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Splicing-dependent NMD does not require the EJC in Schizosaccharomyces pombe

Jikai Wen, Saverio Brogna

Corresponding author: Saverio Brogna, University of Birmingham

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Correspondence 08 June 2009

Thank you for submitting your manuscript for consideration by The EMBO Journal. I have now had the chance to read it carefully and to discuss it with my colleagues. In addition, I have sought expert advice on your study from one of our external advisors. The outcome of these discussions is that we find your manuscript interesting: the fact that none of the current models for NMD can explain your results in S. pombe suggests that new models are required, which may also be applicable in other systems. This will clearly stimulate discussion in the field.
However, both we and our external advisor are hesitant about sending your manuscript out for review as it is, because you do not propose an alternative hypothesis for how NMD may be triggered. It goes without saying that a manuscript will likely fare better under review if it provides a clear explanation for the results, rather than just stating that current models can not explain them.

Our inclination is to send the manuscript out for review in any case, since there is clearly interest in the subject. However, I am writing to offer you the possibility to modify your manuscript before we seek input from referees. If you could provide a model that might account for your observations (and other published results inconsistent with current models), we think it would be in your interests to do so - even if it is only supported by preliminary data.

Please can you let me know how you would like to proceed: if you would like (and are able) to extend your manuscript to incorporate such a model, we will delay sending it out for review until you send a revised version of the manuscript.
However, if you would prefer that we send the manuscript to reviewers in its current format then we can, of course, do this straight away.

I look forward to hearing from you.

With best wishes,

Editor
The EMBO Journal

Additional Correspondence (author) 09 June 2009

Thank you for the suggestion. We can include a figure with a schematic of models which could explain the data, but we are a bit hesitant to do so at this stage because these models would need to be radical different than current ones and we feel that they will attract additional skepticism. Many colleagues in the field perceive NMD as a single and well-defined process and as such they call for a single mechanism that can explain all NMD features and that possibly is conserved across organisms. We think that this point of view is misleading: our data, but also published observations, indicate that NMD may be the consequence of diverse mechanisms even in the same organism. We have articulated this point of view in our recent NSMB Perspective (Brogna & Wen, 2009).

We can definitively provide a reasonable model to explain the splicing-independent component of NMD; this would be based on the "ribosome release" hypothesis we described in the NSMB Perspective (Figure 2). But, how can we explain the effect of splicing on NMD? In the discussion we proposed that the effect may be mediated by some factors that remain associated with the mRNP after splicing, but unlike the EJC these factors are not in direct contact with the exon junction. We have not mentioned it in the current version of the manuscript, but another way to explain the observation is to propose that PTCs are recognised during splicing - we and others have suggest this in the past but clearly the issue is very controversial. We feel that rather than proposing models without strong experimental support we should continue investigating the splicing-NMD link without preconceived hypothesis and for the moment just present the facts. I just hope that the reviewers will appreciate this thorough but somewhat minimalist way of presenting the data.

Thanks a lot and I look forward to hearing from you.

Additional Correspondence (author) 09 June 2009

Thanks for your messages - I think that this will work out for the best during the review process. Obviously I don't want to suggest you propose models that are not backed up by any data, but we think that some speculation as to how your data can be reconciled would be advantageous.
We have withdrawn your original submission, and if you can just resubmit all the files once you have modified the manuscript, I will send it out for review then.

I look forward to receiving the new version.

1st Editorial Decision 23 July 2009

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by three referees, whose comments are enclosed. As you will see, all three referees recognise the potential interest in your results, which call into question current models for NMD. However, the referees vary widely in their enthusiasm for your manuscript. Referee 1 raises no technical issues, but finds that the study presents primarily negative data and does not address how NMD might be triggered in your system. Consequently, he/she is not supportive of publication. Referee 2,

conversely, is generally positive and recommends publication contingent upon a number of issues being resolved. Referee 3 finds your data interesting, but is not convinced by the proposed model, and feels that significant additional analysis of more constructs would be required to and to strengthen your proposal.

Given these rather contradictory reports, I have discussed your study further with my colleagues, including our Executive Editor Pernille R¯rth, and with an external advisor. Following these discussions, we would like to give you the opportunity to revise your manuscript according to the comments of referees 2 and 3. I should stress that it will be particularly important to provide stronger evidence - along the lines of point 2 in referee 3's comments - regarding the importance of distance from the PTC to an intron, and thus to further substantiate your model.

