EMBO reports

Manuscript EMBOR-2015-40084

The RNA surveillance complex Pelo-Hbs1 is required for transposon silencing in the Drosophila germline

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Review timeline:

Submission date:	13 January 2015
Editorial Decision:	02 February 2015
Revision received:	07 May 2015
Editorial Decision:	25 May 2015
Revision received:	27 May 2015
Accepted:	02 June 2015

Transaction Report:

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

Editor: Esther Schnapp

1st Editorial Decision 02 February 2015

Thank you for the submission of your research manuscript to EMBO reports. We have now received the enclosed referee reports on it.

As you will see, all referees agree that a role for the RNA surveillance complex in transposon silencing is potentially interesting, however, they also point out that the current data are not sufficient to support this hypothesis. All referees request that the expression levels of pelo RNA and protein in the different mutants, and the RNA and protein levels of the transposable elements in control, Hbs1 and pelo mutants need to be adequately quantified. Referees 1 and 3 also indicate that it should be investigated whether other transcripts, like mRNAs, are upregulated in pelo mutants. Referee 1 further mentions that it should be addressed why pelo-rescued flies are still sterile, and that the overstatements in the abstract and text need to be toned down. Otherwise, direct evidence for regulation of transposon expression at the translational level needs to be provided.

Given these constructive comments, we would like to invite you to revise your manuscript with the understanding that the referee concerns (as mentioned above and in their reports) must be fully addressed and their suggestions taken on board. Acceptance of the manuscript will depend on a positive outcome of a second round of review. It is EMBO reports policy to allow a single round of revision only and acceptance or rejection of the manuscript will therefore depend on the completeness of your responses included in the next, final version of the manuscript.

Revised manuscripts should be submitted within three months of a request for revision; they will otherwise be treated as new submissions. Please contact us if a 3-months time frame is not sufficient for the revisions so that we can discuss the revisions further. Also, the revised manuscript should not exceed 35,000 characters (including spaces and references) and 5 main plus 5 supplementary figures. Commonly used materials and methods can be moved to the supplementary information, however, please note that materials and methods essential for the understanding of the experiments described in the main text must remain in the main manuscript file.

Regarding data quantification, please specify the number "n" for how many experiments were performed, the bars and error bars (e.g. SEM, SD) and the test used to calculate p-values in the respective figure legends. This information must be provided in the figure legends.

I look forward to seeing a revised version of your manuscript when it is ready. Please let me know if you have questions or comments regarding the revision.

REFEREE REPORTS:

Referee #1:

The repression of transposable elements (TEs) in metazoan germ cells requires small non-coding RNAs, termed piRNAs, that are associated with Piwi proteins. Here, the authors identify the RNA surveillance complex Pelo-Hbs1 as a new component for TE silencing in Drosophila germ cells. Interestingly, the complex mediates TE silencing independently of piRNA pathway.

The authors analyze TE expression in several pelo mutant strains. They find that expression levels of some of TEs are up-regulated in pelo mutant ovaries. This derepression of TEs does not correlate with low levels of piRNAs and components of piRNA pathway. Actually, expression of these piRNA pathway components appears to be unchanged in pelo mutants. This finding suggests that Pelo may function in selective degradation of some TE transcripts, though we aren't assured that other cellular transcripts including protein-coding mRNAs are also up-regulated in pelo mutants.

Given that the authors provide little data suggesting "translational control" of TEs, the statement "possibly through the no-go decay mechanism, to prevent translation of TEs" in Abstract is misleading and should be substantially weakened.

While this paper is important conceptually, additional experiments are required to strengthen the main conclusions of the paper.

(1) On the whole, it is not clear how the authors perform many of experiments shown in the paper. Figure legends are not kind to the reader at all. For example, we aren't even assured that pelo mutants shown in Fig S1 are null or hypomorphic when they are homozygous or trans-heterozygous. The authors should show levels of Pelo protein in these mutants. Another example is found in Fig 2C, in which the authors indicate mutant genotypes with +/- and -/-. What do they mean by "+/-" of Aub[QC42/HN2] and pelo[1/PB60]?

(2) With regard to the data in Fig. 1 that suggest that "Pelo affects protein level more dramatically than mRNA level of TEs", simple comparison of transcript levels measured by RT-PCR and protein expression levels measured by immunostaining is not even sufficient but flawed. We do not know, for example, how TE transcripts measured are (un)stable and we don't know turnover rates of proteins the authors immunostain either.

