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The tudor domain protein Kumo is required to assemble the nuage and to generate germline piRNAs in *Drosophila*

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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 07 August 2011

Thank you for submitting your manuscript for consideration by The EMBO Journal. It has been now been evaluated by two referees and I enclose their reports below. As you will see from their comments they find the study describing a role for Kumo in the germline piRNA pathway to be interesting but they require some further experimental analysis to make the study suitable for The EMBO Journal. The main concerns surround the role of Kumo in nuage and in the nucleus, in the case of the latter it would be beneficial to have a comparison of the effects of loss of Kumo and Rhino. Should you be able to address the concerns we would be happy to consider a revised version of the manuscript for publication in The EMBO Journal.

I should remind you that it is EMBO Journal policy to allow a single round of revision only and that, therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript. When you submit a revised version to the EMBO Journal, please make sure you upload a letter of response to the referees' comments. Please note that when preparing your letter of response to the referees' comments that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website: http://www.nature.com/emboj/about/process.html

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

Yours sincerely,

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Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1:

Review of kumo, a tudor domain protein is required for assembly of nuage and production of germline piRNA in Drosophila" by Anand and Kai

piRNA pathway biology is an intensely studied field in small RNA biology. It appears that this pathway is considerably more complex than the siRNA or miRNA pathways. Largely mysterious is still, how piRNAs are being processed in a controlled way from their precursor transcripts. Recent studies have implicated Tudor proteins in the piRNA pathway and in this manuscript, the authors report an uncharacterized novel Tudor domain containing protein (Kumo) and demonstrate its involvement in the piRNA pathway.
the major findings are:

1. Kumo is required for female fertility and gonad development/oogenesis (kumo mutants display classical polarity and oocyte determination phenotypes)
2. Kumo is an essential piRNA pathway factor with specific involvement in the germline specific ping-pong amplification loop
3. Kumo is an integral nuage component, interacts with other nuage components and appears to be the most upstream factor in nuage organization
4. In the germanium, Kumo appears to be a nuclear protein. Here it appears to be important for piRNA cluster biology and is implicated in sequestering HP1

Experimentally, the paper can be split into three parts:
1. characterization of Kumo as an oogenesis gene and as an integral nuage component
2. evidence that Kumo is required for the piRNA pathway (transposon de-repression and small RNA analysis in Kumo mutants)
3. elucidation of a nuclear function of Kumo in early oogenesis.

Overall, I think that given that this is a novel piRNA pathway component, given that it has an interesting phenotype, the study is of interest to the EMBOJ readership. However, I have quite some issues with the proposed nuclear role of Kumo and I have several comments on the other parts that need to be clarified prior to publication. The data quality for the genetics part is high, the small RNA analysis is medium and the experiments on the nuclear function of Kumo are very weak in my opinion.

major comments:

1.
The proposed nuage hierarchy. I agree that it is per se interesting to examine the hierarchy of nuage organization using genetics and immuno-stainings. Indeed, the authors have initiated this previously and this is of value in the field. My major concern is the following: How can the authors exclude that DNA damage signaling and subsequent Vasa phosphorylation is not impacting nuage organization? In other words, would it be possible that quite some of the effects are not directly related to nuage organization but rather to DNA damage signaling?
I find this particularly important, because of the rescue experiment with Kumo-CT: does this experiment not suggest (because kumo-CT does NOT localize to nuage) that the nuage delocalization defects are possibly an indirect cause (maybe of DNA damage signaling and vasa phosphorylation)?
I suggest to repeat some of these experiments in chk2 mutants to exclude this important aspect.

2.
about the molecular analysis of cluster expression and the chromatin IP data for clusters (HP1):
It is clear that these experiments (if done via PCR) are very dangerous, simply as piRNA clusters are repetitive areas in the genome. Strand specific PCRs are even more challenging and in my opinion, the -RT control does not control for anything. Much more important would be a no-primer control with addition of RT to exclude self-priming or priming by other RNAs in the total RNA prep. Moreover, how can we be sure that the observed effects (which are relatively mild) are not a consequence of distorted ovarian morphology?
Figure 7C: what does normalized expression mean? 42AB1+ levels are 25fold higher than what?

3.
The authors try to find a molecular link that supports a nuclear role of Kumo in early oogenesis. In my opinion, it would be very important to show the specificity of the described phenotypes for Kumo. The authors need to show that the same does not happen in Spn-E or Aub mutants. Conceptually, I have a great problem in understanding how nuclear localization of Kumo very early in oogenesis can have such a strong impact on HP1 localization to clusters if the experiments are done from whole ovary lysates.
The same is true for the molecular interaction between HP1 and Kumo. Hp1 is a nuclear protein. Very few cells in the ovary have nuclear foci for Kumo. How can this result in such a robust interaction via IP? If one looks at the western blots in Figure 7E and F, I really wonder how the stoichiometry of this IP can make sense given the localization of Kumo and HP1. It appears that the IP brings down more HP1 than Kumo, at least in Figure 7F. A reciprocal IP is the very minimum that is required to add some credibility to this claim.
To support the claim that HP1 levels are elevated in Kumo mutants, the authors need to induce mitotic clones in the germline to have heterozygous and mutant cells side by side for a comparison. Alternatively, a quantitative western would also do.

In my opinion, the altered HP1 binding to cluster 42AB in Kumo mutants is highly speculative. Given my arguments above, this is really only believable if being done genome wide via ChIP_seq (due to the repetitive nature of the cluster regions).

The authors must further agree, that the following statement is highly speculative and nor supported by any data:
"This result supports a direct role for Kumo in promoting piRNA precursor transcription by binding to HP1, thereby restricting HP1 binding to piRNA loci."
How can nuclear Kumo in the germanium sequester HP1 during the entire oogenesis?

4.
the manuscript has been prepared in a really sloppy manner. Throughout, the grammar and spelling is poor and the scientific language could be considerably improved.
here are some examples:

"Here we report kumo, a conserved yet uncharacterized component of the germline piRNA pathway in Drosophila"
scientific language

"localizes to nuage; a germline unique structure"
what is a germline unique structure?

"Animal genomes have a special small RNA-based defense system called Piwi interacting RNA (piRNA)"
piRNAs are not a system;

"Drosophila piRNAs can be mapped to discreet clusters"
spelling

"However, some clusters produce piRNA only from one strand (uni-strand clusters), including flamenco which is involved transposon silencing in somatic cells (Desset et al, 2008; Prud'homme et al, 1995; Sarot et al, 2004).
language; in addition, the citations are lacking the entire molecular work on flamenco.