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

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

Yours sincerely,
Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

NMD is an RNA surveillance process that degrades both aberrant and physiological transcripts. Although the phenomenon is apparently universal in eukaryotes and the main factors Upf1-3 required for NMD are conserved, studies in various systems have so far failed to reveal a common fundamental mechanism. The major findings reported here are that NMD in S. pombe can be stimulated by the presence of an intron close to the PTC, regardless as to whether it is up- or downstream of the PTC, and that NMD is independent of an exon junction complex. Furthermore, NMD was shown to be active in mutants that lack the cytoplasmic poly(A)-binding protein, Pab1. Other experiments showed that NMD showed a strong position effect, while the length-dependence experiments had less clear outcomes and no strong conclusions could be drawn. The experiments are technically well carried out, the data are clearly presented and generally the experimental findings support the conclusions well. The experimental data that suggest a dependence upon splicing that is independent of an exon junction complex clearly represent the key dataset. These findings potentially provide a completely new perspective on the basis of NMD. Intriguing as the observations may be that magoh and rnps1 are not required for intron-stimulated NMD, this is negative data and, in its current form, the manuscript provides too little insight into the nature of an alternative "trigger" for NMD.

Referee #2 (Remarks to the Author):

Jikai Wen and Saverio Brogna provide here the first detailed study on NMD in Schizosaccharomyces pombe. Mainly relying on a GFP reporter gene systems, Wen and Brogna focused on the influence of introns (in particular their relative position to the nonsense codon) on NMD and discuss their results in context of the two prevailing NMD models in the field, the "EJC model" and the "Faux 3' UTR model", respectively. They conclude that neither of the models satisfactorily explains their results and propose a new model to explain how splicing could affect NMD.
The main findings are:

- Splicing of a PTC-containing mRNA stimulates the mRNA's susceptibility to NMD.
- Surprisingly and inconsistent with the EJC model, splicing promoted NMD regardless of whether the intron was located upstream or downstream of the PTC.
- Deletion of the S. pombe homologs of the EJC factors MAGO and RNPS1 did not affect NMD.

Their results are intriguing and the apparent inconsistencies with the EJC and the faux 3' UTR models demonstrate our still limited mechanistic understanding of mRNA surveillance processes. Although it is possible that mechanistic differences exist with regard to NMD between S. pombe and other organisms, it seems more likely that the current models are not accurate. The main impact of this work is to introduce S. pombe as an additional model system to the NMD field and to stimulate the ongoing discussion about mechanisms and mRNP features that select an mRNA for NMD.

Provided the following points can be satisfactorily addressed by the authors, I recommend publication of this paper in EMBO Journal:

1. Insertion of the intron into their GFP reporters results in detection of a significant fraction of unspliced reporter RNA from some constructs under some conditions (Fig 2 C-F). Since this unspliced RNA is also an NMD substrate, it may affect the overall NMD efficiency in a cell, for example by saturating the NMD pathway, and hence influence the corresponding spliced reporter RNA levels. Furthermore, potential effects caused by the PTC-generating mutations on splicing efficiencies would also influence the steady-state mRNA levels. It is particularly important to address these concerns in the presented results, because the pre-mRNA levels vary considerably between constructs and between different conditions. For example, much less ivsPTC6 pre-mRNA than ivsPTC27 pre-mRNA is present in wt cells, but in Upf2 deletion strain, pre-mRNA levels of ivsPTC6 and ivsPTC27 are very similar. What is the reason for this and how could it influence the authors' conclusions?
2. In Fig. 2F, a larger part of the autoradiograph that also includes the bands for the pre-mRNA should be shown in the upper panel (as in Fig 2E).
3. The different ivsGFP constructs should be checked for correct splicing. A northern blot gives not enough size resolution to assess that. For example, RT-PCR products could be cloned and sequenced or RNase protection assays could be used.
4. Fig 4B: Plesae clarify what has been quantified here. The legend says "GFP mRNA", but I suspect that in lanes 5 - 8 the unspliced mRNA and in lanes 1 - 4 the spliced mRNA has quantified. That should be clearly pointed out.
5. Addition of a DAPI or Hoechst stain would help to appreciate the cellular distribution of the reporter transcripts in Fig 4C.
6. How do the authors explain the almost 2-fold and over 4-fold mRNA increase of ivsPTC27 and ivsPTC140, respectively, depending on whether the intron is inserted upstream of the stop codon or in the 3' UTR (Fig 6 B)? Furthermore, insertion of the intron into the ORF of ivsPTC27 and ivsPTC140 leads to the detection of a significant population of unspliced transcripts, suggesting that this intron is not spliced out efficiently. A similar accumulation of pre-mRNA is not observed with ivsPTC6 and GFPivs, which raises the same concerns as described above under point 1.
7. Fig 7B: it is pointed out that the 3' UTR extension by102 nucleotides caused a reproducibly higher mRNA lavel of PTC140 compared to the construct with the original 3' UTR. However, is the difference statistically significant given the relative large standard deviations?
8. The nomenclature is inconsistent and confusing and should be revised to facilitate the reading of the paper. I suggest calling the GFP without PTC simply "WT". "GFPivs" would then become "WTivs" and "ivsPTC6" should be called "PTC6ivs". The authors seem to be confused themselves by their current nomenclature: in Fig 4B, the construct GFP construct without PTC and with mutated 5' splice site is called "GFPivs-ss", and in Fig. 4C, the same construct is called "GFPss". According to my suggestion, this construct could be called "WTss" or perhaps more intuitively "WTmut-ivs".
9. You state that S. pombe has no detectable MLN51 homologue. It is my understanding from the EJC core structure that without MLN51, no EJC could be stably bound to the RNA. This should be discussed in the context of whether S. pombe mRNAs harbour EJCs.
10. On page 15, it says "there are cases in which UPF1 and UPF2 are not required for NMD". I believe this is a typo, since UPF1 is always required for NMD. These papers have reported UPF2- and UPF3-independent NMD.