(3) Fig. 3E: The authors conclude that "the P210A mutant Pelo had significantly reduced ability to repress TE levels". But only for 1731 element. Levels of Het-A are unchanged or even more upregulated with P210A. The authors shall consider demonstrating that P210A cannot interact with Hbs1 using an assay shown in Fig 3B.

(4) Fig 4: Expression of mouse Pelo (mPelo) gene driven by nosGal4/UAS in Drosophila is able to

rescue the observed defects found in pelo mutants, including TE depression and defects in spermatogenesis. However, this cannot rescue the sterility of the pelo mutants. Spermatogenesis in flies with mPelo proceeds normally but they are still sterile. Why? The authors find that ubiquitous expression but not germline-restricted expression of mPelo rescues the sterility of the pelo mutants. Why?

Referee #2:

The detection of a role of RNA surveillance complex in transposon silencing seems very interesting. However I have some critical comments.
1.Fig.1A presents the effect of strong null peloPB60 mutation and significantly weaker effect of peloPB60/pelo1 transheterozygous state on transposon up-regulation, while pelo1 was earlier described as a strong mutation. Then the authors switched to study mainly the effects of this transheterozygous state and unfortunately corresponding explanations (or comments) of this choice, as well as short discussion of allele effect differences are absent.
2.In the beginning of this paper related to Fig.1A no indication of the attempts to rescue the effects by polo transgene is indicated and only later in Fig.3F the rescue of peloPB60/pelo1 (but not pelo PB60) is mentioned.
3. p.5, first paragraph- it is mentioned that « the magnitude of TE up-regulation was correlated with the strength of pelo mutants», but who has evaluated this strength? The authors also used the third mutation (Fig.S2), but at RT its effects again differ drastically from peloPB60 effects(Fig.1).Generally, these experiments using different polo mutations are causally described.
4. The results presented in Fig.1B do not allow to conclude that pelo affects I-element protein level more dramatically than mRNA level, because both mRNA and ORF expression is needed to be measured quantitatively in the oocyte.
5. Accumulation of Stellate protein in testes owing to polo 1/BP60 transheterozygous state was shown (Fig.1C), but this story seems to be stopped in its beginning. SuSte piRNA quantities (at least abundant ones) are not analyzed, but it seems significant to be performed in order to accept that piRNA is not involved in this case of Stellate derepression, because the following text is intended to exclude the role of piRNA biogenesis violations in TE up-regulation. But in the end of Discussion the role of piRNA in polo mutation effects is not neglected.
Generally the whole text contains semantic contradictions.
6. I do not understand the conclusion based on the observed moderate activation of heterochromatic rather than euchromatic Stellate transcripts that led authors to conclusion of preferential translational Pelo effects, while heterochromatic Stellates are known to contain perfect ORFs.
7.This paper does not contain any mention concerning the role of pelo mutation effects on GSC maintaining and disturbances of ovarian development.
Generally, this paper contains several «light conclusions» that are needed to be corrected. It would be useful to present some speculation, suggesting possible reasons of RNA surveillance participation in TE silencing.

Referee #3:

The manuscript by Yang et al. reported that Pelo-Hbs1 complex is involved at repression of transposable elements on translational level, possibly through no-go decay (NGD) mechanism. The authors first show the de-repression of transposon RNA and proteins in different pelo mutants. pelo mutants didn't affect piRNA biogenesis nor the expression of many piRNA pathway genes. Authors further showed that Pelo forms a complex with Hbs1, which mutant also moderately increase transposon expression. Overexpression of RpS30s, a NGD downstream protein, rescue the pelo phenotype. Finally, the authors rescue fly pelo mutant phenotypes with mouse pelo protein and proposed the conservation of pelo function in transposon silencing in mammals. The overall findings are novel and interesting, however, the central claim that Pelo silences transposons at translational level is lacking persuasive data. The protein level change was not quantified to support