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"The sense and antisense piRNA thus, derived, overlap by 10 nucleotides (nt) from their 5' ends."
language

"Vas and SpnE are RNA helices;"
grammar

"This is further supported by our observation for Kumo interaction with HP1."
grammar

"Our results suggest that Kumo plays dual role in distinct places for the efficient germline piRNA production;"
scientific language

"In reciprocal experiments, the perinuclear localization of Kumo was not affected in all examined the examined nuage component mutants (Fig. 2B)."
grammar/language

"We observed an approximate 65% decrease in the overall levels of small RNAs levels ranging from 24-29 nt in kumo mutants, indicating (Fig. 4A) that the kumo mutation caused a decrease in piRNA levels."
language

"Schematic diagram showing Kumo variants tagged with FLAG which is used in immunoprecipitation experiments using S2 cell lysate."
language

"we also investigated whether these domains contribute to nuage organization by mediating interactions with other nuage components and retroelement repression"
language

"Figure S2: Kumo localizes to cytoplasmic phase of the nuclear envelop"
spelling

other comments:

1.
conceptually, I am not sure whether it is adequate to split the piRNA pathway in soma versus germline . I would suggest to split it into primary and secondary. for example:
"piRNA in Drosophila gonads can be categorized into germline and somatic piRNAs; they are produced by two independent pathways involving separate sets of genes and function separately in germline cells and somatic cells (Li et al, 2009; Malone et al, 2009; Senti & Brennecke, 2010)."
I would re-phrase this and make clear that the primary pathway from the somatic cells appears to also be active in the germline.
also in the abstract:
"distinct piRNA pathways involving different components function in somatic and germline cells"

2.
when the authors mention cluster 2 and flamenco:
"piRNAs produced from these clusters do not undergo any kind of amplification as germline piRNA and compose the somatic component of piRNA pathway (Li et al, 2009; Malone et al, 2009)."
For cluster 2 this is almost certainly not correct. This cluster is likely to be involved in ping pong in the germline.

3.
Figure S3: single channels are required in monochrome; please add a double stain for another nuage component; this is relevant as in the germanium, also other nuage components form very clear foci. It would be important to show that the nuclear localization of Kumo is not a general feature of nuage

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components.

4.
Figure 2: The AGO3 an Mael staining in the kumo mutant appear to be identical images. is that possible?

5.
Where are the primers for Fig 1C located? please add to Figure 1A.

6.
What about Spn-E and Mael localization in the rescued animals? (Figure 2)

7.
Figure 3: The de-silencing of transposons are by far the strongest ones reported to date. To put this into perspective, I suggest to perform this QPCR side by side with one or two other known pathway members such as spn-E or tejas.

8.
I have several comments on the bioinformatic analysis of the small RNA datasets:
normalization: the authors write:
"The sequencing data was filtered for non-coding RNA and small RNAs that result from RNA degradation, and was normalized using genome matching reads."
this does not make any sense; it is fine to remove degradation products from abundant RNAs such as tRNAs, rRNAs, etc. But the normalization to genome matching reads does not make any sense as the genome mappers also include the piRNA fraction. The normalization should be done with a set of bona fide endogenous small RNAs that are not piRNAs such as endo-siRNAs or miRNAs.
also the following quote does not make much sense in this respect. Is the data normalized for siRNAs or for genome mating reads then?
"Data among two libraries were normalized to the total number of perfect genome-mapping reads, and non-coding RNA and endosiRNAs, which are derived from two major hairpins (Okamura et al, 2008)."

for the length profile of the small RNAs:
why does the profile not go to essentially zero at 30nt? There are hardly any piRNAs having 30nt in length. How do the authors explain this?

Split Fig 4A in sense and antisense small RNAs

Fig 4B: it appears that these density plots also include siRNAs. This is indicated by the internal density of small RNAs mapping to the genomic minus strand, which is a sign of siRNAs. Please restrict this analysis to piRNAs only.

Fig5: ZAM small RNA profile: Two things are very strange: (1) why is the density starting so abruptly at 24nt? (2) why is the 21mer fraction still antisense biased? As these are siRNAs, there should be an equal number of sense and antisense small RNAs. I just wonder whether there is a general flaw in the computational analysis.

"Thus, sequencing analysis of piRNAs confirms the importance of kumo for the production of both germline primary and ping-pong derived piRNAs, but not for that of somatic piRNAs."
how can the authors conclude that the primary pathway in the germline is affected in kumo mutants? Have they stained for Piwi as delocalization of Piwi seems to correlate with defects in the primary pathway?

about the ping-pong analysis: the authors conclude that Kumo is required for ping-pong but that a sub-population of ping-pong pairs is independent of Kumo. I think that this analysis needs a statistically better controlled analysis:
"However, piRNA levels with 10-nt overlap for TARTA and I-element were not completely abolished in kumo mutant ovaries (Fig. 5). In addition, only a small reduction in that of roo and blood was observed, implicating that kumo may not be necessary for production of a subset of piRNAs in ping-pong cycle (Fig. 5, S6)."

9.
Figure 6C: what are the black marks in the upper panels?
lower panels: why does the western blot to the left look so much worse than the one to the right? on the left blot it is very difficult to agree that the experiment has indeed technically worked. where are the molecular size markers?

10.
about the sDMA analysis: the authors can only conclude that Kumo does not interact with the published peptide in an sDMA independent manner. this does not mean that Aub has no additional sDMA residues, which could be essential for the interaction.

11.
naming: I suggest to call "precursor piRNAs" cluster transcripts to avoid confusion with the mature piRNAs.

12.
citations:
"Animal genomes have a special small RNA-based defense system called Piwi interacting RNA (piRNA) that can counter the transposon threat in the gonads (Brennecke et al, 2007; Saito et al, 2006; Vagin et al, 2006)."
animal?? all citationsrefer to flies; several key citations (even for fly) are missing; for example the entire genetics on transposon control.

"Many other proteins that localize to nuage like Vasa (Vas), Spindle-E (Spn-E), Tejas (Tej), Krimper (Krimp), and Maelstrom (Mael), have been shown to play a role in maintaining nuage organization, and transposon repression via germline piRNA pathway (Chen et al, 2007; Li et al, 2009; Lim & Kai, 2007; Malone et al, 2009; Patil & Kai, 2010).
where is the maelstrom citation? why is cutoff cited?

Referee #2:

This manuscript describes a new gene, kumo, that acts in the piRNA pathway of Drosophila. The authors performed a fairly thorough study of its effects in oogenesis. As most of the previously described members of this pathway, a mutation in kumo affects germline determination, and results in moderately ventralized eggs which are sterile. Retrotransposons are deregulated in the mutant, and piRNAs are depleted. Interestingly, the Kumo protein localizes both to the nuage as well as the nucleus. The authors show that the transcription of the piRNA precursor transcripts is affected in the mutant. This reduction is relatively mild and cannot explain the more dramatic loss of piRNAs, indicating that the loss (or detachment?) of the nuage that is observed in the kumo mutant may have a further severe effect on the process. Finally, the authors show that Kumo interacts with HP1, and that HP1 is somewhat enriched at the piRN locus 42AB when Kumo is lost.

Overall this is an interesting study which describes a factor with a dual function in the pathway. While the exact biochemical function of Kumo is not known, the manuscripts delineates the roles of the protein, where the activity in the nucleus seems particularly intriguing and novel.

There are, however, a number of questions that should be addressed before publication. In particular, a previous report by Klattenhoff et al (2009) presented the factor Rhino as the first protein that is involved in the transcription of the primary piRNA precursor clusters. The authors only mention this study in passing, but a more thorough comparison between the effects of Rhino and Kumo, and an investigation on interactions would seem necessary to allow the field to make sense of the two reports (see point 2 and 7 below). This would definitely improve the impact of the manuscript.