Referee #3 (Remarks to the Author):

The nonsense-mediated mRNA decay (NMD) pathway is an mRNA quality control mechanism that detects and degrades mRNAs with premature translation termination (PTC) codons. Study of NMD in different species revealed that several mechanisms exist to define PTCs as opposed to natural stops. The best documented mechanism is the splicing-dependent NMD observed in mammals where premature codons are identified when they are located at least 50 nt upstream of an exon-exon boundary. The position of the exon-exon boundary is communicated to translating ribosomes by the exon junction complex (EJC) that recruits the Upf proteins. In other species like S. cerevisiae or D. melanogaster, stop codons are considered as premature when positioned to far upstream of the 3'-end polyA tail that binds the protein PABP (faux 3'-UTR model). Recent data showed that the two models are not exclusive as observed in plants and mammals and are not sufficient to explain the degradation of all PTC-containing mRNAs. In this context, the study presented by Wen and Brogna tempt to dissect mechanism(s) of PTC recognition in S. pombe, organism in which almost nothing is known about NMD.
To study PTC recognition in pombe, the authors constructed numerous GFP reporters containing PTC and/or intron at different positions. They also analyzed the endogenous intron-containing ypt3 gene with or without PTC. Measurement of the expression of all these reporters, mainly by northern blots, conducted the authors to draw the following conclusions : (i) NMD presents a polar phenotype because PTC close to the 5' extremity triggers stronger decay than PTC close the 3' end. (ii) In some cases, lengthening of the 3'-UTR induced NMD but the faux 3'-UTR model is most likely not the major mechanism because NMD does not require PABP. (iii) The presence of a spliceable intron increases the efficiency of NMD of close PTCs (either upstream or downstream) and this, independently of the EJC components Magoh and RNPS1. These results show for the first time that, like in other eukaryotes, several mechanisms co-exist in pombe to detect PTC-containing mRNA and that splicing influences with NMD, a link that has been shown exclusively in human and zebrafish cells. For these reasons, this manuscript brings an interesting piece of data in the field. Overall, the manuscript is well written and the experiments are of good quality and mostly adequately controlled. However, I consider that data are missing to reach the proposed model concerning the mechanism by which splicing influences NMD and moreover, this model is hard to conceptualize.

Major comments :
1 - Splicing has pleiotrophic effect on mRNA metabolism and notably a positive effect on translation that may indirectly alters NMD efficiency. The authors said that they "found no correlation between changes in translation yields attributable to the position of the intron and NMD". The western blot presented in Figure 6E is not sufficient to reach this conclusion. Translation efficiency of reporters with or without introns must be measured more precisely.

2 - The authors proposed that an intron influences NMD only when the PTC is close to the intron and this, on either side in contrast to what has been clearly observed in mammals. Not enough data are presented to generalize this model and some results are contradictory. In Figure 6B, the mRNA ivsPTC140 (85%) must be degraded more efficiently than the mRNA ivsPTC27 (44%) in which the PTC is at a greater distance. The model is mainly based on the reporters shown on Figure 6A. Similar experiments should be done with a PTC located upstream of the intron (and not only downstream like PTC 140) and with insertions of variable length also upstream of the intron. Analysis of additional PTCs located on either side of the introns of the endogenous gene ypt3 would also strengthen the model.