this idea, and the authors need to provide more convincing data to support their hypothesis.
Major concerns:
1. In Fig. 1A, authors claim that the magnitude of transposon up-regulation was correlated with the strength of pelo mutations. However, expression level of pelo RNA or protein is not shown to support this correlation. Considering that pelo[1] was previously characterized as a strong or null mutant (Xi et al 2005 and Fig.1C), the impact on transposon up-regulation by pelo[1]/[PB60] is very minor (<5-fold increase). Same as Fig. 1C, the Stellate crystal only appeared at some tricky conditions (partial depletion but not null mutants), the expression of pelo in RNAi and rescue groups should also be quantified.
2. Fig. 1B and 1C are the only experiments in this manuscript suggesting that the Pelo mainly suppress transposons at translational level. This statement is weak, because immunostaining of I-element ORF1 or Stellate is not quantitative. Authors should show the protein level change either by western blots or by reporter assays with available fly lines containing transposon-fused quantifiable reporter. Besides, because in the immunostaining, permeabilization of cell membrane is variable among individual egg chambers, authors should calculate the probability of I-element ORF1 positive egg chambers in Fig. 1B to show it is a general effect.
3. In Fig. 3C Hbs1 showed a moderated increase of transposon expression in mRNA level. How about the protein level? It will be more persuasive to claim Hbs1 as a functional partner of Pelo in transposon repression if more significant change in protein level can be shown. The same comment also goes to the following experiments. The authors didn't track protein level in any following experiment, instead the minor mRNA change was shown.
4. If this pathway is piRNA independent, how does this no-go decay system specifically targets transposon RNA but not host genes? Author need to address this question and provide a possible explanation.
Minor concerns:
1. The double mutant shown in Fig. 3D actually doesn't support the hypothesis that Pelo and Hbs1 work as a complex. It can be two parallel pathways that additively suppress Het-A expression.

1st Revision - authors' response 07 May 2015

Response to referees' comments

Referee #1:

The repression of transposable elements (TEs) in metazoan germ cells requires small non-coding RNAs, termed piRNAs, that are associated with Piwi proteins. Here, the authors identify the RNA surveillance complex Pelo-Hbs1 as a new component for TE silencing in Drosophila germ cells. Interestingly, the complex mediates TE silencing independently of piRNA pathway.

The authors analyze TE expression in several pelo mutant strains. They find that expression levels of some of TEs are up-regulated in pelo mutant ovaries. This derepression of TEs does not correlate with low levels of piRNAs and components of piRNA pathway. Actually, expression of these piRNA pathway components appears to be unchanged in pelo mutants. This finding suggests that Pelo may function in selective degradation of some TE transcripts, though we aren't assured that other cellular transcripts including protein-coding mRNAs are also up-regulated in pelo mutants.

Given that the authors provide little data suggesting "translational control" of TEs, the statement "possibly through the no-go decay mechanism, to prevent translation of TEs" in Abstract is misleading and should be substantially weakened.

While this paper is important conceptually, additional experiments are required to strengthen the main conclusions of the paper.

-We agree with the reviewer that the conclusion for “translational control” is overstated. It is true that we do not have direct evidence to support that Pelo functions at the translational level, and we can only speculate that this could be the case based on the known function of Pelo-Hbs1 in no-go decay and the ability of RpS30a expression in suppressing the phenotype. In this revised manuscript, the conclusion for “translational control” has been substantially weakened by correcting or rewriting the related descriptions throughout the manuscript.

(1) On the whole, it is not clear how the authors perform many of experiments shown in the paper. Figure legends are not kind to the reader at all. For example, we aren't even assured that pelo mutants shown in Fig S1 are null or hypomorphic when they are homozygous or trans-heterozygous. The authors should show levels of Pelo protein in these mutants. Another example is found in Fig 2C, in which the authors indicate mutant genotypes with +/- and -/-. What do they mean by "+/-" of Aub[QC42/HN2] and pelo[1/PB60]?

-We have added detailed information about pelo alleles used this study in the text, materials and methods and the supplemental figure S1. pelo[1] is a P-element insertional allele, and RT-PCR analysis suggests that this allele produces a C-terminal truncated product. Pelo[PB60] and pelo[PA13] are deletion/ insertion alleles generated by imprecise excision of P-element insertions: pelo[PB60] has a 107 base pair deletion in the 2nd exon which creates an early stop codon and can be considered as a genetic null allele; pelo[PA13] has a 558 base pair insertion in the 5’UTR, and RT-PCR analysis suggests that the mutation causes approximately 80% reduction of gene product. The nature of molecular lesions associated with each allele indicates the following order of allele strength for the loss-of-function, from the strongest to the weakest: pelo[PB60]>pelo[1]>pelo[PA13]. Interestingly, the strength of the reduced ovary size phenotype in homozygous females also follows this order (data not shown), indicating that this order is objectively reflective of the allele strength.