Major questions/concerns:

1) Does kumo affect stem cell maintenance? This would be expected to result in empty ovarioles in females older than 20 days? Or is there another reason for the decrease in egg laying? Is the fusome affected? (which would explain the oocyte determination defects). Staining with alpha-spectrin antibody would answer this question and would allow placing kumo either among the genes that have mostly postmitotic germline defects versus the ones that already affect the stem cells.

2) Does Kumo co-localize with Rhino? Rhino has been described to have essentially the same function as Kumo in the nucleus, but loss of Rhino gives a stronger phenotype with respect to loss of cluster transcription. Does Kumo interact with Rhino? A Rhino-GFP construct is available.

3) Results p. 8 and Figure S5, C: the authors claim that Gurken protein is not localized correctly in the mutant. But in the picture they provide, Gurken is completely normally accumulating in the region directly adjacent to the oocyte nucleus! One can easily discern the nucleus in the mutant picture - the egg chamber is misoriented and points with the dorsal side up, but the accumulation of Gurken is clearly asymmetric around the nucleus, and if the authors had rolled the egg chamber by 90 degrees, the picture would have looked very similar to the control. Normally in the spindle mutants, the oocyte nucleus, i.e. the primary dv polarity, is normal, as is Gurken localization, but Gurken translation is reduced, leading to lower levels of protein and therefore a reduction in dorsal cell fates. This is probably the case here as well, and may be the reason why the authors thought the protein was "only anterior" but it would require careful quantitation to allow the firm conclusion. It would be completely surprising if Gurken protein were mislocalized in a band along the anterior, as the authors claim, since this would lead to a dorsalized (K10 or squid-like) egg shell phenotype!!

4) Nuage (results p. 8): The authors claim that Kumo is 'the most upstream" in the organization of the nuage and participates in the organization of the nuage. However, the pictures shown in Fig. 2A show striking particles in all the mutants that are not "small foci" - but as large as the normal nuage particles, strongly suggesting that Kumo's role is to anchor the nuage particles to the nuclear envelope, and not necessarily for the "organization" of the particles. The authors need to formulate this much more carefully, since they provide no evidence that the other nuage proteins are not forming the appropriate particles that may well be organized "normally", just not anchored to the nuclear membrane (all the proteins still seem to colocalize in those foci, no reason to think that they are "disorganized").

5) Results p. 9: I did not understant the normalization of the libraries "perfect genome-mapping reads". It would be better to use just other small RNAs (miRNAs) that are not affected by the pathway to normalize the samples. Would not the "perfect genome-mapping reads"be affected by the mutant?

6) Results p. 14, Fig. 7D: Are the differences between wildtype and the kumo mutant significant in all cases for cluster 42A/B? It may be that only regions B2 and B5 show a significant increase? Please specify statistics for the various regions in the Figure/Figure legend.

7) How do all the various results compare to the results of loss of Rhino? (which piRNAs are affected? which cluster transcripts?) A careful and explicit comparison would be useful. - Also, did the authors use the same primers for the analysis of the cluster transcription as Klattenhoff et al.?

Minor comments:

The manuscript contains numerous typographical and grammatical errors, for instance (these are just a few examples):
p.3 discreet clusters: correct is "discrete clusters"
(discreet means "tactful")
p.5 This is further supported by our observation for Kumo interaction with HP1.
Correct: ..our observation for an interaction of Kumo with HP1
p.5 Our results suggest that Kumo plays dual role:
Correct: Kumo plays a dual role
p. 11 Consistent with observation in another piRNA pathway mutants, rhino and krimper..
correct: observation in other piRNA pathway mutants...
p. 11 kumo mutation causes a great reduction

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correct: The kumo mutation causes ...

It would probably be good to have a native English speaker to read carefully through the manuscript.

1st Revision - authors' response 13 October 2011

Response to reviewers' comments

General response: We thank the reviewers for their interests and positive recommendation on our work. We deeply appreciate their thoughtful and constructive comments. We have made changes to the manuscript according to the comments and we hope that this improved manuscript is now acceptable for publication in EMBO Journal. Please find our response to the comments below.

Referee #1:

major comments:

1.
The proposed nuage hierarchy. I agree that it is per se interesting to examine the hierarchy of nuage organization using genetics and immuno-stainings. Indeed, the authors have initiated this previously and this is of value in the field. My major concern is the following: How can the authors exclude that DNA damage signaling and subsequent Vasa phosphorylation is not impacting nuage organization? In other words, would it be possible that quite some of the effects are not directly related to nuage organization but rather to DNA damage signaling?
I find this particularly important, because of the rescue experiment with Kumo-CT: does this experiment not suggest (because kumo-CT does NOT localize to nuage) that the nuage delocalization defects are possibly an indirect cause (maybe of DNA damage signaling and vasa phosphorylation)?
I suggest to repeat some of these experiments in chk2 mutants to exclude this important aspect.

Response: We appreciate the reviewer for raising this important issue. We cannot rule out the relation between DNA damage signalling and piRNA pathway, as having a DNA damage signalling pathway mutant in background of piRNA pathway component mutant rescues the polarity defects (Klattenhoff et al, 2007). However, the checkpoint mutations fail to rescue the double strand breaks in the piRNA pathway mutants, suggesting that persistent DSBs/DNA damage in the piRNA pathway mutant ovaries takes place independent of the meiotic DSBs and are caused by active transposition in the piRNA mutants.
To analyse if DSBs/DNA damage has a role for nuage organization, we examined localization of some nuage components in *kumo* and *meiW68* double mutants, but *meiW68* mutation did not rescue any of the nuage protein localizations affected by the loss of *kumo* function (Figure S7). We also analysed localization some of nuage components which occupy high, middle and low position in the hierarchy like organization, Kumo, Tejas and Krimper in the mutants of DNA damage signalling pathway components, *chk2*, *mei41* and *meiW68*. All these components remain localized to the nuage in these examined mutant ovaries (Figure S7); strongly supporting that loss of canonical DNA damage signalling pathway (and/or subsequent phosphorylation of Vas) does not affect nuage organization.
We have included these data and arguments as follows (Page 6, Line 17);

> "We also investigated whether the hierarchy of nuage organization is also dependent on DNA damage as it has been shown previously that mutations in the DNA damage signalling pathway rescue the polarity defects in some piRNA pathway mutants (Klattenhoff et al 2007). Immunostaining reveals that nuage components Aub, Krimp and Kumo are not affected in the DNA damage pathway mutants (*chk2*, *mei41* and *meiW68*), indicating that these do not play a role in nuage organization (Fig. S7)."

Although Kumo-CT does not localize to nuage, it rescues the localization of other nuage components and the sterility of *kumo* mutant, to a large extent. These results suggest that cytoplasmic Kumo-CT sufficiently functions in germline cells, possibly by mediating a formation of

macromolecular complex. However, we cannot exclude the possibility that inconspicuous amount of Kumo-CT may exist and function in nuage.
In this manuscript, we provided our arguments for this issue (Page 14, Line 14);

> "The results suggest that cytoplasmic Kumo-CT is likely sufficient to function by mediating the formation of the macromolecular complex at the perinuclear region. However, it also may be possible that a modest amount of Kumo-CT is also present at perinuclear nuage."