Minor comments :
1 - Given that deletion of Upf2 stabilizes the wt mRNA (Figure 1G), why not presenting the results obtained with Upf1 deletion?
2 - Can we really discuss about a polar effect with 3 PTCs (Figure 1)?
3 - It may help the reader if the complete name of each construct is indicated on the top of the gels (see notably Figure 6A & B).

Response to the referees' comments.

Referee 1
No specific points are raised. As for the criticism that our conclusion that MAGO and RNPS1 are not required for NMD is based on negative data, it is not clear how differently we should have done it. The hypothesis was that the two proteins are required for NMD, to test it we did some straightforward experiments and the results (which we have shown) indicate that neither protein is required. To us these seem positive data like the rest of the results. As for the critique that the manuscript provides too little insight, we think it is not a fair evaluation: we provide for the first time an extensive characterization of NMD in S. pombe and the results call into question the two main models championed by the field, the EJC and faux 3' UTR. However, we think the manuscript is interesting not just because it raises a red flag for how the NMD phenomenon is currently rationalized, but also because it introduces a novel model system which we think will be particular useful in understanding the link with splicing. We find particularly insightful the finding that splicing strongly enhances NMD only when the intron is not too far from the PTC, regardless of whether the intron is before or after the PTC (we present additional supporting data in this revised manuscript). This novel finding provides a clear indication that there is a direct link between splicing and NMD which is independent of the EJC or similarly defined complexes. Of course, our study opens new questions; our model is an attempt to rationalize our current data and to guide future studies. Future work should be aimed at testing the generality of our model in other organisms and at identifying which trans-acting factors are involved.

Referee 2
1. While it is true that pre-mRNA accumulation is apparent with some of the reporters (see answer to point 6), the pre-mRNA is just a small fraction of the spliced product and we did not think that this might saturate the NMD capacity of the cell by exhausting the pool of free NMD factors for example. In addition pre-mRNA accumulation is most apparent with the PTC27 and PTC140 reporters, yet NMD is very efficient with both reporters. Initially, we were concerned that overexpression could saturate the NMD pathway; that is one of the reasons why we also checked whether NMD efficiency is different with integrated reporters, which produce about tenfold less mRNA than the plasmids. However, we found no differences, which argues that a high level of substrate does not saturate the NMD pathway. Instigated by the Referee's comments, we have further addressed the issue by making two more constructs (WTnmt1 & PTC6nmt41) with a stronger promoter (nmt1) that produces about eightfold more mRNA than nmt41, which was used in the previous reporters. We found that that PTC6 still produces strong NMD: thus NMD is not saturated even when the reporter is expressed at very high level; in fact NMD efficiency is very similar between reporters that that differ almost two order of magnitude in expression (we refer to this additional data in the revised manuscript; see supplementary Fig 2). As for the more specific issue of why there is pre-mRNA accumulation with some reporters, please see answer to point 6.

2. We have changed Fig 2F as suggested. Now we show a film in which the pre-mRNA bands are clearly visible.

3. We had not tested splicing accuracy by RT-PCR because there was no indication of problems: splicing accuracy is indicated by the fact that in the PTC-less controls produce GFP, if splicing was not accurate the GFP ORF would be mutated and no functional protein produced. Yet it is possible that splicing is affected in the PTC reporters. On the suggestion of the referee we have RT-PCR amplified the GFP transcripts produced by different intron-containing reporters (WT-ivs, PTC140-ivs and WT-3'ivs); we have sequenced 4 plasmid clones of the PCR products and found that all sequences were spliced correctly. While it is possible that a small fraction of transcripts are not spliced correctly, the fact that we have not seen evidence of it in the clones we have sequenced suggests that most of the transcripts seen by Northern blot are correctly spliced.

4. The Referee is correct: in Fig 4B, we quantified the spliced mRNA in lanes 1-4, and the unspliced transcript in lanes 5-8. We have now specified this in the figure legend.

5. The reason why we didn't stain with DAPI or Hoechst dyes is that the microscope we used didn't

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have the right filter cube to do this. Given that the mRNA seems to be present throughout the cell regardless of the reporter - in none of the cells we detected a clear dot-like signal - we thought that visualization of the nucleus would not add more information to the experiment anyway.