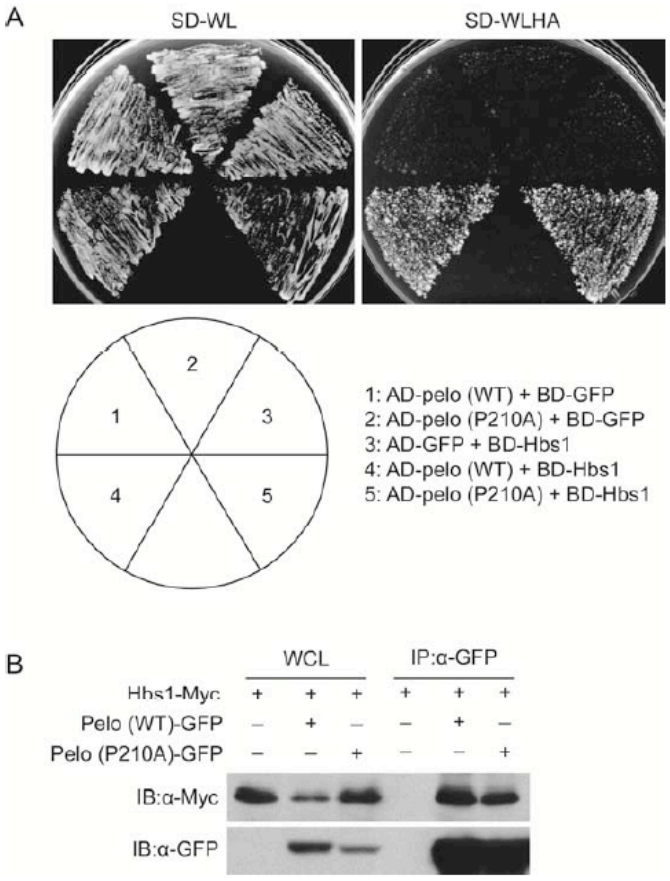
Figure legends were carefully checked for clarity and essential information where is needed was incorporated. Incorrect or incomplete annotations were fixed.

(2) With regard to the data in Fig. 1 that suggest that "Pelo affects protein level more dramatically than mRNA level of TEs", simple comparison of transcript levels measured by RT-PCR and protein expression levels measured by immunostaining is not even sufficient but flawed. We do not know, for example, how TE transcripts measured are (un)stable and we don't know turnover rates of proteins the authors immunostain either.

-This is a good point and we agree that our previous statement is flawed. It is indeed difficult to compare whether pelo affects mRNA level more than protein level, especially given the fact that it is largely unknown about stability of TE transcripts and protein products. Because it is technically difficult to measure the stability of TE transcripts and protein products, and the antibodies that we currently have are not performing very well in western blot, it is therefore impossible for us to determine whether pelo mutation affects more on the transcript level or the protein level. We therefore decided to remove this conclusion throughout the manuscript, and the revised manuscript emphasizes only the fact that pelo mutation caused TE up-regulation at both mRNA and protein levels.

(3) Fig. 3E: The authors conclude that "the P210A mutant Pelo had significantly reduced ability to repress TE levels". But only for 1731 element. Levels of Het-A are unchanged or even more upregulated with P210A. The authors shall consider demonstrating that P210A cannot interact with Hbs1 using an assay shown in Fig 3B.

-We thank the reviewer for raising this point, as our previous description on the functional property of Pelo (P210A) was inaccurate. Structural and biochemical studies in yeast suggest that the PGF motif of Dom34 is important for its binding to Hbs1, but disruption of this binding activity requires mutation of all three residues (van den Elzen et al., 2010), and P to A mutation alone is not sufficient to disrupt the interaction (Chen et al., 2010), indicating that it is the motif interface that mediates the protein-protein interaction. However, Dom34 with P to A mutation indeed significantly reduces the no-go decay activity (Passos et al., 2009), implying that the mutation reduces the function of Pelo-Hbs1 without disrupting their interaction. We studied the interaction between Pelo (P210A) and Hbs1 by Y2H and co-IP experiments. As shown below, both studies suggest that Pelo (P210A) is still able to interact with Hbs1. We have corrected the related statements in the text and conclude that Pelo (P210A), which has reduced no-go decay activity, also showed reduced ability to repress TE levels.