2.
about the molecular analysis of cluster expression and the chromatin IP data for clusters (HP1): It is clear that these experiments (if done via PCR) are very dangerous, simply as piRNA clusters are repetitive areas in the genome. Strand specific PCRs are even more challenging and in my opinion, the -RT control does not control for anything. Much more important would be a no-primer control with addition of RT to exclude self-priming or priming by other RNAs in the total RNA prep. Moreover, how can we be sure that the observed effects (which are relatively mild) are not a consequence of distorted ovarian morphology? Figure 7C: what does normalized expression mean? 42AB1+ levels are 25fold higher than what?

Response: We would like to highlight that Klattenhoff et al 2009 have successfully assessed cluster transcription using a PCR based approach, and we used the same primers reported by them. These primers have also been used in other credible published studies as well (Rangan et al, 2011; Zamparini et al, 2011). In addition we also designed new two sets of the primers, and confirmed the specificity of their amplification by agarose gel and sequencing of the unique PCR products (data not shown). Please refer Table S2 and supplementary M&M section describing strand specific RT-PCR. We would be happy to provide those data if necessary.
As suggested, we performed a 'no-primer control', and as described by Klattenhoff et al 2009, we also observed very subtle amplification. The small value was deducted from amplification obseved in the samples with the PCR primers. The value was normalized by *rp49*, and the expression levels of cluster transcripts were depicted using an arbitrary scale to compare with those in the heterozygous controls. In this revised manuscript, we have provided the expression levels at Y axis as suggested by the reviewer.
The value was normalized by by *rp49*, and the expression levels of cluster transcripts were depicted using an arbitrary scale to compare expression levels between mutants and heterozygous controls. In this revised manuscript, we have provided the levels at Y axis as suggested by the reviewer.
As to the distortion of ovarian morphology, indeed, the aged *kumo* mutant ovaries contain smaller ovarioles and fewer number of late stage egg chambers. However, the structure of germarium and early stage egg chambers are not much affected (as shown in Figure S6 in the revised manuscript, please refer Point 1 by the Reviewer #2). Such distortion becomes prominent only after 4-5 days of eclosion. For IP experiments throughout in this study, we used young flies those are yet in the early ages to avoid being compromised by such distortion. Though we cannot absolutely exclude the possibility that the ovarian morphology may have contributed to the results, we believe Kumo function for cluster transcription is strongly supported by the nuclear localization of Kumo, high HP1 occupancy at germline piRNA loci in *kumo* mutant ovaries and the direct interaction of Kumo and HP1, as presented in this study.

3.
The authors try to find a molecular link that supports a nuclear role of Kumo in early oogenesis. In my opinion, it would be very important to show the specificity of the described phenotypes for Kumo. The authors need to show that the same does not happen in Spn-E or Aub mutants.

Response: As suggested by the reviewer, we examined the cluster transcription in the other piRNA pathway mutants, *krimp* and *tej*. These mutants did not show any significant difference in the cluster transcription compared to controls. We have included the data in supplementary figures (Figure S12).

Conceptually, I have a great problem in understanding how nuclear localization of Kumo very early in oogenesis can have such a strong impact on HP1 localization to clusters if the experiments are done from whole ovary lysates.
The same is true for the molecular interaction between HP1 and Kumo. Hp1 is a nuclear protein. Very few cells in the ovary have nuclear foci for Kumo. How can this result in such a robust

interaction via IP? If one looks at the western blots in Figure 7E and F, I really wonder how the stoichiometry of this IP can make sense given the localization of Kumo and HP1. It appears that the IP brings down more HP1 than Kumo, at least in Figure 7F. A reciprocal IP is the very minimum that is required to add some credibility to this claim.
To support the claim that HP1 levels are elevated in Kumo mutants, the authors need to induce mitotic clones in the germline to have heterozygous and mutant cells side by side for a comparison. Alternatively, a quantitative western would also do.

Response: By immunostaining, we observed high expression of HP1 in *kumo* mutants in germarium and the early stage egg chambers, but not in the late stage egg chambers. To support this observation, as suggested by the reviewer, we conducted quantitative western blot analysis. While we could not detect significant upregulation of HP1 in *kumo* mutant using whole ovarian extract (data not shown), we observed a 2.4-fold higher expression of HP1 in the *kumo* mutant using lysates prepared from hand-dissected germaria and early stage egg chambers. This experiment has been included in the revised manuscript (Figure S16B).
We thank the reviewer for pointing out the stoichiometry issue in Figure 7F. In this particular Western blotting, a trace amount of input was loaded. We have replaced the gel image with another blot with an appropriate loading (in 1/100 proportion). In addition, as suggested by the reviewer, we performed a reciprocal IP using the ovarian lysates (Fig 7E). Kumo was successfully pulled down with HP1 from the ovarian lysates.

In my opinion, the altered HP1 binding to cluster 42AB in Kumo mutants is highly speculative. Given my arguments above, this is really only believable if being done genome wide via ChIP_seq (due to the repetitive nature of the cluster regions).

Response: We would like to highlight again that many studies in the past have successfully studied changes in protein binding at piRNA clusters using ChIP (Klattenhoff et al, 2009; Moshkovich & Lei, 2010; Rangan et al, 2011; Yin & Lin, 2007). In this study, we used the same primer sets reported by Klattenhoff et al 2009, which were used for PCR to amplify regions of cluster 42AB in their ChIP experiments. In addition, we designed two primer sets in our hands and ensured specific amplifications for each primer set by electrophoresis through agarose gel (data not shown).
Although the genome-wide analysis of epigenetic markers in piRNA pathway components mutants would be a great interest in the field, we think it is out of scope in this study, where we first describe a robust requirement of *kumo* for germline piRNA production in two ways, one at the perinuclear nuage in the cytoplasm and the other in the nucleus for cluster transcription. We are hoping to report it in future manuscript.
The authors m
ust further agree, that the following statement is highly speculative and nor supported by any data:
"This result supports a direct role for Kumo in promoting piRNA precursor transcription by binding to HP1, thereby restricting HP1 binding to piRNA loci."
How can nuclear Kumo in the germanium sequester HP1 during the entire oogenesis?

Response: We have changed the above sentences and have written it in a speculative manner. We did not claim that HP1 is perturbed during all stages of oogenesis in the *kumo* mutant ovaries. Quantitative Western Blot and immunostaining analyses supports that HP1 is mostly affected in the early stages of the germline development. Kumo may support the cluster transcription only in the early stages. However, high HP1 occupancy in the *kumo* mutant ovaries and interaction between Kumo and HP1 do make some ground for this speculation.

4.
the manuscript has been prepared in a really sloppy manner. Throughout, the grammar and spelling is poor and the scientific language could be considerably improved.
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what is a germline unique structure?

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piRNAs are not a system;

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"However, some clusters produce piRNA only from one strand (uni-strand clusters), including flamenco which is involved transposon silencing in somatic cells (Desset et al, 2008; Prud'homme et al, 1995; Sarot et al, 2004).
language; in addition, the citations are lacking the entire molecular work on flamenco.

