is RNA-bound and depends on Detector components Upf2 and Upf3, and another that is RNA-free." The two forms are also depicted in **Fig. 7G**.

2) Nmd4 or Ebs1 deletion (or combined deletion) has only a mild effect on NMD. Did the authors test the function of Nmd4 or Ebs1 in a strain that lacks proteins of the decapping machinery? Or are Nmd4 or Ebs1 required to stimulate the decapping activity of Upf1-bound decapping proteins during NMD?

The mild, but highly correlated, effect seen when either *NMD4* or *EBS1* were deleted was substantially more important under conditions in which NMD activity is provided by the over-expressed Upf1-HD-Cter fragment (**Fig. 5E**). We used a Dcp2-degron destabilized strain to see if the changes in the levels of NMD substrates in these conditions was dependent on decapping. As shown in **Fig. EV4**, the NMD effect of Upf1-HD-Cter on pre-L28 was dependent on the presence of Dcp2. Our interpretation of these results is that the partial complementation of NMD obtained with Upf1-HD-Cter occurs through stimulation of decapping. Its dependence of the presence of either *NMD4* or *EBS1* is compatible with a potential stimulation of the decapping activity by the corresponding proteins.

3) Do Nmd4 and Ebs1 affect the production of 3' RNA fragments that have been detected for Upf1 ATP hydrolysis mutants (Serdar et al., Nature Communications 2016, Volume 7, doi:10.1038/ncomms14021)? Could Ebs1 and Nmd4 be involved in the recycling of Upf1 or the unwinding of mRNPs by Upf1?

A role of Ebs1 and Nmd4 in the recycling of Upf1 is definitely possible although we currently lack an experimental system to test this hypothesis. We did not test yet the effect of Nmd4 or Ebs1 on the production of 3' RNA fragments since the effects of deleting any of the corresponding genes on stabilization of NMD substrates were modest. This is definitely an interesting direction for future studies.

4) What is the role of Ebs1 in NMD? Does it impact on the helicase activity of Upf1? Does it bind directly to Upf1? Both could be tested in vitro using recombinant proteins.

Before the identification of a molecular function of Ebs1 in NMD, we will probably need to set up a system that allows to test the decapping activity of the Upf1 or Nmd4-associated complexes *in vitro*, as mentioned in the answer to the general comments of reviewer #2 (**Answer 5** and **6**, page 2).

5) The authors report that Nmd4 contains (or consists almost entirely of) a PIN domain. Has the potential endonuclease activity of Nmd4 been tested in vitro or in cells? Is it possible to identify and test putative catalytic residues required for endonucleolytic activity?

The presence of the PIN domain in Nmd4 was one of the reasons we concentrated our efforts on the protein, once it became clear it was a strong and specific binder of Upf1. We have not yet tested the potential endonuclease activity of Nmd4 *in vitro*. We are in the process of identifying a more robust phenotype for the loss of function of Nmd4, which will allow, as suggested by the reviewer, the test of mutations into candidate catalytic residues. The fact that Nmd4 can stabilize Upf1 binding to RNA already provides a potential molecular role for the PIN domain. A structural role has been previously proposed for the PIN domain of Dis3/Rrp44, the core exosome protein. Mutations of the catalytic residues of the Dis3 PIN domain were tolerated much better than the absence of the domain, which is involved in protein-protein interactions (Schneider et al., Nucl Acids Res 2009 PMID 19129231). Since endonucleolytic activities associated with yeast NMD have not been described yet, it is possible that this activity was lost in favor of a structural role.

6) SMG5-7 interact with human UPF1 mainly via phosphorylated Serine and Threonine residues. Could the potential 14-3-3 domain of Ebs1 also confer binding to Upf1 in a phosphorylation-dependent manner? How do mutations of the 14-3-3 domain affect the interaction between Ebs1 and Upf1?

As mentioned above, we focused most of our efforts on the study the dynamics of the involved complexes and on the description of Nmd4, its potential role in NMD and its interactions with Upf1. To our knowledge, there is no strong evidence that phophorylation of Upf1 plays a role in yeast NMD. A version of Upf1 lacking the C-terminal poorly conserved domain is still able to destabilize and endogenous NMD substrate when over-expressed, albeit with reduced efficiency (**Fig. EV4A**).

7) NMD function is abolished in Upf2 or Upf3 deletion strains. However, the global interaction partner profile of Upf1 is unchanged (except for Upf2 and Upf3 of course). How does this fit with the detector/effector model? Should one not observe a loss of Upf1 interaction with Nmd4, Ebs1 and the decapping machinery upon depletion of the detector complex? Or is the Upf1-Nmd4-Ebs1-DCP complex the stable "end-point" NMD formation that needs to be remodeled/recycled by Upf2-Upf3 in order to engage a new RNA substrate?

This is a very good observation, in line with comments from **reviewer #3** about the interpretation of polysome profiles for Nmd4-TAP in the absence of Upf1 and Upf2 (**Fig. 7B** to **E**) and the lack of major changes in the composition of the purified complexes in the absence of Upf2 or Upf3 (**Fig. 6A**, **B**). It is indeed possible that a major part of Upf1 is engaged in RNA-free or RNA-labile complexes together with Dcp1, Dcp2, Edc3, Nmd4, and possibly, Ebs1. However, at least a fraction of these complexes is also bound to RNA, as shown by the ultracentrifugation

results mentioned above and by the enrichment of an NMD substrate in association with Nmd4 (**Fig. 7A**). Further fractionation experiments will be needed to clarify the number, composition and dynamics of the involved complexes.

Minor comments

8) Fig 2a-f; 3b-e; 4a-b; 6a-b and e: Please improve the labels of the x-axis, because they are rather difficult to read.

The revised version of the figures includes thorough changes of the font faces and size to improve legibility.

9) I do not think the Figure 2h contributes to understanding of the data. To me there seem to be a lot of arrows in all possible directions. In some cases, interactions vary in their RNAse-dependence, depending on the direction.

We would like to conserve the 2h panel, now labeled **2I**, because it is the most compact representation of most of our biochemical fractionation results, which cannot be provided by any other method of visualization. Its role is also to show that using tagged proteins can be tricky, especially in the case of interactions that are RNA-dependent. We believe that the variations in the observed results are inevitable artifacts of using tagged proteins to study RNA-protein complexes by affinity purification followed by mass-spectrometry. This cautionary message that the panel conveys is an important one, especially for scientists who are not experienced with the type of experiments shown in the manuscript.

Referee #2:

This manuscript describes the results of a large quantitative mass spectrometry-based characterization of UPF1 complexes in yeast. Unlike previous mass spec-based identifications of protein interactors, Dehecq and colleagues employed a rigorous and quantitative experimental scheme, in which for each co-purified protein the enrichment score was determined, rather than just an abundance score. By this normalization of the IPs to the cellular abundance of a protein, typical contaminations of high abundant proteins could be eliminated and specific interactions of low abundant proteins that previously were lost in the noise, could now be reliably detected. Using this experimental approach, with and without RNase A treatments, for the TAP-tagged yeast NMD factors Upf1, Upf2 and Upf3, and additional Upf1-associated factors (Nmd4, Ebs1, Dcp1), the authors could identify two distinct Upf1-containing protein complexes, which they termed the detector (Upf1, Upf2, Upf3) and the effector (Upf1, Ebs1, Nmd4, decapping enzyme) complexes. Most interesting was the finding that interaction with Upf1 of Upf2 and Upf3 appeared to be mutually exclusive with the interaction of the effector complex constituents with Upf1. This contrast with current models, in particular with the widespread SURF-DECID model, which proposes that Upf2/3 would remain associated with Upf1 after recruitment of the downstream effector factors (SMG6-7 in metazoans).