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-We believe this is because Pelo could have a role in somatic supporting cells as well during spermatogenesis. It is known that proper differentiation of the germline requires coordinated action of the surrounding somatic cyst cells. Nos-Gal4 only allows germline expression of Pelo, but tub-Gal4 allows expression of Pelo in both germline and somatic cyst cells. Failed rescue of male fertility by nosgal4 indicates that Pelo is likely required in somatic cyst cells for proper spermatogenesis. The reason that the fly pelo but not the mouse Pelo transgene is able to rescue of male fertility with nos-Gal4 is likely because the leaky expression of pelo associated with this UAS-pelo transgene (weak pelo expression without the presence of Gal4), which has been observed and described by our previous studies (Xi et al., 2005). These transgenes are randomly inserted into the genome, and whether the transgene has leaky expression or not is dependent on the chromosome location where it is inserted. We have made necessary changes in the main text and Figure legends in order to avoid confusion.

Referee #2:

The detection of a role of RNA surveillance complex in transposon silencing seems very interesting. However I have some critical comments.
1.Fig.1A presents the effect of strong null peloPB60 mutation and significantly weaker effect of peloPB60/pelo1 transheterozygous state on transposon up-regulation, while pelo1 was earlier described as a strong mutation. Then the authors switched to study mainly the effects of this transheterozygous state and unfortunately corresponding explanations (or comments) of this choice, as well as short discussion of allele effect differences are absent.

-As also replied to the reviewer 1, we have added detailed information of pelo alleles used this study in the text, materials and methods and the supplemental Figure S1.

Based on these added information, the Pelo[1]/Pelo[PB60] transheterozygote would represent a moderate loss-of-function of Pelo, but the strength is stronger than Pelo[1]/Pelo[1]. In addition, because Pelo[1] and Pelo[PB60] are derived from strains with different genetic background, the use of this transheterozygote would be ideal to minimize any possible effect from background mutations.

2.In the beginning of this paper related to Fig.1A no indication of the attempts to rescue the effects by polo transgene is indicated and only later in Fig.3F the rescue of peloPB60/pelo1 (but not pelo PB60) is mentioned.

-We have performed the rescue of Pelo[PB60]/Pelo[PB60] by pelo transgene. As shown in Figure 1B, the transgene expression significantly suppressed the TE up-regulation in Pelo[PB60]/Pelo[PB60] ovaries.

3. p.5, first paragraph- it is mentioned that « the magnitude of TE up-regulation was correlated with the strength of pelo mutants», but who has evaluated this strength? The authors also used the third mutation (Fig.S2), but at RT its effects again differ

drastically from peloPB60 effects(Fig.1).Generally, these experiments using different polo mutations are causally described.

-As also replied to the reviewer 1, the strength of each pelo allele was evaluated by molecular lesion, transcript abundance and ovary phenotypes. pelo[1] is a P-element insertional allele, and RT-PCR analysis suggests that this allele produces a C-terminal truncated product. Pelo[PB60] and pelo[PA13] are deletion/ insertion alleles generated by imprecise excision of P-element insertions: pelo[PB60] has a 107 base pair deletion in the 2nd exon which creates an early stop codon and can be considered as a genetic null allele; pelo[PA13] has a 558 base pair insertion in the 5'UTR, and RT-PCR analysis suggests that the mutation causes approximately 80% reduction of gene product. The nature of molecular lesions associated with each allele indicates the following order of allele strength for the loss-of-function, from the strongest to the weakest: pelo[PB60]>pelo[1]>pelo[PA13]. Interestingly, the strength of the reduced ovary size phenotype in homozygous females also follows this order (data not shown), indicating that this order is objectively reflective of the allele strength.

Pelo[PA13], the weakest allele was used for experiments shown in Fig. S2. The choice of this allele is because that *pelo* is also important for egg development at late stages oogenesis, and unlike pelo[PA13] mutants, other strong pelo mutants have very small ovaries. This allele will allow us to determine whether TE silencing is compromised in pelo mutants when overall size and morphology of ovary is not altered. As shown in Fig. S2, pelo[PA13] homozygous females has no obvious ovary phenotype when cultured at 25℃ or 29℃, but at 29℃, *HeT-A* mRNA is significantly increased.

4. The results presented in Fig.1B do not allow to conclude that pelo affects I-element protein level more dramatically than mRNA level, because both mRNA and ORF expression is needed to be measured quantitatively in the oocyte.

-We agree that as also replied to the reviewer 1, our previous statement is flawed. It is indeed difficult to compare whether pelo affects mRNA level more than protein level, especially given the fact that it is largely unknown about stability of TE transcripts and protein products. Because it is technically difficult to measure the stability of TE transcripts and protein products, and the antibodies that we currently have are not performing very well in western blot, it is therefore impossible for us to determine whether pelo mutation affects more on the transcript level or the protein level. We therefore decided to remove this conclusion throughout the manuscript, and the revised manuscript emphasize only the fact that pelo mutation caused TE up-regulation at both mRNA and protein levels.