"The sense and antisense piRNA thus, derived, overlap by 10 nucleotides (nt) from their 5' ends."
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"Vas and SpnE are RNA helices;"
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"In reciprocal experiments, the perinuclear localization of Kumo was not affected in all examined the examined nuage component mutants (Fig. 2B)."
grammar/language

"We observed an approximate 65% decrease in the overall levels of small RNAs levels RNAs levels ranging from 24-29 nt in kumo mutants, indicating (Fig. 4A) that the kumo mutation caused a decrease in piRNA levels."
language

"Schematic diagram showing Kumo variants tagged with FLAG which is used in immunoprecipitation experiments using S2 cell lysate."
language

"we also investigated whether these domains contribute to nuage organization by mediating interactions with other nuage components and retroelement repression"
language

"Figure S2: Kumo localizes to cytoplasmic phase of the nuclear envelop"
spelling

Response: We thank the reviewer for pointing out these mistakes, and we have incorporated the suggestions with proper amendments.

other comments:
1.
conceptually, I am not sure whether it is adequate to split the piRNA pathway in soma versus germline . I would suggest to split it into primary and secondary. for example: "piRNA in Drosophila gonads can be categorized into germline and somatic piRNAs; they are produced by two independent pathways involving separate sets of genes and function separately in germline cells and somatic cells (Li et al, 2009; Malone et al, 2009; Senti & Brennecke, 2010)." I would re-phrase this and make clear that the primary pathway from the somatic cells appears to also be active in the germline. also in the abstract:"distinct piRNA pathways involving different components function in somatic and germline cells"

Response: We have changed the statements throughout the manuscript as suggested by the reviewer.

2.
when the authors mention cluster 2 and flamenco:"piRNAs produced from these clusters do not undergo any kind of amplification as germline piRNA and compose the somatic component of piRNA pathway (Li et al, 2009; Malone et al, 2009)."For cluster 2 this is almost certainly not correct. This cluster is likely to be involved in ping pong in the germline.

Response: We thank reviewer for pointing this out, we also did not intend to mention cluster 2 as somatic cluster, we believed this cluster produces piRNA from predominantly from single strand, we have changed it accordingly in the text.

3.
Figure S3: single channels are required in monochrome; please add a double stain for another nuage component; this is relevant as in the germanium, also other nuage components form very clear foci. It would be important to show that the nuclear localization of Kumo is not a general feature of nuage components.

Response: As suggested by the reviewer, we have included immunostaining of Krimp co-stained with Myc-Kumo and Otefin, and provided monochrome channels, supporting that Kumo foci, but not Krimp, are present in the nucleus in germarium (Fig S3).

4.
Figure 2: The AGO3 an Mael staining in the kumo mutant appear to be identical images. is that possible?

Response: The particular images were same optical section where we co-stained Ago3 and Mael in the *kumo* mutant ovaries. As we described in this study, *kumo* mutation affects the localization of Ago3 and Mael to perinuclear nuage, but they still co-localize (also see comment 4 from reviewer #2). Therefore they may have given an impression as if they looked identical, but they were not. To avoid such a fallacious impression, however, we have provided a different image of Mael staining (Fig 2).

5.
Where are the primers for Fig 1C located? please add to Figure 1A.

Response: We have provided an updated image.

6.
What about Spn-E and Mael localization in the rescued animals? (Figure 2)

Response: As suggested by the reviewer, we have included images of SpnE and Mael localization in *kumo* rescued ovaries (Figure 2).
7.
Figure 3: The de-silencing of transposons are by far the strongest ones reported to date. To put this into perspective, I suggest to perform this QPCR side by side with one or two other known pathway members such as spn-E or tejas.

Response: We agree with the reviewer for that observed derepression levels for *HetA* is quite strong in *kumo* mutant ovaries (approximately 1000 fold compared to that in heterozygous control). We would like to highlight that the other piRNA pathway mutants also exhibits a strong derepression of *HetA* among the other reteroelements (Klattenhoff et al, 2009; Li et al, 2009; Patil & Kai, 2010). To confirm this, as suggested by the reviewer, we have performed Q-PCR to analyse derepression of several transposons in *mael* and *spnE* mutants. Consistent with those previously reported, we observed a huge derepression for of *HetA* in the *spnE* mutants as well, and provided the data in the revised manuscript (Figure S8). However, the derepression of other reteroelements was in the range reported for *rhi* (Klattenhoff et al, 2009) and *ago3* mutants (Li et al, 2009).

8. I have several comments on the bioinformatic analysis of the small RNA datasets:
normalization: the authors write:

"The sequencing data was filtered for non-coding RNA and small RNAs that result from RNA degradation, and was normalized using genome matching reads."
this does not make any sense; it is fine to remove degradation products from abundant RNAs such as tRNAs, rRNAs, etc. But the normalization to genome matching reads does not make any sense as the genome mappers also include the piRNA fraction. The normalization should be done with a set of bona fide endogenous small RNAs that are not piRNAs such as endo-siRNAs or miRNAs.
also the following quote does not make much sense in this respect. Is the data normalized for siRNAs or for genome mating reads then?
"Data among two libraries were normalized to the total number of perfect genome-mapping reads, and non-coding RNA and endosiRNAs, which are derived from two major hairpins (Okamura et al, 2008)."

Response: We thank the reviewer for raising this important point. As mentioned in the supplementary materials and methods, to find out suitable normalization strategy, we compared two normalization methods; one using the non-coding RNA;, and the other with siRNA, as employed in other credible publications (Klattenhoff et al 2009, Malone et al 2009). After comparing the two normalization methods, we employed one with the endosiRNA for our analysis which gave us the most reliable and comparable results to the other credible published studies. We have amended the results to clearly state our normalization strategy.

for the length profile of the small RNAs:
why does the profile not go to essentially zero at 30nt? There are hardly any piRNAs having 30nt in length. How do the authors explain this?

Response: We thank the reviewer for pointing this out. It was a simple labelling mistake, and we have corrected it.

Split Fig 4A in sense and antisense small RNAs

Response: We have split the figure as suggested by the reviewer.

Fig 4B: it appears that these density plots also include siRNAs. This is indicated by the internal density of small RNAs mapping to the genomic minus strand, which is a sign of siRNAs. Please restrict this analysis to piRNAs only.

Response: We greatly appreciate that the reviewer pointed this out. Although the plots did not contain siRNA, we found some technical mistakes, and that is why the plots appeared differently from what they were supposed to be. We also checked other density plots as well, to make sure no further mistakes. We provided amended plots in Figure 4B of the revised manuscript.

Fig5: ZAM small RNA profile: Two things are very strange: (1) why is the density starting so abruptly at 24nt? (2) why is the 21mer fraction still antisense biased? As these are siRNAs, there should be an equal number of sense and antisense small RNAs. I just wonder whether there is a general flaw in the computational analysis.

Response: We do not think both issues are due to any flaw in our analysis as our amended plots are quite consistent with those in other credible articles. As to the first point, however, we agree that ZAM piRNAs abruptly starts at 24nt, and that is not the case in other transposons. We suspect that in our libraries, most of the small RNAs against *ZAM* may be piRNAs and contain very few endo-siRNAs (we notice similar trend for small RNA against ZAM in cuff heterozygous library (Pane et al, 2011). This could explain the first issue. As the percentage of sense and antisense endo-siRNAs against transposons varies (Ghildiyal et al 2008), it is reasonable to see such bias in that population. Consistent with this view, small RNA profile for *ZAM* in the *rhino* heterozygous and mutant ovaries also showed high number of 21nt antisense RNA species, but a small population of corresponding sense RNA (Klattenhoff et al 2009).