Using Upf1 fragments, the authors demonstrated that Upf2/3 as well as the decapping complex and Hrr25 all interact with the CH domain of Upf1 in an RNase-resistant manner, whereas Ebs1 and Nmd4 interact with the helicase domain (HD) and the C-terminal part of Upf1 in an RNase-resistant manner.

While a previous study from the Lingner lab (Luke et al., 2007) already showed a function for Ebs1 in NMD, Nmd4 has been originally identified by the Jacobson lab in a screen for NMD factors but not

further been followed up because they later couldn't find an effect on NMD in Δnmd4 strains. In this manuscript here, the authors also find only minor effects on NMD in Δnmd4 strains (Fig. 5). To see a greater effect, the authors needed to "weaken" NMD by expressing a truncated Upf1 version. Interestingly, the affected transcripts in Δnmd4 overlapped considerably with those detected in Δebs1, providing additional evidence that Nmd4 indeed is involved in NMD.

The last part of the manuscript addresses the question, if formation of the effector complex depends on previous formation of the detector complex. The authors chose a rather indirect way to address this hypothesized switch (Fig. 7), the results of which I do not find conclusive. The straight-forward way to address this would have been to test if in Δupf2 or Δupf3 strains, effector complex can still be detected to the same extent as in WT yeast. The authors state that they tried this but that it could not be done because the increased levels of NMD targets rendered enrichment calculations and comparisons to the WT situation unreliable. I don't understand this argument, since enrichment calculations involve the normalization to the input level, which should eliminate the problem. In any case, the authors should provide additional results, ideally from more direct assays, to better support their hypothesis of the switch from the detector to the effector complex. With the currently presented data, this part of the model remains speculative.

In summary, the proteomics approach is probably the most thorough that has so far been done with yeast NMD factors, the results are compelling and led to the identification of two distinct Upf1-containing complexes. The subsequent investigation of the functional relationship between these two complexes is much less thorough and the proposed model does conceptually not very much differ from previously proposed models, except that the model proposed by Dehecq and colleagues excludes Upf2 and Upf3 from complexes further downstream in the NMD pathway. With regards to Ebs1 and Nmd4, this study confirms a function for Ebs1 in NMD and for the first time also shows a function for Nmd4.

> We agree with the reviewer that testing the importance of Detector components on the formation of Effector is important. Our experiments, shown in **Fig. 6**, addressed directly this question and led to the unexpected result that Upf2 and Upf3 were interdependent for their binding to Upf1. However, the absence of either of these two protein did not change the protein composition of Effector. This result, together with the partial loss of Nmd4 from the monosomal and polysomal fractions in the absence of Upf2 (**Fig. 7B** to **E**), suggest that Effector can form without the action of Upf2 and Upf3, and that it consists of both an RNA-bound (as shown in **Fig. 7A**) and an RNA-free form. To explicitly mention these two forms, and following the brief description of the Detector/Effector model (**Fig. 7F**, **G**), we added the sentence to the text of the manuscript: "This model includes two forms of Effector: one that is RNA-bound and depends on Detector components Upf2 and Upf3, and another that is RNA-free." We are currently developing an experimental setup to address the succession of Detector and Effector proteins on the same RNA target *in vivo*. The strategy will require probably several months, and we do not know yet if it will be technically feasible.

Specific points to address:

- Fig. 1: Is there an explanation why in the case of Upf1-TAP, co-IP of UpfF2 and Upf3 is RNase sensitive, while in the case of Upf2-TAP and Upf3-TAP, co-IP of the other two partners is RNase resistant?

An explanation of the different sensitivity to RNase depending on which protein of the complex was used for pull-down would be that the tags change the stability of the protein-protein interactions.

Another possibility is that we are not looking at the same type of interactions when using Upf1 or Upf2/3 for the purifications. Based on the fact that Upf2/3 are less abundant than Upf1, we can imagine that, in the case of Upf2/3 purification, we enrich the fraction of Upf1 which is directly interacting with the tagged protein. No RNase effect is seen. When using tagged Upf1, it is possible that a large fraction of the co-purified Upf2/3 is enriched through interactions mediated by RNA, since it is known that several Upf1 molecules can reside on a single 3' UTR (Kurosaki & Maquat, PNAS 2013 PMID 23404710). In this case, the net effect of an RNase treatment will be a decrease of Upf2/3 in the purified fraction, even if the interactions are still present.

- The methods section, although referred to it several times in the text, is missing from the manuscript.

We are very sorry that, by error, the version of the manuscript sent for review was missing the **Materials and Methods** section. An updated version was uploaded as soon as the editor told us about the error. The revised version has a Materials and Methods section, with more details in the Appendix.

- Fig. 3: Why was the Upf1-HD fragment not used to show that Nmd4 specifically binds to the helicase domain? This would have made the authors conclusion more compelling.

The *in vitro* interaction experiments were performed with a 220-851 yeast Upf1 fragment that corresponds to the most conserved region of the protein, which comprises the helicase domain. This region interacts with Nmd4 (**Fig. 4D**) and its binding to RNA is enhanced in the presence of Nmd4 (**Fig. 4E**). The interaction of Nmd4 with Upf1 HD domain *in vivo* was detected as well in the purification using Upf1-HD alone (**Fig. 3E**).

- The authors suggest that yeast Ebs1 represents a functional equivalent of the mammalian SMG5/SMG7 heterodimer, and Nmd4 a functional equivalent of SMG6. While it is an attractive hypothesis and the respective factors appear to contact similar regions on Upf1, it should however also be mentioned that the PIN domain of Nmd4 is unlikely to have endonuclease activity, because one of the three conserved glutamates in the catalytic center of SMG6-PIN is a lysine in Nmd4. In that sense, Nmd4 is more similar to SMG5 than to SMG6.

We agree with the reviewer that in the absence of direct structural and biochemical data on Nmd4, it is difficult to conclude whether its PIN domain is more similar to the PIN domain of SMG6 or the inactive PIN domain of SMG5. It is indeed likely that the PIN domain of Nmd4 does not function as an endonuclease. However, in addition to sequence conservation, our data indicate that Nmd4 binds to yeast Upf1 in the same region as the one described for the binding of SMG6 to UPF1. Please see **Answer 4** (page 2, line 14) for the additions to the revised version of the manuscript regarding this question.

- The Discussion is to a large part a repetition of the result section. It should be shortened and emphasize on the comparison of the results with other studies rather than summarizing the results. Regarding the proposed "revised universal NMD model", it should be acknowledged that similar ideas can already be found in the literature. For example, that EJC-independent and EJC-enhanced

NMD are mechanistically very similar has already been proposed by the Muhlemann lab ten years ago (Muhlemann et al., BBA 2008; Stalder and Muhlemann, TiCB 2008).

The revised discussion is shorter by 20% and recapitulates only briefly the main findings to put them into the literature context. It has now only four sections directly related to the new findings and their interpretation in relation with previous work: **a)** discuss affinity purification and how the described results fit previous association data; **b)** explain how our data are compatible or different from previous NMD models in terms of the detection of aberrant translation termination events; **c)** highlight the importance of the C-terminal region of Upf1 and its potentially conserved interactions with Smg-like factors, and **d)** describe the switch from Detector to Effector and its potential importance in understanding NMD. We now acknowledge the previous proposition of a common molecular framework for EJC-enhanced and EJC-independent NMD:

"Similar unified NMD models that include EJC-enhanced and EJC-independent NMD as two aspects of the same molecular mechanism have been proposed earlier (Stalder & Mühlemann, 2008), but differ from the extended Detector/Effector model in terms of the order of interactions during NMD substrate detection and of the importance of Smg1 in the process."

Minor points:

- Abstract: Modify sentence "...characterization of yeast NMD complexes in yeast"

The sentence:

"We used affinity purification coupled with mass spectrometry and an improved data analysis protocol to obtain the first large-scale quantitative characterization of yeast NMD complexes in yeast (112 experiments).

was replaced by:

"We used affinity purification coupled with mass spectrometry and an improved data analysis protocol to characterize the composition and dynamics of yeast NMD complexes in yeast (112 experiments)."