5. Accumulation of Stellate protein in testes owing to polo 1/BP60 transheterozygous state was shown (Fig.1C), but this story seems to be stopped in its beginning. SuSte piRNA quantities (at least abundant ones) are not analyzed, but it seems significant to be performed in order to accept that piRNA is not involved in this case of Stellate derepression, because the following text is intended to exclude the role of piRNA biogenesis violations in TE up-regulation. But in the end of Discussion the role of piRNA in polo mutation effects is not neglected.
Generally the whole text contains semantic contradictions.

-We have performed northern blot analysis for Su(ste) piRNA expression in pelo mutants. As shown in Fig. 2E, Su(ste)-4 piRNA production in testis was almost disappeared in Aub mutants but remained largely unaltered in pelo-RNAi mutants. Our results suggest that pelo is not required for piRNA biogenesis, but it remains possible that piRNAs could mediate TE decay by Pelo-Hbs1, a speculated mechanism proposed by this study. The genetic mutants in the piRNA pathway used in the study

disrupts the ping-pang cycle and largely but not completely abolishes piRNA biogenesis, so it is possible that the trace amount of piRNAs remained in the piRNA pathway mutants could be sufficient to mediate TE decay. Therefore in the discussion, we wish to make it clear that this possibility is not completely excluded.

6. I do not understand the conclusion based on the observed moderate activation of heterochromatic rather than euchromatic Stellate transcripts that led authors to conclusion of preferential translational Pelo effects, while heterochromatic Stellates are known to contain perfect ORFs.

-We actually observed moderate activation of euchromatic stellate transcripts rather than heterochromatic stellate transcripts (Fig. 1F). Because transcriptional repression is important for transposon silencing and we do not see reactivation of heterochromatic stellate, indicating that pelo may not regulate transposon silencing at the transcriptional level. It is therefore reasonable to speculate that moderate increase of euchromatic stellate transcripts and stellate crystal formation may be caused at post-transcriptional or translational level.

7.This paper does not contain any mention concerning the role of pelo mutation effects on GSC maintaining and disturbances of ovarian development.
Generally, this paper contains several «light conclusions» that are needed to be corrected. It would be useful to present some speculation, suggesting possible reasons of RNA surveillance participation in TE silencing.

-We have analyzed the ovary phenotype in various pelo mutants and the phenotypic severity was used as one criteria for the determination of allele strength. We agree that there were many “light conclusions”, and in this revised manuscript, these improper statements were either removed or corrected.

Referee #3:

The manuscript by Yang et al. reported that Pelo-Hbs1 complex is involved at repression of transposable elements on translational level, possibly through no-go decay (NGD) mechanism. The authors first show the de-repression of transposon RNA and proteins in different pelo mutants. pelo mutants didn't affect piRNA biogenesis nor the expression of many piRNA pathway genes. Authors further showed that Pelo forms a complex with Hbs1, which mutant also moderately increase transposon expression. Overexpression of RpS30s, a NGD downstream protein, rescue the pelo phenotype. Finally, the authors rescue fly pelo mutant phenotypes with mouse pelo protein and proposed the conservation of pelo function in transposon silencing in mammals. The overall findings are novel and interesting, however, the central claim that Pelo silences transposons at translational level is lacking persuasive data. The protein level change was not quantified to support this idea, and the authors need to provide more convincing data to support their hypothesis.
Major concerns:
1. In Fig. 1A, authors claim that the magnitude of transposon up-regulation was correlated with the strength of pelo mutations. However, expression level of pelo RNA or protein is not shown to support this correlation. Considering that pelo[1] was previously characterized as a strong or null mutant (Xi et al 2005 and Fig.1C), the impact on transposon up-regulation by pelo[1]/[PB60] is very minor (<5-fold increase).

Same as Fig. 1C, the Stellate crystal only appeared at some tricky conditions (partial depletion but not null mutants), the expression of pelo in RNAi and rescue groups should also be quantified.