"Thus, sequencing analysis of piRNAs confirms the importance of kumo for the production of both germline primary and ping-pong derived piRNAs, but not for that of somatic piRNAs."
how can the authors conclude that the primary pathway in the germline is affected in kumo

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mutants? Have they stained for Piwi as delocalization of Piwi seems to correlate with defects in the primary pathway?

Response: We thank the reviewer for pointing this out. By 'reduction in primary germline piRNA' we referred to reduction in the piRNA matching to dual strand clusters, which are product of primary processing. This was also supported by observed reduction in expression of Piwi in *kumo* mutant germline cells (but not in somatic cells), suggesting there may be some defects in primary processing in germline. In the revised manuscript, we included Piwi staining and amended the text as follows (Page 6, Line 11).

> "In addition, we also observed a slight reduction of Piwi in *kumo* mutant germline cells, indicating that the primary processing may also be affected."

about the ping-pong analysis: the authors conclude that Kumo is required for ping-pong but that a sub-population of ping-pong pairs is independent of Kumo. I think that this analysis needs a statistically better controlled analysis:

"However, piRNA levels with 10-nt overlap for TARTA and I-element were not completely abolished in kumo mutant ovaries (Fig. 5). In addition, only a small reduction in that of roo and blood was observed, implicating that kumo may not be necessary for production of a subset of piRNAs in ping-pong cycle (Fig. 5, S6).

"Response: The ping-pong signatures for *TART-A* and *I element* (which are predominantly suppressed by germline piRNA) are significantly reduced in *kumo* mutant ovaries (more than 80% compared to heterozygous controls). The statement "not completely abolished" was written in the comparison with *Het-A* (which showed a near complete abolition of pig-pong pairs, Fig 5). We deleted the statement regarding to *TART-A and I-element*, which may have misled the reviewer.

As to *roo,* consistent with the observations in in *krimp* and *aub* mutants, we detected a mild reduction of piRNAs including those with the ping-pong signature, in the *kumo* mutant (Malone et al, 2009). Observation for *blood*, however, is curious, as there is an increase in sense piRNAs in both *kumo* and *rhi* mutants matching to *blood* (Klattenhoff et al 2009; Figure S9). Concomitantly, we do not observe a reduction in the 10-nt overlapping species in the both mutants. To confirm, as suggested by the reviewer, we extended our analysis to the other retroelements. Indeed, we observed this trend not only for *blood,* but also for *mclintock*, *mdg3* and some others, in *kumo* mutant ovaries (Figure S9). Hence it is tempting to speculate that *kumo* may not be required for production of a subset of ping-pong derived piRNAs. We provided those arguments in this revised manuscript as follows (Page 15, Line 1);

> "While most of ping-pong amplification for *TART-A*, *I element* and *Het-A* was collapsed, for *roo,* a germline dominant transposon, only a mild reduction (~40%) in piRNA with 10-nt overlap was observed. This observation, however, is consistent with those reported in the other germline piRNA pathway component mutants, *aub* and *krimp* (Malone et al 2009). In addition, for a group of transposons such as *blood, mclintock* and *mdg3,* we observed only a small or no reduction in the piRNAs with 10-nt overlap. This may be due to an increase in sense piRNAs of those transposons in *kumo* mutant ovaries. A similar profile for *blood* and other transposons has been reported in the *rhi* mutant (Klattenhoff et al 2009)."

9.

Figure 6C: what are the black marks in the upper panels? lower panels: why does the western blot to the left look so much worse than the one to the right? on the left blot it is very difficult to agree that the experiment has indeed technically worked. where are the molecular size markers?

Response: We believe that the black marks in the upper panels are some background from chemi-luminiscence. However, as they do not overlap with Myc-tagged proteins, they should not cause any interference and not result in misinterpretation. For the lower left blot, we provided a better image of the same blot. Although some background signal can be seen in the blot, the specific bands are abundantly present, indicating that Myc-kumo variants were indeed expressed and were immunoprecipitated. In the revised Figure 6C, we have included size markers as suggested.

10.

about the sDMA analysis: the authors can only conclude that Kumo does not interact with the published peptide in an sDMA independent manner. this does not mean that Aub has no additional sDMA residues, which could be essential for the interaction.

Response: Previously we have mutated the four arginine residues (Patil and Kai 2010), which were proposed as sites for the sDMA by (Nishida et al, 2009). In their study, they analysed Aub by mass spec for sDMA sites and have not reported only above four sites. However, we could not exclude small possibility that there may be other sDMA sites, as coverage in their mass spec analysis was not 100%, hence we modified our conclusion in accordance with reviewer's comment as follows (Page 10, Line 19);

> "suggesting that the interaction is not dependent on the sDMA of Aub at the four arginine residues, at least in S2 cells."

11.
naming: I suggest to call "precursor piRNAs" cluster transcripts to avoid confusion with the mature piRNAs.

Response: As suggested by reviewers, we have changed 'precursor piRNA' to 'cluster transcripts'.

12.
citations
"Animal genomes have a special small RNA-based defense system called Piwi interacting RNA (piRNA) that can counter the transposon threat in the gonads (Brennecke et al, 2007; Saito et al, 2006; Vagin et al, 2006)."
animal?? all citationsrefer to flies; several key citations (even for fly) are missing; for example the entire genetics on transposon control.
"Many other proteins that localize to nuage like Vasa (Vas), Spindle-E (Spn-E), Tejas (Tej), Krimper (Krimp), and Maelstrom (Mael), have been shown to play a role in maintaining nuage organization, and transposon repression via germline piRNA pathway (Chen et al, 2007; Li et al, 2009; Lim & Kai, 2007; Malone et al, 2009; Patil & Kai, 2010).
where is the maelstrom citation? why is cutoff cited?

Response: We have included the citations as suggested by the reviewer, and deleted the cutoff citation.

Referee #2

Major questions/concerns:

1) Does kumo affect stem cell maintenance? This would be expected to result in empty ovarioles in females older than 20 days? Or is there another reason for the decrease in egg laying? Is the fusome affected? (which would explain the oocyte determination defects). Staining with alpha-spectrin antibody would answer this question and would allow placing kumo either among the genes that have mostly postmitotic germline defects versus the ones that already affect the stem cells.

Response: We appreciate that the reviewer raised an important issue which may be of an interest in the field. We did not observe any empty ovarioles of *kumo* mutant even at 20 days after eclosion. Hence the loss of oocyte in *kumo* mutant ovaries is likely due to mitotic defect. Immunostaining with alpha-spectrin also did not indicate any loss of stem cells in 15 days-old females. We have included this observation in the revised manuscript as follows (Page 5, Line 11);

> "However, even after 15 days of eclosion, in *kumo* mutant ovaries, we observe germline stem cells harboring round fusome and differentiating cysts with branched fusome (Fig. S6). This observation suggests that the *kumo* mutation does not cause defects in the maintenance or differentiation of germline stem cells."