- P. 4: Modify incomprehensible sentence "First, even if most of the RNA decay factors, and the key NMD proteins …"

The phrase reads now: "First, the SURF/DECID model proposes crucial roles for factors or events that are not conserved in all eukaryotes, even if the key NMD proteins are present from yeast to humans."

Referee #3:

General summary:

The authors are interested in the regulation of the NMD pathway in yeast and have used epitope-tagged alleles of all three UPF genes to search for co-purifying proteins that might expand our understanding of the factors involved in NMD. Tagged alleles of the genes encoding co-purifying proteins were also utilized to examine their respective interactors, and mass spec analyses were compared to protein abundances to establish relative enrichment scores for the proteins of interest. Ultimately, the respective candidate genes were tested for possible involvement in NMD regulation.

The authors found the expected interactions between Upf1, Upf2, and Upf3, as well as expected interactions between Upf1 and components of the decapping complex. Additional interactors with Upf1 included Nmd4 (a potential NMD regulator identified previously in a Upf1 two-hybrid screen) and Ebs1, a protein previously identified as a potential NMD regulator by virtue of its homology to Smg5/7. The appearance of two possible "sets" of Upf1 interactors (namely Upf2 and Upf3 in one set, and Nmd4, Ebs1, Hrr25, and decapping factors in a second set) led the authors to propose the existence of two NMD complexes that they call the "detector" and "effector" complexes.
The experiments of the paper encompass a substantial amount of work and the results are potentially interesting. However, several substantial concerns, itemized below, limit the reliability of the conclusions, i.e., additional work needs to be done to provide suitable validation of the results.

Major concerns:

1. The experiments of this manuscript utilize numerous TAP-tagged alleles in various formats, e.g., 5' or 3' tagging, expression from plasmids or chromosomal loci, and different degrees of expression level. **None of these alleles has been shown to leave NMD intact,** and this is unacceptable. For example, overexpression of a particular tagged allele could have a dominant-negative effect on NMD and thus lead to the recruitment of interacting proteins that are not relevant. Hence, it must be shown that all tagged alleles used in the study do not affect NMD, i.e., they do not elevate the levels of known NMD substrates. (more on that test in item #2, below).

> Please see general **Answer 2** (page 1, line 31), which fully addresses these concerns. In addition to the functionality of the tagged strains, please note that the enrichment of specific interacting factors in the fraction purified in association with an over-expressed N-terminal tagged Upf1 was very similar with the values obtained when using a chromosomal C-terminal tagged Upf1(**Fig. EV2B**). Thus, at least for tagged Upf1, the affinity purification results were very robust.

2. While most of the protein co-purification experiments are convincing, the experiments showing that certain factors, e.g., Nmd4, have a role in NMD are very weak. One would expect that deletion of an NMD regulator would yield substantial increases in the levels of known NMD substrates. This is the type of experiment that was done previously in multiple labs to identify all three Upfs, and to demonstrate roles for Dcp1, Dcp2, and Xrn1 in NMD. Here, the authors have used RNA-Seq to address this point and their results largely point to very modest effects (e.g., Fig. 5b). The use of IGV browser "shots" as validation (e.g., Fig. 5e) is simply a reuse of the same RNA-Seq data in a different format. Fortunately for the authors, decades of prior studies in other labs have provided the necessary tests, i.e., northern blotting assessments of the levels of known NMD substrates in cells that are WT or mutant for the putative factor. The authors need to use these definitive (and very reliable) tests on cells that are WT or mutant for NMD4, EBS1, etc. in order to prove that these are bona fide NMD factors.

> It is not clear why the reviewer thinks that 'deletion of an NMD regulator would yield substantial increases in the levels of known NMD substrates'. First, we never used the term "regulator" for Nmd4 or Ebs1, as we consider them best described as "accessory factors" in NMD. Second, such accessory factors can play subtle roles in specific processes and their absence might not lead to the strong phenotype seen in the absence of **core** factors.

An example of a situation that is similar to what we see for Nmd4 and Ebs1 is the decapping enhancer Edc3. Its absence has a limited effect on the transcriptome of yeast cells, with visible changes only for YRA1 and RPS28B RNAs, two transcripts that are subject to specific decapping-dependent auto-regulation mechanisms. (Badis et al., Mol Cell 2004 PMID 15225544, Dong, Li, et al., Mol Cell, 2007 PMID 17317628). However, Edc3 is conserved from yeast to humans and form a conserved complex with the essential decapping factors Dcp1 and Dcp2. Edc3 is thus a *bona fide* decapping co-factor, despite the extremely mild phenotype seen in strains deleted for the corresponding gene.

Regarding the IGV screenshots that we have used to illustrate reads distribution on specific loci, they were not intended as 'validation' figures. Such screenshots or equivalent illustrations are common in most, if not all articles describing sequencing results (see, for example Fig.1D, E in the Mao et al., Genome Res 2017, PMID 28912372, or Fig. 3C, D in the seminal paper from Ingolia et al., Science 2009, PMID 19213877). These screenshot help the reader see if the signal was homogeneous or biased and provide examples of the global sequencing results presented in panel **B**, **Fig. 5**.

We followed the reviewer's suggestion to use Northern blots for a known NMD substrate to look at effects of deleting *NMD4* or *EBS1*. The results (**Appendix Fig. S4**) were similar to those obtained by RT-qPCR, presented in the initial version of the manuscript (now **Fig. 5E**). As shown in a previous study on EBS1 and in excellent agreement with our own results, the magnitude of the effects seen in these mutants is modest, and can only be increased in specific conditions (complementation of *upf1*Δ by a fragment lacking the CH domain). As explained above, the effects are weak but highly specific and should be judged in the context of previously published results and of the extensive biochemical data presented in the manuscript. In isolation, such modest results were overlooked by scientists interested in NMD, and we believe it is the merit of the biochemical results presented here to bring Ebs1 and Nmd4 in a more visible position for NMD research.

3. Recent studies (Feng et al, Mol Cell 67: 239, 2017; Kuroha et al, JBC 288: 28630, 2013) point to a role of Upf1 in proteasome-mediated degradation. Hence, some of the variations in polypeptide recoveries seen here may reflect protein decay roles, not RNA decay roles, and may also account for some of the differential recoveries of specific proteins. The authors must provide some indication that they have considered this potential complication and ruled it out.

It would be extremely interesting to investigate whether some protein composition changes observed in our experiments were due to the activity of Upf1 as a potential E3 ubiquitin ligase. We did not consider this hypothesis because we did not detect any specific enrichment of the proteasome components in the purifications. Moreover, the only E2 ubiquitin-conjugating enzyme detected, but not enriched, in association with Upf1, was Ubc4, and not Ubc3, the E2 protein previously proposed to interact with Upf1 (Takahashi et al., RNA 2008 PMID 18676617).

We agree with the reviewer that protein degradation should be taken into account as a possible mechanism by which proteins may appear 'lost' from a complex, when, in reality, the protein of interest is no longer present in the total extract. We specifically addressed this issue whenever it was technically possible. Thus, we show that the loss of Upf3 in association with Upf1 in the

strain lacking Upf2 (**Fig. 6A**), was not due to a loss of Upf3 in the total extract (**Fig. 6C**). The same control is shown for the reciprocal situation (**Figs. 6B** and **6D**). We also show the interesting stabilization effect for Nmd4-HA when Ufp1 fragments that can bind Nmd4 were over-expressed (**Fig. 3F**). Ideally, for every situation in which a mutant strain is used, we should be able to globally test all the proteins of interest for their level and stability. While this is extremely challenging today, the latest developments in mass spectrometry sensitivity and speed (Meier et al., Nat Methods 2018 PMID 29735998) might allow such control experiments in the near future.