-As replied to both reviewers 1 & 2, we have added detailed information about pelo alleles used this study in the text, materials and methods and the supplemental figure S1. pelo[1] is a P-element insertional allele, and RT-PCR analysis suggests that this allele produces a C-terminal truncated product. Pelo[PB60] and pelo[PA13] are deletion/ insertion alleles generated by imprecise excision of P-element insertions: pelo[PB60] has a 107 base pair deletion in the 2nd exon which creates an early stop codon and can be considered as a genetic null allele; pelo[PA13] has a 558 base pair insertion in the 5'UTR, and RT-PCR analysis suggests that the mutation causes approximately 80% reduction of gene product. The nature of molecular lesions associated with each allele indicates the following order of allele strength for the loss-of-function, from the strongest to the weakest: pelo[PB60]>pelo[1]>pelo[PA13]. Interestingly, the strength of the reduced ovary size phenotype in homozygous females also follows this order (data not shown), indicating that this order is objectively reflective of the allele strength.
The Pelo[1]/Pelo[PB60] transheterozygote represents a moderate loss-of-function of Pelo, but the strength is stronger than Pelo[1]/Pelo[1]. In addition, because Pelo[1] and Pelo[PB60] are derived from strains with different genetic background, the use of this transheterozygote would be ideal to minimize any possible effect from background mutations.

It is true that stellate crystals only appears at certain conditions when pelo function is only partially compromised. We think this is because Stellate can only be expressed at late stages of spermatocyte differentiation, and strong or null pelo mutants all have spermatocyte differentiation arrested at early stages when Stellate are not able to be expressed. We have measured the RNAi efficiency, and the results showed that RNAi decreases pelo transcripts to approximately 10% of wild type level (Supplemental Fig. S1B).

2. Fig. 1B and 1C are the only experiments in this manuscript suggesting that the Pelo mainly suppress transposons at translational level. This statement is weak, because immunostaining of I-element ORF1 or Stellate is not quantitative. Authors should show the protein level change either by western blots or by reporter assays with available fly lines containing transposon-fused quantifiable reporter. Besides, because in the immunostaining, permeabilization of cell membrane is variable among individual egg chambers, authors should calculate the probability of I-element ORF1 positive egg chambers in Fig. 1B to show it is a general effect.

-We agree, as replied to the reviewers 1 & 2, that the conclusion for the translational control is weak. It is true that we do not have direct evidence to support that Pelo functions at the translational level, and we can only speculate that this could be the case based on the known function of Pelo-Hbs1 in no-go decay and the ability of RpS30a expression in suppressing the phenotype. It is also difficult to compare whether pelo affects mRNA level more than protein level, especially given the fact that it is largely unknown about stability of TE transcripts and protein products. Because it is technically difficult to measure the stability of TE transcripts and protein products, and the antibodies that we currently have are not performing very well in western blot, it becomes impossible for us to determine whether pelo mutation affects more on the transcript level or the protein level. We therefore decided to remove this conclusion throughout the manuscript, and the revised manuscript emphasize only the fact that pelo mutation caused TE up-regulation at both mRNA and protein levels.

The depression of ORF1 and Stellate crystals is a general effect, and the penetrance is 100%, although the protein level varies among different egg chamber or testis. The quantitative data is included in the manuscript.

3. In Fig. 3C Hbs1 showed a moderated increase of transposon expression in mRNA level. How about the protein level? It will be more persuasive to claim Hbs1 as a functional partner of Pelo in transposon repression if more significant change in protein level can be shown. The same comment also goes to the following experiments. The authors didn't track protein level in any following experiment, instead the minor mRNA change was shown.

-This is a good point. Hbs1 is a well studied Pelo/Dom34 partner in NGD, however its has much weaker phenotype in NGD compared to its partner Pelo. One possible explanation is that Hbs1 is a member of the GTPases family including ski7, eRF3, etc. which may partially compensate Hbs1 function when Hbs1 is depleted. We did not observe the expression of I-element ORF1 in hbs1 mutant ovaries or Stellate crystals in Hbs1 mutant testis, possibly because that the phenotype is too weak to be detected at protein levels, due to function redundancy. Based on inefficient rescue of Pelo by Pelo (P210A), it is reasonable to speculate that Pelo, in complex with Hbs1 or other small GTPases, are probably involved in transposon silencing.

4. If this pathway is piRNA independent, how does this no-go decay system specifically targets transposon RNA but not host genes? Author need to address this question and provide a possible explanation.

-Whether piRNA is involved in this pathway is still an open question. In ago3 or aub mutants, the amplification cycle of piRNAs is compromised, but piRNAs are not eliminated. Although we find that Pelo-mediated TE repression still occurs in ago3 mutants, it remains possible that trace amount of piRNAs in ago3 or aub mutants is sufficient to guide Pelo-mediated TE repression. Because at the moment it is impossible to genetically eliminate all piRNAs in vivo, we wish to make it clear in the discussion that this possibility is not completely excluded.