2) Does Kumo co-localize with Rhino? Rhino has been described to have essentially the same function as Kumo in the nucleus, but loss of Rhino gives a stronger phenotype with respect to loss of cluster transcription. Does Kumo interact with Rhino? A Rhino-GFP construct is available.

Response: We thank the reviewer for raising this important issue. Rhino appear to localize to the nucleus throughout oogenesis while Kumo localization to nucleus is restricted to region 2 of germarium. We have performed immunostaining to examine co-localization of Kumo and Rhino using an unpublished anti-Rhino (a kind gift from Dr. William Theurkauf). Although some foci of those proteins occasionally appear to be juxtaposed in germline nuclei, they were not overlapped

(Supple X). In addition, as suggested by the reviewer, we performed immunoprecipitation experiment using ovarian lysates to examine physical interaction between Rhi-Kumo. Even after several trials, we, however, could not detect any interaction (supple X).Our results suggest that Rhino and Kumo probably are not present in the same complex, and not in the same pathway for precursor transcription. Instead, *kumo* likely functions to balance the heterochromatin state for cluster transcription via interaction with HP1. We have included our argument in the revised manuscript as follows (Page 12, Line 1);

> "Rhi, a HP1 homologue expressed in germline, has been reported to function for cluster transcription (Klattenhoff et al 2009). However, the exact mechanism how *rhi* promotes the cluster transcription remains unknown. To study whether Kumo functions for cluster transcription via Rhi, we examined their interaction by immunostaining and coimmunoprepicitation. By immunostainingwe observed some juxtaposed Kumo and Rhi foci but not a significant number of overlapping foci (Fig. S13A). Furthermore, Rhi did not co-immunoprecipitate with Myc-Kumo from the ovarian lysates (Fig. S13B), suggesting that Rhi and Kumo are not present in the same complex."

3) Results p. 8 and Figure S5, C: the authors claim that Gurken protein is not localized correctly in the mutant. But in the picture they provide, Gurken is completely normally accumulating in the region directly adjacent to the oocyte nucleus! One can easily discern the nucleus in the mutant picture - the egg chamber is misoriented and points with the dorsal side up, but the accumulation of Gurken is clearly asymmetric around the nucleus, and if the authors had rolled the egg chamber by 90 degrees, the picture would have looked very similar to the control. Normally in the spindle mutants, the oocyte nucleus, i.e. the primary dv polarity, is normal, as is Gurken localization, but Gurken translation is reduced, leading to lower levels of protein and therefore a reduction in dorsal cell fates. This is probably the case here as well, and may be the reason why the authors thought the protein was "only anterior" but it would require careful quantitation to allow the firm conclusion. It would be completely surprising if Gurken protein were mislocalized in a band along the anterior, as the authors claim, since this would lead to a dorsalized (K10 or squid-like) egg shell phenotype!!

Response: We truly appreciate that the reviewer pointed this out. Considering the possibility that we might have overlooked the defect due to the lower Gurken level in *kumo* mutant, we carefully re-examined Gurken localization in 2-3 days old *kumo* mutant flies where egg chambers at late stages are still present. Using confocal microscope, we scanned oocytes from the top to the bottom to determine how Gurken is really localized regardless of the oocytes' angles. This experiment reveals that 65.6% of *kumo* mutant ovarioles had proper Gurken localization, and 34.4 % showed defective in it (either weak expression or localized only to anterior). This is consistent with our quantitative analysis showing on the milder defect in appendages of the eggs (Table S1; 42.1% of the eggs from *kumo* mutant mother show the normal appendages versus 94.4% of the eggs from control mother are normal). We have taken out the images of the Gurken localization, but instead provided the statistics of Gurken localization supporting the polarity defect in *kumo* mutant ovaries as follows (Page 5, Line 26);

> "While Gurken was properly localized at the anterior-dorsal region in the most of control oocytes, it was mislocalized in 34.4% of *kumo* mutant ovarioles in 2-3 days old females, indicating a mild defect in the polarity formation at least in young *kumo* mutant ovaries. Hence, *kumo* mutant ovaries exhibit the shared defects with other nuage components, such as oocyte fate determination, karyosome compaction the defect in the polarity formation, implicating that *kumo* may function in the same pathway as the other nuage components."

4) Nuage (results p. 8): The authors claim that Kumo is 'the most upstream" in the organization of the nuage and participates in the organization of the nuage. However, the pictures shown in Fig. 2A show striking particles in all the mutants that are not "small foci" - but as large as the normal nuage particles, strongly suggesting that Kumo's role is to anchor the nuage particles to the nuclear envelope, and not necessarily for the "organization" of the particles. The authors need to formulate this much more carefully, since they provide no evidence that the other nuage proteins are not forming the appropriate particles that may well be organized "normally", just not anchored to the nuclear membrane (all the proteins still seem to colocalize in those foci, no reason to think that they are "disorganized").

Response: We thank the reviewer for raising this important issue. Indeed, the examined nuage components appear to form cytoplasmic 'aggregates' in *kumo* mutant ovaries, as rightly pointed by

the reviewer. In addition, they appear to co-localize in such aggregates (data not shown). Furthermore, in the previous study, we have shown that Tej can interact with SpnE and Aub in the absence of germline factors when expressed in S2 cells, supporting that at least interactions of some nuage components do not require *kumo* function (Patil et al., 2010). Therefore we could not exclude the possibility that the assembly of a macromolecular complex is not affected, and instead the engagement of such a complex in the processing of germline piRNA at perinclear region may be perturbed in the absence of *kumo* function. To include our argument, we amended the statement regarding to this issue throughout the revised manuscript as follows.

> Page 6, results,
> *confirming that Kumo is required for localization of other piRNA pathway proteins to the perinuclear nuage*.
>
> Page 14 in discussion,
> Out study also demonstrates that the tudor domains of Kumo mediate the interaction with other nuage components and this interaction of other nuage components with Kumo is central for their localization on nuage (Fig. 6C, S8A).

5) Results p. 9: I did not understant the normalization of the libraries "perfect genome-mapping reads". It would be better to use just other small RNAs (miRNAs) that are not affected by the pathway to normalize the samples. Would not the "perfect genome-mapping reads"be affected by the mutant?
Response: Please refer our response to point 8 raised by the reviewer #1 for more details. We normalized the small RNA sequencing data with the endosiRNA (supplementary materials and methods). We have amended the results and now clearly state the normalization strategy.

6) Results p. 14, Fig. 7D: Are the differences between wildtype and the kumo mutant significant in all cases for cluster 42A/B? It may be that only regions B2 and B5 show a significant increase? Please specify statistics for the various regions in the Figure/Figure legend.
Response: We thank the reviewer for bringing up this point. As mentioned in our response to Point 2 and 3 in the main comments from the reviewer 1, the piRNA clusters are indeed repetitive in nature hence it is not possible to design primers to examine all the regions spanning the clusters. In our study, in addition to those described by Klattenhoff et al 2009 (positions mentioned in the Figure 7A, also see table S2 and sup M&M), we were able to design two primer sets those worked nicely. We would like to highlight that those primers covers the regions in the 42AB cluster which most actively contribute to piRNA production, and we observed significant increase in HP1 enrichment in *kumo* mutant ovaries. As suggested by the reviewer, we now have included a statistics of increase in HP1 occupancy at the regions examined (in the legend for Fig.7).