4. The manuscript has a considerable amount of unprofessional boasting that needs to be toned down substantially. Use of unnecessary terms like "first extensive characterization," and the frequent ignoring of previously published work which makes similar points is not appropriate for EMBO J. In the same vein, the NMD field already has a surfeit of names for various complexes (surveillance complex, SURF complex, DECID complex) and all of them are unnecessary and may simply reflect the frequently discussed transition of the Upf proteins from recognition of a substrate to recruitment of decay enzymes, and perhaps even to recruitment of the proteasome. I strongly recommend that the authors abandon their use of "detector" and "effector" and adopt the more conventional thinking about a "pathway."

The term "first extensive characterization of NMD complexes in yeast" was used only once, in the "Synopsis" section. The only other reference to our study being the first of this size and depth was done in the initial "Abstract", and we rephrased the original sentence to make it easier to comprehend, as suggested by Reviewer #2. The sentence:"We used affinity purification coupled with mass spectrometry and an improved data analysis protocol to obtain the first large-scale quantitative characterization of yeast NMD complexes in yeast (112 experiments)." was replaced by: "We used affinity purification coupled with mass spectrometry and an improved data analysis protocol to characterize the composition and dynamics of yeast NMD complexes in yeast (112 experiments)."

We do not understand why the reviewer thinks that the manuscript is "ignoring previously published work which makes similar points". We tried as much as possible, and within the limits of our own knowledge of the field, to cite the original studies that analyzed proteins or interactions that we identified or quantified in our experiments or were related with our results. We can include other references suggested by the reviewer.

Concerning the names of the various complexes involved in NMD, it is easier to refer to a group of interacting proteins by a descriptive name. We agree that in using the names SURF and DECID, the initial authors used only limited evidence about the existence of separate complexes. The identity of SURF was mostly based on immunoprecipitation followed by focused immunoblots and on the study of a Upf1 variant that is no longer capable of participating to NMD. The situation is different about the surveillance complex, composed of Upf1, Upf2 and Upf3, as the association of the three protein has been shown by different methods, including the analysis in the current manuscript. However, the term "surveillance" was originally used in a specific context, when the prevalent proposed model involved the translocation of this complex along the 3' UTR of an NMD target, in search of a downstream element (Czaplinski et al., Genes & Dev 1998, PMID 9620853). A different nomenclature, that avoids history-charged confusion, is proposed in the current manuscript. It reflects a

biochemical reality, helps in communicating our findings and is a common practice that allows thinking in terms of functional units instead of individual proteins.

An example that illustrates the importance of calling complexes by specific names is the Mediator, a large transcription-associated molecular machine composed of 25 to 30 proteins with a major role in transcription regulation in eukaryotes (reviewed in Souturina J, Nature Rev Mol Cell Biol, 2017, PMID 29209056). It is much easier to think about this complex in terms of its role in linking RNA polymerase II to regulatory factors, than to refer to each of its subunits individually. There are countless other examples in which a complex has a name because it works as a molecular unit for a given task.

In conclusion, we use the Detector and Effector names as pragmatic shortcuts to two experimentally defined complexes, to help readers (and ourselves) cope with the relatively large numbers of involved proteins.

5. It would be very helpful if the authors could provide relative abundances of the different proteins that comprise the different complexes. The demonstration of stoichiometric relationships would certainly support their arguments.

We agree with the reviewer that having stoichiometric data about the isolated complexes would be extremely useful. However, the technology that we used does not have enough accuracy to compare the levels of proteins of different size and amino acid composition - a problem in the field since the early days of protein identification by this method (see, for example Shalit et al., J of Proteome Res, 2015 PMID 25780947). In addition to this technical limitation, the purification conditions affect to a different extent the presence of proteins in the final fraction. A possible solution to circumvent this second problem is the use of split-tag purification approaches, in which the complex associated with a tagged protein is further fractionated by a second purification of another tagged factor. This is an interesting and useful approach that we intend to use in the future, but will require a considerable scaling up of the purification conditions.

While we do not have yet access to stoichiometry information about the complexes, the mass spectrometry data was effective in comparing the levels of the same protein in different conditions, and our manuscript presents several such comparisons that are highly informative.

6. The authors state that the C-terminus of Upf1 is able to complement a upf1 deletion and restore NMD. This needs to be validated because the results in the literature are inconsistent.

The complementation of a *upf1*Δ strain through over-expression of a variant of Upf1 lacking the CH region (aa 62-152) was shown by measuring the ratio between pre-CYH2, an NMD substrate and CYH2(RPL28) as a control (Weng, Czaplinksi & Peltz, Mol Cell Biol 1996 PMID 8816462, Fig. 1B in that article). This Upf1 variant was able to decrease the CYH2 precursor to 28% of the levels found in a *upf1*Δ strain. We reproduced these results using a larger domain deletion for Upf1 (aa 1-208) and showed that the effect was dependent on the presence of Upf2, Upf3 (**Fig. 3**, **Fig. EV4** and **Appendix Fig. S4**), the decapping machinery and Xrn1 (**Fig. EV4**). It is thus very likely that the initial observation and our results reflect the ability of Upf1 lacking the CH domain to elicit NMD when over-expressed. We are not aware of literature data showing a different type of result. The apparent inconsistency signaled by the reviewer could be linked with the observation that the C-terminal Upf1 fragment lacking amino acids 1-289 is no longer able to destabilize an NMD substrate (He, Ganesan & Jacobson, Mol Cell Biol 2013

PMID 24100012) *in vivo*. However, the extended domain deletion in this case removes part of the 1B domain, which could affect RNA binding or ATPase activity of the protein fragment. Thus, the situation is not comparable with the Weng et al. 1996 paper, or with our results.

Minor concerns:

7. The authors' interpretation of their polysome data appears to be incorrect. They state that most of the Nmd4 signal was in monosomes in the Upf WT strain (and therefore state that it is NMD substrate-associated, by their reference to Heyer, et al), but this is not the case because 20% was in the polysome fraction, and 9% was in the 80S. They also claim that the upf2 deletion had similar effects as the upf1 deletion on Nmd4 polysome association. While polysome/80S association was down in the upf2 deletion, there was still twice as much Nmd4 associated with polysomes/80S as there was in upf1 deletion. This indicates that Nmd4 requires Upf1 to bind to the ribosome, and that this interaction is stabilized by Upf2. They also state that Upf1 and Nmd4 are part of their "effector" complex, but there is no evidence that Upf1/Nmd4 interaction occurs in the same complex as Upf1/Dcp1/2. They also don't reconcile this with Fig. 6a/b in which they showed that Upf1 interaction with Nmd4 is unaffected by deletion of Upf2 or Upf3. This suggests that the Upf1/Nmd4 interaction that they observe in their IP is probably occurring off of the ribosome.

Our analysis of the distribution of Nmd4 signal in the polysome gradient fractions mentioned that "While most of Nmd4 sedimented in the upper part of the gradient, a fraction of the protein was found in the polysomes, specifically the monosomal fraction…". The reviewer is right that the sum of the signal in the polysomal region for Nmd4 is larger than the one found in the monosomal fraction. To avoid any misunderstanding, we changed the original phrase to "While most of Nmd4 sedimented in the upper part of the gradient, a fraction of the protein was found in the polysomal and monosomal fractions…".

Concerning our "claim that ufp2 deletion had similar effects as the upf1 deletion on Nmd4 polysome association", as the reviewer explained, this claim is valid, even if the magnitude of redistribution observed is lower in the case of *UPF2* deletion than in the case of *UPF1* deletion.

We are sorry that the evidence that Nmd4 is part of the Upf1 complex that binds Dcp1/2 was not more visible. As shown in **Fig. 2D**, the purification of Nmd4-TAP led to a strong and specific enrichment of Upf1, Dcp1 and Dcp2, and these interactions were resistant to an RNase treatment. Conversely, we show that both Upf1 and Nmd4 were specifically enriched in a Dcp1-TAP purification (**Fig. 2F**). Thus, Nmd4 and the decapping factors were present in the same complex.