6. We think that the PTC27 and PTC140 do not trigger strong NMD in the reporters with the 3' UTR intron because in both transcripts the PTC is too distant from the splice site (664 nt in PTC27-3'ivs and 321 nt in PTC140-3'ivs); instead in the reporter with the intron in the ORF, the PTCs are closer to the intron (250 nt in PTC27ivs and 89 nt in PTC140ivs). As for the significance of the pre-mRNA accumulation, we agree: the intron is probably not very efficiently spliced in the PTC27 and PTC140 reporters. As can be seem in the manuscript, we observed this in many experiments, but in some instances we see no clear difference between control and PTC reporters (see Fig 2E for example). What does it mean? Maybe it means that a PTC can affect splicing? The issue is controversial and at this stage we prefer not to speculate; but we have mentioned this pre-mRNA accumulation in the new manuscript (beginning of page 7). As for the PTC6 pre-mRNA, we think that there is less pre-mRNA because this transcript is subjected to splicing-independent NMD - PTC6 induces very strong NMD of the mRNA of the intro-less reporter (Fig 1) and of the unspliced transcript with a mutant 5' splice site (Fig 4).

7. In all Northern blots we have seen more mRNA in the reporter with the 102nt extension. We agree the difference is not big and it may not be statistically significant; we just reported what we have seen in many experiments. The mean level of the PTC140 mRNA with the 102-nt insert is more than the highest level observed for the transcript without the insertion. However, the sample size is too small to do any meaningful statistic test. In the manuscript we have changed "significantly" to "reproducibly" when referring to this variation, to prevent misinterpretations (page 11).

8. Thank you. This nomenclature could improve the reading: we have changed it as suggested.

9. We agree, on the basis on what is known about the EJC it is unlikely that an EJC can be formed without MLN51 - now we mention it in the Discussion.

10. That was typo; we have changed it - thank you.

Referee 3

1 We agree that there is substantial evidence to indicate that splicing can enhance translation in many systems. Although there is no experimental evidence for it in S. pombe, we were initially as concerned as the Referee that an intron might enhance NMD indirectly. To address this issue further we have made some more reporters in which a GFP construct is joined at the 3' end with the coding region of firefly luciferase (new Fig 6F); these reporters allow to compare translation yields more accurately. Surprisingly, we found that the presence of an intron reduced translation efficiency to some extent rather than enhancing it (new Fig 6G). Whether this is a general feature in S. pombe at this stage is premature to say, but these observations indicate that splicing does not enhance translation of our reporters. More specifically with these new reporters we could compare the translation efficiency of the reporters with different insertions (new Fig 6C) more accurately than with the Western blot. We found that the translation efficiency of the spliced mRNAs is similar and that the reporters with the inserts are not translated less efficiently; these results are in agreement with the Western blot (Fig 6E). We think that the additional data exclude that the lengthening of the distance between intron and PTC reduces NMD because translation efficiency is reduced.

2. Yes we propose that an intron strongly enhances NMD only when it's not too far from the PTC, we think this is an important finding and to be more sure of this conclusion we have followed the Referee's suggestions and made several new reporter constructs. Altogether, the results are consistent with the earlier conclusion. In a set of new constructs we have placed a spacer between the stop codon and the 3'UTR intron (see new Fig 5D). As we have shown before, a 3' UTR intron causes strong NMD when close to the stop codon; instead, in the reporter with the insert we found that the mRNA level is partially restored (new Fig 5E, lane 2 vs 4) - in this experiment the mRNA levels were estimated also by real-time RT-PCR, which confirmed that the mRNA with the spacer is about 2.5 fold more abundant (see supplementary Fig 3); we used qRT-PCR because the Northern bands were appreciably smeared (perhaps the intron can influence 3' end formation) and we felt that the quantification with the phosphoroimager underestimates the amount of transcript. In agreement