Minor concerns:
1. The double mutant shown in Fig. 3D actually doesn't support the hypothesis that Pelo and Hbs1 work as a complex. It can be two parallel pathways that additively suppress Het-A expression.

-This is true but we have additional evidence to support that Pelo and Hbs1 work as a complex, such as the physical interaction between Pelo and Hbs1 and the functional property of Pelo (P210A). So the hypothesis is based on several lines of evidence.

2nd Editorial Decision 25 May 2015

Thank you for the submission of your revised manuscript to our journal. We have now received the enclosed reports from the referees that were asked to assess it. Referee 1 still has a few suggestions that I would like you to address and incorporate in a final version before we can proceed with the official acceptance of your manuscript.

I noticed that scale bars are missing in the supplementary figures. Please also explain * and specify the test used to calculate p-values in the respective figure legends.

I look forward to seeing a final version of your manuscript as soon as possible. Please let me know if you have any questions.

REFEREE REPORTS:

Referee #1:

It is an important contribution to our understanding of how transposable elements (TEs) are repressed in Drosophila ovaries. Studies of this sort are good references and resources for further comparisons. On the whole it has been significantly improved and the authors have attempted to address the various criticisms.

However, the statement "Pelo-Hbs1 might function at the translational level -----" in Abstract is still misleading. As it is mentioned in the text (page 10 in the second paragraph) that "---, possibly through the NGD pathway to cleave TE transcripts that -----", Pelo-Hbs1 could direct cleavage of TE transcripts and as a consequence, the protein products of TE transcripts might not be produced. Thus, Pelo-Hbs1 may control the abundance of transcripts by cleaving them but not their translation, per se.

The authors should also provide a possible explanation of how this NGD system specifically targets TE transcripts but not cellular genes. At least, the authors should discuss about where the specificity comes from.

The manuscript needs proofreading to correct typos etc ("house keep genes" in page 8, for example).

Referee #2:

My comments largely coincided with the findings of other reviewers. The authors have removed from the text several inadequate conclusions. Although this paper failed to present strong direct evidence in favor of the main ideas of the authors, the presented data are interesting and may attract the attention of researchers.

2nd Revision - authors' response 27 May 2015

Referee #1:

It is an important contribution to our understanding of how transposable elements (TEs) are repressed in Drosophila ovaries. Studies of this sort are good references and resources for further comparisons. On the whole it has been significantly improved and the authors have attempted to address the various criticisms.
-We really appreciate the reviewer's kind comments.

However, the statement "Pelo-Hbs1 might function at the translational level -----" in Abstract is still misleading. As it is mentioned in the text (page 10 in the second paragraph) that "---, possibly through the NGD pathway to cleave TE transcripts that -----", Pelo-Hbs1 could direct cleavage of TE transcripts and as a consequence, the protein products of TE transcripts might not be produced. Thus, Pelo-Hbs1 may control the abundance of transcripts by cleaving them but not their translation, per se.
-As the reviewer suggested, we have further weakened our statement on translational control in the abstract. The reason that we point out the likelihood of translational level of

regulation by Pelo-Hbs1 is because this protein complex is known to function at the translational level: it only recognizes mRNAs that have loaded on ribosomes and might have entered the translational process. Therefore, Pelo-Hbs1 might provide a mechanism to stop translation of TE mRNAs that have escaped from post-transcriptional silencing, and subsequently degrade them through the No-go decay pathway.

The authors should also provide a possible explanation of how this NGD system specifically targets TE transcripts but not cellular genes. At least, the authors should discuss about where the specificity comes from.
-How the loaded TE mRNAs are recognized by the NGD machinery is unclear, but our favorite hypothesis is still the involvement of piRNAs, whose binding to the loaded TE mRNAs may cause ribosome stalling, a possibility that is worthy of future investigation. We have added this to the discussion.

The manuscript needs proofreading to correct typos etc ("house keep genes" in page 8, for example).
-The manuscript was thoroughly checked for typos and errors.

Referee #2:

My comments largely coincided with the findings of other reviewers. The authors have removed from the text several inadequate conclusions. Although this paper failed to present strong direct evidence in favor of the main ideas of the authors, the presented data are interesting and may attract the attention of researchers.
-We thank all the reviewers for their constructive comments and positive evaluation of this study.

3rd Editorial Decision 02 June 2015

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.