The reviewer was puzzled by the lack of a change in the observed association between Nmd4 and Upf1 when *UPF2* or *UPF3* were deleted. We agree that the interpretation of the results would have been much easier if the formation of the Effector complex were completely dependent on Upf2 or Upf3 presence. As suggested by Reviewer #1 as well, it is likely that the Effector exists both as an RNA bound and as an RNA-free complex. The fact that the absence of Upf2 and Upf3 does not lead to a change in the protein composition of the Effector suggests either that the RNA-bound fraction is minor or that its protein composition does not vary between the two states (RNA-free and RNA-bound). We thus added a sentence, just after mentioning the Detector/Effector model (**Fig. 7F, G**), which reads: "This model includes two

forms of Effector: one that is RNA-bound and depends on Detector components Upf2 and Upf3, and another that is RNA-free."

8. Fig 2G: It would be helpful if the authors included a positive control for a protein whose interaction with Upf1 is sensitive to RNase, e.g., Pat1.

We tested the reviewer's suggestion by mixing three strains that expressed Upf1-TAP and Pat1-HA, Lsm1-HA (two proteins that showed a dependence on RNA for their association with Upf1 in the mass-spectrometry results) and Edc3-HA, as a control showing no dependence on RNA. The advantage of mixing strains before the purification is that all the complexes were subject to identical conditions of RNase treatment. Edc3-HA was recovered to similar levels both with and without RNase treatment, as expected. Recovered Pat1-HA was about half of the amount purified without an RNase treatment, while Lsm1-HA was lost to levels that could not be detected by immunoblot. These data are presented now in **Fig. 2H**.

9. Fig. 1E arrows are pointing at the wrong bars.

Thank you for pointing out this error. It has been corrected.

2nd Editorial Decision 9th july 2018

Thank you for submitting a revised version of your manuscript. It has now been seen by all three original referees and their comments are shown below.

As you will see the referees appreciate that the revision has addressed several of the original concerns but they remain unconvinced about the proposed sequential role for Effector and Detector complexes. I appreciate that the experiments proposed by ref #2 to directly test this model are technically difficult but in the absence of more conclusive evidence I am afraid the referees find that the final model should be revised and that the names 'Effector' and 'Detector' should be changed. In addition, ref #3 asks for clarification on the mRNA stability assay in EDS1- and NMD4-depleted cells.

Given these remaining concerns from the referees, I ran an additional consultation session with all three of them and the outcome is that they all highlight the value and importance of the dataset generated here and that they do want to see the manuscript published in The EMBO Journal. However, you will have to tone down the claims for a sequential role for the two complexes and instead acknowledge/discuss that they complexes may act in parallel rather than in a sequential manner. The referees also agree that the names 'Detector' and 'Effector' are not helpful and should be changed.

In light of the overall positive recommendations from the referees I would like to invite you to submit a final revision of the manuscript in which you address the concerns listed above as well as the following editorial points regarding text and figures.

REFEREE REPORTS.

Referee #1:

In my opinion, the authors have done a good job and significantly improved the manuscript. It can now be published in EMBO J.

Referee #2:

While the UPF1 interactome part of the manuscript was already compelling in the initial version, the authors have added in the revised manuscript additional evidence suggesting that Ebs1 and Nmd4 are indeed NMD factors in yeast (Fig. 5F-H). In my view, this is fine and strengthens the point. That the effect of Δnmd4 alone is rather weak should not devalue the conclusion; depletion of SMG7 alone in the mammalian system gives similarly weak effects, pointing towards the existence of redundancies.
My main criticism in the initial version of the manuscript was the missing evidence for their conclusion that the Detector complex is subsequently remodeled/switched to the Effector complex. As the authors themselves state, if this was the case, one would expect a marked decrease in Effector complex in Δupf2 and Δupf3 strains (conditions that prevent Detector formation). However, this is clearly not the case (Fig. 6A,B). The authors explain this apparent contradiction to their model by postulating the existence of two types of Effector complexes, one RNA-bound and one not bound to RNA. While this is possible, no experimental evidence for this speculation is provided. Nevertheless, the prediction that the detection of RNA-bound Effector should be abolished in Δupf2 and Δupf3 strains remains valid. The authors state that they could not test this directly, "because deletion of UPF2 or UPF3 leads to a massive stabilization of NMD-sensitive RNAs and renders RNA enrichment calculations and comparisons with the wild-type situation unreliable", an argument that I do not comprehend since such enrichment calculations by definition are normalized to input. Instead, the authors performed polysome gradient fractionations (Fig. 7B-D) from which they claim that the amount of Nmd4 found in mono- and polysome fractions is reduced in Δupf2 compared to WT strains. To me the distribution of Nmd4 in these gradients looks very similar (compare Fig. 7B with 7D) and hence rather supports the notion that formation of RNA-bound Effector is not dependent on UPF2. Thus, in the revised manuscript, the claim that Effector is formed from

Detector remains unsubstantiated. In fact, the authors did not add any new data addressing this issue, despite of the concerns raised by two reviewers. Without convincing data for their Detector → Effector model (Fig. 7G), I would request that the model and all mentioning of it should be removed from the manuscript or at least declared as highly speculative. Whether the then remaining UPF1 interactome study is suitable for publication in EMBO J is eventually an editorial decision.

Specific point:
Page 1, line 22: delete "(112 experiments)"
Page 13, line 10: "since Nmd4 was lost from large RNA-associated complexes in the absence of UPF1" is a misleading statement given the data shown in Fig. 7B and D (see my comment above).

Referee #3:

The revised manuscript from Dehecq et al. includes a substantial amount of new information that will undoubtedly be of interest and use to everyone trying to understand the mechanism of NMD. Many of the questions raised by the reviewers have been addressed satisfactorily, but one key issue is still very troubling, namely the question of whether or not NMD4 and EBS1 mutations affect the half-life of an mRNA known to be an NMD substrate. This issue is addressed in Fig. 5 F-H. In this figure the authors have expressed an HA-tagged NMD-targeted mRNA under the control of a TET-off promoter. In Fig. 5G, they show that levels of the expressed protein are slightly higher in nmd4Δ cells and ebs1Δ than in WT cells, and even higher in the double mutant. These are interesting observations, but definitely not results that point exclusively to changes in mRNA abundance via a change in mRNA stability. Clearly, they could also result from unanticipated changes in mRNA translatability, or other mechanisms. To address the mRNA stability issue directly, the authors present Fig. 5H, in which they exploit a TET-off promoter to measure the levels of the mRNA in question after inhibiting its transcription in WT or nmd4Δ/ebs1Δ cells. They observe a 5' delay in mRNA decay in the double mutant, but immediate mRNA decay in WT cells, and interpret the delay as indicating a significant difference in the mRNA decay behavior of the WT and doubly mutant cells. Unfortunately, this is not the correct interpretation. Transcriptional shut off experiments are notorious for variations in the precise timing of the shut off and, consequently, the correct approach to measuring the decay rates of the mRNA in the two cell types is to examine the respective slopes of mRNA disappearance from the point at which decay has begun. For the WT cells, this means that the slope of interest would be the data points from t=0' to t=30'. However, for the doubly mutant cells, the slope of interest is that from t=5' to t=30'. (Note: this is the method employed in ~ten different papers from the Parker lab). When these two slopes are compared there is no significant difference in WT and doubly mutant cells, i.e., the mRNA has the same decay rate in both types of cells. This failure to demonstrate a direct effect on mRNA decay means that it is essential that the authors modify all their statements/conclusions about the roles of NMD4 and EBS1, i.e., they cannot imply that these proteins influence mRNA decay rates.
Other points to note:
-My previous objection to the use of the new terms "detector" and "effector" still stands. The additional terminology being used here and elsewhere are just guesses about mechanism that will only complicate the literature unnecessarily. Evidence in support of this conclusion is the title to Fig. 3, in which the authors themselves mistake Detector for Effector.
-Wilmes et al. (Mol. Cell 32: 735-746, 2008) have demonstrated positive genetic interaction between NMD4 and UPF1. This should be acknowledged.
-In Fig. 4E, several "+" marks are in the wrong places.
-Fig. EV4 is labeled as a second Fig. EV3.