with the mRNA quantifications, the reporter with the spacer yields more GFP (new Fig 5G, compare WT-3'ivs and WT-147-3ivs), suggesting that lengthening the distance not only suppresses NMD but it also does so without impairing steady-state translation efficiency. In another new construct we have moved the intron to the very beginning of the GFP coding region and found that at this position (417 nt from the PTC) the intron does not enhance NMD of the PTC140 mRNA - yet the intron is spliced very well, we see no pre-mRNA accumulation (see new Fig 6A and B). As suggested by the Referee we have made a set of reporters with a PTC before the intron (PTC88) and different inserts, unexpectedly, we found that PTC88 does not trigger NMD. This is the only PTC we have found that does not induce NMD even if located close to an intron. This is an exception to the rest of the data, but there may be specific reasons for why this PTC behaves differently. We suspect that PTC88 does not induce NMD because there is an AUG 10 nt after PTC88 (unfortunately we had not noticed it when planning the constructs), which probably suppresses NMD by driving translation re-initiation as suggested by previous studies (for a discussion on how re-initiation could suppresses NMD see Brogna&Wen, 2009). In summary, we think that altogether our data justify our conclusion that the closer the intron is to the PTC the stronger is NMD. NMD, however, is most likely the consequence of different mechanisms; therefore there may be other situations in which PTCs do not cause NMD even if located close to an intron; and some PTCs can induce NMD independently of splicing as we have shown; different mRNA features can affect NMD positively or negatively. As suggested we have also analysed an additional PTC in the last exon of the ypt3 gene (PTC183); we found that NMD is enhanced in the intron-containing reporter compared to the cDNA derivative (see new Fig 3C and 3E). As for the criticism that some of our results are contradictory. We think this is a misunderstanding; the results are as predicted and in line with the other observations in the manuscript. In the reporter with a 3'UTR intron, our model predicts that neither PTC27 nor PTC140 mRNAs is affected by splicing because the intron is too far from the PTCs (664 nt and 321 nt respectively): the PTC27 mRNA is degraded more than PTC140 because it is subjected more to splicing-independent NMD because it is closer to the beginning of the coding region.

Minor points

1. We have not measured mRNA stability in Upf1 as precisely as in Upf2 ; however, based on what we have seen in Northern blots of steady-state RNA, deletion of Upf1 seems also to stabilize the PTC-less control; we think that it is this mRNA stabilization that results in enhanced GFP expression in both mutants (see Fig 1H). Throughout the study we mostly used the Upf2 deletion because it grows better, which probably is due to Upf1 having additional functions separate from NMD - for example published data suggest an involment of Upf1 in DNA replication, preliminary work in our lab indicates that this might also be true in S. pombe.

2. We agree, to address the polar issue we should test more positions. It is unlikely that the polar effect is linear, that is the reason we refer to polar between inverted commas.

3. We have changed the labelling of the constructs as advised by Referee 2, we hope it is clearer now.

Additional correspondence (editor) 01 March 2010

Many thanks for submitting the revised version of your manuscript EMBOJ-2009-71693R. It has now been seen again by referees 2 and 3, who both find the revised manuscript to be substantially strengthened and are now fully supportive of publication (their comments are appended below). Therefore, I am pleased to tell you that we will be able to accept your manuscript for publication in the EMBO Journal.

However, there are just two minor issues that need to be dealt with before we can accept the study. Firstly, I notice that in Figure 2D, the gel seems to have been rotated and/or cropped, and it's not quite clear what has been done here. I would therefore ask you to replace this panel with a properly cropped image that doesn't cut off the bottom of the bands; could you please also send us the original, uncropped scan for our records? Secondly, we are trying to encourage authors to include a clear statement as to author contributions in their acknowledgements section. Could you please modify the text file to include this?

I suggest that the easiest way to make these minor changes would be for you just to send us the new files by e-mail; we can then upload them in place of the originals. Once we have these, we should then be able to accept your manuscript without further delay.

Thanks again for all the effort that you and Jikai put into the revision of this manuscript - it's a really nice study and I'm happy to be publishing it!

Referee 2:

Wen and Brogna have carefully revised their manuscript and improved it according to the referees advice. All my previously raised issues have been satisfactorily solved, in particular my concerns about saturation of the NMD pathway. By expressing their reporter genes from promoters with different strength, the authors could demonstrate that under the conditions used throughout the manuscript, the NMD pathway is not saturated.

In my view, this paper will receive much attention in the RNA surveillance community for two reasons: 1.) it provides the first extensive study about NMD in S. pombe, and 2.) it challenges current working models in this field.

Referee 3:

The authors have included additional data in response to the most important comments of the reviewers 1 & 3 and they revised the manuscript. They notably included the study of new constructs containing PTC at different positions and they have adequately controlled the effect of PTCs on translation efficiency. I consider that the manuscript is now acceptable for publication.

Additional correspondence (author) 03 March 2010

Thanks a lot for the good news and the nice comments.

The line across the picture in Fig 2D is just an artifact of our phosphorimager scanner that sometime create these shadows across the filter. We have not done any cropping and pasting, or any other manipulation. We will send you a picture of the full scan as an additional supplementary figure as soon as possible. I have attached the new manuscript file with the amended acknowledgments.

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