Manuscript: “Nonsense-mediated mRNA decay involves two distinct Upf1-bound complexes” by M. Dehecq *et al*.

Answers to the editor’s questions

Question: As you will see the referees appreciate that the revision has addressed several of the original concerns but they remain unconvinced about the proposed sequential role for Effector and Detector complexes. I appreciate that the experiments proposed by ref #2 to directly test this model are technically difficult but in the absence of more conclusive evidence I am afraid the referees find that the final model should be revised and that the names 'Effector' and 'Detector' should be changed. In addition, ref #3 asks for clarification on the mRNA stability assay in EBS1- and NMD4-depleted cells.

Given these remaining concerns from the referees, I ran an additional consultation session with all three of them and the outcome is that they all highlight the value and importance of the dataset generated here and that they do want to see the manuscript published in The EMBO Journal. However, you will have to tone down the claims for a sequential role for the two complexes and instead acknowledge/discuss that they complexes may act in parallel rather than in a sequential manner. The referees also agree that the names 'Detector' and 'Effector' are not helpful and should be changed.

Thank you for the detailed analysis and criticism of the manuscript. To comply to the referee's requests:

1. We changed the title from "*Nonsense-mediated mRNA decay involves distinct and successive Upf1-bound complexes*" to “*Nonsense-mediated mRNA decay involves two distinct Upf1-bound complexes*”.

2. Changed the manuscript text by replacing "Detector" with "Upf1-23" and "Effector" with "Upf1-decapping", as descriptive names of the observed complexes, which do not imply a specific molecular role.

3. Carefully analysed the text of the manuscript to ensure that every time we mentioned the possibility that Upf1-23 and Upf1-decapping are successive complexes, it was clearly stated that this is a hypothesis.

Solutions to the editorial points:

E1. Please make sure the database accession numbers for the RNAseq and mass spec data are listed in the main manuscript file. Ideally, we recommend that you include a separate data availability paragraph in the Materials and Methods section (e.g. by moving it from the Appendix file).

The data availability paragraph is now included in the Materials and Methods section.

E2. 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 labeled 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.

The original gel and immunoblot images are now included as source data files for all the main figures. We also updated the figures to include the individual data points, in addition to average and SD values. Each panel is now accompanied by the corresponding numerical values.

E3. Our production/data editors have asked you to clarify several points in the figure legends (see attached document). Please incorporate these changes in the attached word document and return it with track changes activated. The data editors also suggested that using colour in the figures would make it easier to highlight differences between the samples (note that we do not charge extra for colour figures).

The number of replicates for each experiment are now indicated in the figure legends. We made a minor change to Figure 1 and used color to make it easier for the reader to spot the important elements.

E4. 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.

A file with a potential image downsized to the required dimensions is included now in a synopsis document, along with four one-sentence bullet points that highlight the main findings.

Referee comments and answers to comments and questions:

Referee #2

While the UPF1 interactome part of the manuscript was already compelling in the initial version, the authors have added in the revised manuscript additional evidence suggesting that Ebs1 and Nmd4 are indeed NMD factors in yeast (Fig. 5F-H). In my view, this is fine and strengthens the point. That the effect of Δnmd4 alone is rather weak should not devalue the conclusion; depletion of SMG7 alone in the mammalian system gives similarly weak effects, pointing towards the existence of redundancies.

My main criticism in the initial version of the manuscript was the missing evidence for their conclusion that the Detector complex is subsequently remodeled/switched to the Effector complex. As the authors themselves state, if this was the case, one would expect a marked decrease in Effector complex in Δupf2 and Δupf3 strains (conditions that prevent Detector formation). However, this is clearly not the case (Fig. 6A,B). The authors explain this apparent contradiction to their model by postulating the existence of two types of Effector complexes, one RNA-bound and one not bound to RNA. While this is possible, no experimental evidence for this speculation is provided. Nevertheless, the prediction that the detection of RNA-bound Effector should be abolished in Δupf2 and Δupf3 strains remains valid. The authors state that they could not test this directly, "because deletion of UPF2 or UPF3 leads to a massive stabilization of NMD-sensitive RNAs and renders RNA enrichment calculations and comparisons with the wild-type situation unreliable", an argument that I do not comprehend since such enrichment calculations by definition are normalized to input. Instead, the authors performed polysome gradient fractionations (Fig. 7B-D) from which they claim that the amount of Nmd4 found in mono- and polysome fractions is reduced in Δupf2 compared to WT strains. To me the distribution of Nmd4 in these gradients looks very similar (compare Fig. 7B with 7D) and hence rather supports the notion that formation of RNA-bound Effector is not dependent on UPF2. Thus, in the revised manuscript, the claim that Effector is formed from Detector remains unsubstantiated. In fact, the authors did not add any new data addressing this issue, despite of the concerns raised by two reviewers. Without convincing data for their Detector → Effector model (Fig. 7G), I would request that the model and all mentioning of it should be removed from the manuscript or at least declared as highly speculative. Whether the then remaining UPF1 interactome study is suitable for publication in EMBO J is eventually an editorial decision.

We thank the reviewer for the comprehensive and constructive criticism of the revised version of the manuscript. It is true that we concentrated our efforts on the confirmation by additional experiments of the role of Nmd4 and Ebs1 in yeast NMD, since this was a problem that was raised by the three reviewers and was the major point to be clarified after the first round of review for our manuscript.

The reviewer is right that we could not observe a change in the composition of purified Upf1-decapping complex in the absence of UPF2 or UPF3. In hindsight, it was only a small probability that we would. There is a large difference in abundance between the proteins involved in these complexes. Upf2 and Upf3 are between 3 and 10 times less abundant than either Upf1 or Nmd4. Thus, the newly formed Upf1-decapping complex, if formed through a shift from a Upf1-23 complex, can only represent a fraction of the Upf1-decapping assemblies present in a cell. The results, showing no change in protein composition in the absence of UPF2 or UPF3, were not without interest because we learnt that Upf1 can associate with Nmd4 and decapping factors independent of Upf2 and Upf3. Still, this observation does not preclude the existence of a fraction of Upf1-decapping that forms from Upf1-23. Such a succession of complexes is suggested by published data and by results from this manuscript: a) decapping is

the first step in NMD substrate degradation in yeast and previous data and our own results indicate a strong specific binding of Upf1 to decapping factors; b) binding of decapping and Upf2/3 seem to be mutually exclusive and occurs via the same domain of Upf1; c) Upf1-decapping specific factor Nmd4, like Upf2 and Upf3, co-sediments with monosome and polysomes and specifically associates with NMD substrates; d) in the absence of UPF2 or UPF3, rapid decapping of NMD substrates no longer occurs.

We did not provide additional results about the proposed shift form the Upf1-23 complex to the Upf1-decapping complex on RNA because obtaining unambiguous data on this issue depends on answering to the still unsolved question of Upf1 recruitment and role on RNA. In the following paragraphs, we discuss why the experiment proposed by the reviewer has to be done in a well controlled and large-scale setting to get to robust conclusions, which is beyond the scope of this manuscript. Why the specificity of association of Nmd4 with NMD substrates in the presence or absence of Upf2 cannot answer whether the Upf1-23 and Upf1-decapping are successive on RNA ?

1. First, estimates of the association of Nmd4 to NMD substrates in the absence or presence of UPF2 or UPF3 can also be obtained by looking at how Upf1 binds to specific RNA substrates as a function of UPF2 and UPF3 presence. This statement is based on two observations: a) the only connection between Nmd4 and the Upf1-decapping complex is Upf1, since in the absence of Upf1, the purification of Nmd4 showed that all the other factors of the complex were lost (Fig. 4B). b) The enrichment of RNA NMD substrates in Nmd4-associated fractions (Fig. 7A) is similar to the enrichment observed in association with Upf1 and it is thus very likely that the NMD substrates have a specific association with Upf1-decapping, which includes Nmd4.

If Upf1 no longer binds NMD substrates in the absence of UFP2, we can conclude that UFP2 participates both to the formation of the Upf1-23 complex and of the Upf1-decapping complex on RNA.

2. The above question, about the specificity of Upf1 binding to NMD substrates and its dependence on other factors, has been addressed in previous publications. The selectivity of Upf1 for binding to NMD substrates (the specific enrichment of NMD substrates over a control RNA) is dependent on UPF2 and UPF3, as shown by experiments done in *C. elegans* by the Anderson lab (Johns et al., 2007). Please note, however, that the input RNA is very different in a wild type or upf2Δ strain - NMD substrates are enriched in the mutant strain by a factor of 2 to 20. Thus, if the amount of intracellular Upf1 is limiting in comparison with the bound substrates, its redistribution to the available substrate RNA, when these substrates are strongly enriched in the absence of UPF2, will lead to an **apparent loss of specificity**. The interpretation of the results of such experiments is thus ambiguous - either Upf1 depends on Upf2 for binding to the substrates or it redistributes on the available population of RNA, with the net result that its specificity of binding to NMD substrates is seen as decreased. A similar situation might occur during the proposed redistribution of Upf1 to coding regions, as observed when translation is inhibited in human cells (Zund et al., 2013; Hurt et al., 2013).

3. As explained in the paragraph 2, only a careful estimation of the changes in the entire population of RNA that is bound by Upf1 in different conditions will eventually clarify whether a fraction of Upf1 (including the Nmd4 containing Upf1-decapping complex) depends on Upf2

for its binding to NMD substrates. Obtaining such estimations is, we believe, beyond the scope of the current manuscript.

4. For all the reasons stated above, polysome association of Upf1 and Nmd4 is a more reliable indication about the potential role of Upf2 in the formation of an RNA bound Upf1-decapping complex. Upf1 association to the monosome and polysome fraction is weakened in the absence of UPF2 (Atkin et al., 1997) and we observed the same effect for Nmd4, marker of the Upf1-decapping complex. There is an excellent correlation between the loss of proteins associated with Nmd4 (including the interactions that are clearly mediated by RNA, Fig. 4B) in the absence of UPF1 and the decrease of Nmd4 from the RNA-bound fraction (Fig. 7, compare C with B, with quantitations in E).

In conclusion, we do not claim that the formation of a Upf1-decapping complex could not occur independent of Upf2 or Upf3. We fully agree with the reviewer that, alone, the polysome gradient distribution changes for Nmd4 in the absence of Upf1 and Upf2 are not enough to decide whether the Upf1-23 and Upf1-decapping complexes are mandatory successive. However, the dependence of NMD on both decapping and Upf2/3, together with all our results, support the hypothesis of successive Upf1-23 and Upf1-decapping complexes. Proving this hypothesis right or wrong will advance in the future our knowledge of NMD mechanisms.

To answer the reviewer concerns, we adjusted the text of the manuscript to eliminate any strong reference to a mandatory succession of the two NMD complexes in yeast and modified both the title and the abstract.

Specific point:

Page 1, line 22: delete "(112 experiments)"

Since the abstract is the part of the manuscript that will be read most frequently, we believe it is important that the reader understands that this was a systematic effort, and the number of experiments is an important indicator of that. We would thus prefer to maintain this number in the abstract section.

Page 13, line 10: "since Nmd4 was lost from large RNA-associated complexes in the absence of UPF1" is a misleading statement given the data shown in Fig. 7B and D (see my comment above).

The line now reads: "since a significant fraction of Nmd4 was lost from large RNA-associated complexes in the absence of UPF1". We added computed *p-values* for the observed change in the relative distribution of the Nmd4 signal in the ultracentrifugation gradients (Fig. 7E) to support this statement.

Referee #3:

The revised manuscript from Dehecq et al. includes a substantial amount of new information that will undoubtedly be of interest and use to everyone trying to understand the mechanism of NMD. Many of the questions raised by the reviewers have been addressed satisfactorily, but one key issue is still very troubling, namely the question of whether or not NMD4 and EBS1 mutations affect the half-life of an mRNA known to be an NMD substrate. This issue is addressed in Fig. 5 F-H. In this figure the authors have expressed an HA-tagged NMD-targeted mRNA under the control of a TET-off promoter. In Fig. 5G, they show that levels of the expressed protein are slightly higher in nmd4Δ cells and ebs1Δ than in WT cells, and even higher in the double mutant. These are interesting

observations, but definitely not results that point exclusively to changes in mRNA abundance via a change in mRNA stability. Clearly, they could also result from unanticipated changes in mRNA translatability, or other mechanisms. To address the mRNA stability issue directly, the authors present Fig. 5H, in which they exploit a TET-off promoter to measure the levels of the mRNA in question after inhibiting its transcription in WT or nmd4Δ/ebs1Δ cells. They observe a 5' delay in mRNA decay in the double mutant, but immediate mRNA decay in WT cells, and interpret the delay as indicating a significant difference in the mRNA decay behavior of the WT and doubly mutant cells. Unfortunately, this is not the correct interpretation. Transcriptional shut off experiments are notorious for variations in the precise timing of the shut off and, consequently, the correct approach to measuring the decay rates of the mRNA in the two cell types is to examine the respective slopes of mRNA disappearance from the point at which decay has begun. For the WT cells, this means that the slope of interest would be the data points from t=0' to t=30'. However, for the doubly mutant cells, the slope of interest is that from t=5' to t=30'. (Note: this is the method employed in ~ten different papers from the Parker lab). When these two slopes are compared there is no significant difference in WT and doubly mutant cells, i.e., the mRNA has the same decay rate in both types of cells. This failure to demonstrate a direct effect on mRNA decay means that it is essential that the authors modify all their statements/conclusions about the roles of NMD4 and EBS1, i.e., they cannot imply that these proteins influence mRNA decay rates.

From the referee's criticism, we understand that we failed to provide the required technical details for the RNA decay experiment. We thus added a detailed paragraph in the *Materials and Methods* section that state that the two cultures (wild-type and double mutant strain) were processed in parallel. The figure shows now also the individual results of replicated experiments. The moment of addition of doxycycline, to block the synthesis of the reporter RNA, was virtually the same, within a 10 seconds time frame, for the two cultures that were compared (wt vs mutant). In view of the fast turnover of the used NMD reporter, the 5 minutes delay observed in the double mutant is significant, and corresponds to approximately 40% decrease in the levels of measured RNA under wild type conditions. The "RNA decay assay" section in the updated Materials and Methods is the following:

"Yeast strains were transformed with a single-copy plasmid expressing an NMD reporter RNA that uses the TET-off transcriptional repression system (Garí et al, 1997). Cells were grown overnight in a medium lacking uracil to maintain plasmid selection, diluted in YPD medium, grown for 2 to 4 hours at 30°C and shifted for 1 hour at 20°C, then split in several flasks in preparation for the time course experiment (20°C). Doxycycline was added to the wild type and mutant strain cultures at a final concentration of 10 µg/ml to ensure the presence of the drug in the cultures for 0 to 40 minutes. After repression, the flasks were incubated at the same time with agitation for 5 minutes in an ice-containing water bath, the cultures were next centrifuged at 4°C and the cell pellets kept at -80°C until RNA extraction. Reporter RNA levels were tested by reverse transcription followed by quantitative PCR with oligonucleotides that are specific to positions close to the 5' end of the mRNA (CS1127 and CS1128, amplified region between 40 and 120 nucleotides downstream the potential transcription start site) and normalized to RIM1, an endogenous mRNA."

The observed lag in RNA degradation, specific to the double mutant strain, could arise from two different mechanisms, since, as explained above, it is unlikely to be a simple experimental artifact: 1. Repression of transcription in the TET-off system might be delayed specifically in the

double mutant strain. To our knowledge, such an effect has not yet been reported for the TET-off system. 2. RNA degradation in the double mutant follows a kinetics that is more complex than first-order kinetics. Deviations from first-order kinetics have been observed in large-scale analyses of RNA decay, where measurements were best fitted by a model containing two independent parameters (Sorenson et al., 2018), although that analysis might not be best applied to our case (it mostly applies to biphasic degradation curves in which a fast decay is followed by a slower one). We agree with the reviewer that, after the initial lag, the decay rate of the RNA is similar to the wild type situation. However, it would be very unusual to ignore a 5 minute period when analysing the data for the double mutant and not do the same for the wild type strain. In previous publications (many of them from the Parker lab), when a time lag was observed in RNA decay experiments, the same time adjustment was used when estimating the half-life of the tested RNA in two different strains.

If we ignore the point at 5 minutes as an outlier, the fitted exponential decay curves correspond to an RNA half life of 6.5 minutes for the wild type condition and 11.6 minutes for the double mutant. Given all the above explanations and the other results presented in the manuscript, the simplest explanation for the observed lag in the degradation of an NMD RNA reporter in a *nmd4*Δ/*ebs1*Δ strain is that the corresponding proteins have a role in RNA decay. Whether the delay in the decrease of RNA signal in the double mutant is due to the accumulation of heterogeneous RNA species with different half-life values or with different accessibility to the degradation machinery remains unclear. To avoid any ambiguity, we replaced the term "degradation" with "decrease in RNA levels" in the paragraph describing the experiment. Please note, that the final conclusion of the paragraph refers to the RNA decay experiment in the context of the other results presented in the manuscript. The paragraph now reads:

"Since the strongest effect was seen in the double mutant strain, we tested the decrease in the reporter NMD RNA levels at different times after transcription shut-off in this condition. Compared with the wild type, the *nmd4*Δ/*ebs1*Δ strain showed a delay in the decrease of RNA levels, best seen at 5 minutes after doxycycline addition (**Fig. 5H**). These data suggest that Ebs1 and Nmd4 are NMD factors that affect the degradation of NMD substrates through their specific interactions with the Upf1 helicase and C-terminal domain."

Other points to note:

-My previous objection to the use of the new terms "detector" and "effector" still stands. The additional terminology being used here and elsewhere are just guesses about mechanism that will only complicate the literature unnecessarily. Evidence in support of this conclusion is the title to Fig. 3, in which the authors themselves mistake Detector for Effector.

It is known that RNA decapping is the first step in the Upf1-dependent degradation of NMD substrates in yeast, and our data and previous publications show a strong physical link between Upf1 and decapping factors, which led to the name Effector for the complex of Upf1 and decapping factors. We agree that, for the moment, the enzymatic activity of Dcp2 bound to Upf1 was not yet demonstrated. We thus replaced "Detector" with "Upf1-23" and "Effector" with "Upf1-decapping" throughout the manuscript. The reasons we used descriptive names for the complexes is that these biochemically defined entities contain additional factors, have a defined composition, and can be recovered by using several of the identified factors in reciprocal purifications.

-Wilmes et al. (Mol. Cell 32: 735-746, 2008) have demonstrated positive genetic interaction between NMD4 and UPF1. This should be acknowledged.

We thank the reviewer for pointing out the E-MAP data set, which includes tests done with strains deleted for UPF1, UPF2, UPF3 and NMD4. The positive genetic interaction between the deletion of NMD4 and UPF1 was present, but only ranked 122nd in the UPF1 screen and 40th in the NMD4 screen (out of 552 tested deletion or DAMP strains). When looking at the Wilmes et al. data set we observed an interesting correlation between results obtained with DAMP strains (in which a long 3' UTR extension may render an mRNA susceptible to NMD) in combination with NMD inactivation through deletion of UPF1, UPF2 and UPF3 and the situation of these DAMP strains in combination with NMD4 deletion. These observations are now mentioned in the manuscript:

"The deletion of the NMD4 gene shows a weak positive genetic interaction with the deletion of UPF1 and modifies the growth phenotype of several mutant strains expressing essential gene alleles for which the corresponding mRNAs were sensitive to NMD (Wilmes et al, 2008)."

-In Fig. 4E, several "+" marks are in the wrong places.

The displaced marks are now aligned.

-Fig. EV4 is labeled as a second Fig. EV3.

The correct label on the figure is now used. Thank you for pointing out these errors.

REFERENCES for the answers to reviewers:

Atkin AL, Schenkman LR, Eastham M, Dahlseid JN, Lelivelt MJ & Culbertson MR (1997) Relationship between yeast polyribosomes and Upf proteins required for nonsense mRNA decay. *J. Biol. Chem.* **272:** 22163-22172

Hurt JA, Robertson AD & Burge CB (2013) Global analyses of UPF1 binding and function reveal expanded scope of nonsense-mediated mRNA decay. *Genome Res.* **23:** 1636-1650

Johns L, Grimson A, Kuchma SL, Newman CL & Anderson P (2007) Caenorhabditis elegans SMG-2 Selectively Marks mRNAs Containing Premature Translation Termination Codons. *Mol. Cell. Biol.* **27:** 5630-5638

Sorenson RS, Deshotel MJ, Johnson K, Adler FR & Sieburth LE (2018) Arabidopsis mRNA decay landscape arises from specialized RNA decay substrates, decapping-mediated feedback, and redundancy. *Proc. Natl. Acad. Sci. U. S. A.* **115:** E1485-E1494

Zund D, Gruber AR, Zavolan M & Mühlemann O (2013) Translation-dependent displacement of UPF1 from coding sequences causes its enrichment in 3’ UTRs. *Nat. Struct. Mol. Biol.* **20:** 936-943

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Corresponding Author Name: Saveanu
Journal Submitted to: The EMBO Journal
Manuscript Number: EMBOJ-2018-99278

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

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?	We were not looking for a pre-specified effect size. Every experiment was performed at least three times independently, when quantitative values were to be compared.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	This study did not involve animals.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
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.	NA
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.	NA
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?	Yes.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We did not specifically test the error structure of our data. However, we applied both parametric and non-parametric tests to ensure that the obtained conclusions were robust independent on any assumption about normality.
Is there an estimate of variation within each group of data?	
Is the variance similar between the groups that are being statistically compared?	

C- Reagents

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http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repo

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
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http://www.consort-statement.org/checklists/view/32-consort/66-title

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

http://datadryad.org

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http://biomodels.net/miriam/
http://jjj.biochem.sun.ac.za
http://oba.od.nih.gov/biosecurity/biosecurity_documents.html
http://www.selectagents.gov/

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).	Anti-HA monoclonal antibodies (3F10) coupled with peroxidase and Peroxidase-anti-Peroxidase (PAP) complexes have been previously validated as specific reagents for yeast cell extracts.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	No cell lines were used, only yeast strains.

* for all hyperlinks, please see the table at the top right of the document

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.	NA

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for '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	References for mass-spectrometry and RNA sequencing results were deposited and referenced.The accession number for the sequencing data reported in this paper is GEO: GSE102099. The MS proteomics data that support the findings of this study have been deposited in the ProteomeXchange Consortium via the PRIDE repository (Vizcaíno et al, 2016) with the dataset identifier PXD007159.
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).	No other large scale data sets were generated in this work.
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
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G- Dual use research of concern

22. 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.	NA