7) How do all the various results compare to the results of loss of Rhino? (which piRNAs are affected? which cluster transcripts?) A careful and explicit comparison would be useful. - Also, did the authors use the same primers for the analysis of the cluster transcription as Klattenhoff et al.?
Response: As mentioned above, we have used the same primer sets described by Klattenhoff et al, 2009 in addition to ones we designed (Point 2 in the major comments by the reviewer #1). While *rhi* mutation abolished most of the cluster transcription, we observed a moderate decrease in cluster transcription from the same regions in *kumo* mutant ovaries (1.6- to 7.6-fold reductions compared to those in the heterozygous control, Figure 7C). In addition, similar to the *rhi* mutant, *kumo* mutant ovaries exhibit reduction of piRNAs from all germline piRNA clusters (Table S4). However, as our newly added data suggested that *rhi* and *kumo* does not appear to be in the same complex, and not in the same pathway for precursor transcription (please refer Point 2), we did not include comparisons of those mutants.

Minor comments:

The manuscript contains numerous typographical and grammatical errors, for instance (these are just a few examples):
p.3 discreet clusters: correct is "discrete clusters"
(discreet means "tactful")
p.5 This is further supported by our observation for Kumo interaction with HP1.

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Correct: ..our observation for an interaction of Kumo with HP1
p.5 Our results suggest that Kumo plays dual role:
Correct: Kumo plays a dual role
p. 11 Consistent with observation in another piRNA pathway mutants, rhino and krimper..
correct: observation in other piRNA pathway mutants...
p. 11 kumo mutation causes a great reduction
correct: The kumo mutation causes ...

It would probably be good to have a native English speaker to read carefully through the manuscript.

Response: We thank reviewer for pointing these errors, and we have incorporated all corrections above and the others as well.

References:
Klattenhoff C, Bratu DP, McGinnis-Schultz N, Koppetsch BS, Cook HA, Theurkauf WE (2007) Drosophila rasiRNA pathway mutations disrupt embryonic axis specification through activation of an ATR/Chk2 DNA damage response. *Dev Cell* **12:** 45-55

Klattenhoff C, Xi H, Li C, Lee S, Xu J, Khurana JS, Zhang F, Schultz N, Koppetsch BS, Nowosielska A, Seitz H, Zamore PD, Weng Z, Theurkauf WE (2009) The Drosophila HP1 homolog Rhino is required for transposon silencing and piRNA production by dual-strand clusters. *Cell* **138:** 1137-1149

Li C, Vagin VV, Lee S, Xu J, Ma S, Xi H, Seitz H, Horwich MD, Syrzycka M, Honda BM, Kittler EL, Zapp ML, Klattenhoff C, Schulz N, Theurkauf WE, Weng Z, Zamore PD (2009) Collapse of germline piRNAs in the absence of Argonaute3 reveals somatic piRNAs in flies. *Cell* **137:** 509-521

Malone CD, Brennecke J, Dus M, Stark A, McCombie WR, Sachidanandam R, Hannon GJ (2009) Specialized piRNA pathways act in germline and somatic tissues of the Drosophila ovary. *Cell* **137:** 522-535

Moshkovich N, Lei EP (2010) HP1 recruitment in the absence of argonaute proteins in Drosophila. *PLoS Genet* **6:** e1000880

Nishida KM, Okada TN, Kawamura T, Mituyama T, Kawamura Y, Inagaki S, Huang H, Chen D, Kodama T, Siomi H, Siomi MC (2009) Functional involvement of Tudor and dPRMT5 in the piRNA processing pathway in Drosophila germlines. *EMBO J* **28:** 3820-3831

Pane A, Jiang P, Zhao DY, Singh M, Schupbach T (2011) The Cutoff protein regulates piRNA cluster expression and piRNA production in the Drosophila germline. *EMBO J*

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Rangan P, Malone CD, Navarro C, Newbold SP, Hayes PS, Sachidanandam R, Hannon GJ, Lehmann R (2011) piRNA Production Requires Heterochromatin Formation in Drosophila. *Curr Biol* **21:** 1373-1379

Yin H, Lin H (2007) An epigenetic activation role of Piwi and a Piwi-associated piRNA in Drosophila melanogaster. *Nature* **450:** 304-308

Zamparini AL, Davis MY, Malone CD, Vieira E, Zavadil J, Sachidanandam R, Hannon GJ, Lehmann R (2011) Vreteno, a gonad-specific protein, is essential for germline development and primary piRNA biogenesis in Drosophila. *Development* **138:** 4039-4050

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2nd Editorial Decision 27 October 2011

Thank you for submitting your revised manuscript for consideration by the EMBO Journal. It has now been seen by the two original referees whose comments are enclosed. As you will see they find that the study has been significantly improved, there are a few things that need to be clarified and importantly the grammar and spelling within the manuscript must be improved. Referee #1 suggests the potential removal of the section describing the nuclear role of Kumo but I leave this to your discretion.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: http://www.nature.com/emboj/about/process.html

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

Yours sincerely,

Editor
The EMBO Journal

REFEREE COMMENTS

Referee #1:

In their revised manuscript, the authors have attempted to address most of the points raised by the two reviewers.

considering my previous split of the manuscript into three parts:

1. characterization of Kumo as an oogenesis gene and as an integral nuage component
2. evidence that Kumo is required for the piRNA pathway (transposon de-repression and small RNA analysis in Kumo mutants)
3. elucidation of a nuclear function of Kumo in early oogenesis.

Significant improvements have been made on section 1 (mostly based on comments from reviewer #2) and section 2.
I still think, that the computational small RNA analysis has a few irregularities. For example in Figure 4A: The legend says, that this plot represents piRNAs. But the plot ranges from 20nt to 30nt, so it does include also siRNAs. In addition, the 21mer population is essentially exclusively antisense. Given the distributions of small RNAs mapping to individual elements (Figure 5), this comes as a surprise as many elements do contain sizable sense populations as well, as would be expected from siRNAs.
For the calculations of changes in piRNA levels, the authors use the 24-30nt pool, although 23mers should be included as bona fide piRNAs as well.
Finally, as stated in my previous report, I continue to emphasize that the ping-pong analysis should be approached in a more statistically controlled manner. At the moment, the manuscript suggests that some elements are participating in ping-pong independently of Kumo. But as pointed out by the authors, an increase in sense piRNAs might increase the 10nt overlap population. However, if that sense piRNA increase is random, it will also increase the 9mer, 8mer, etc overlap population. A properly controlled ping-pong analysis will in this case still report a reduced ping-pong signal.

The third part of the manuscript (concerning the nuclear role of Kumo) is still rather speculative in my opinion.

The newly added data supports a nuclear localization of Kumo in early oogenesis. Furthermore, no

co-localization and interaction with Rhino, a known regulator of germline piRNA clusters has been observed. As pointed out in my previous report, PCR analysis of cluster transcripts or chromatin-IP experiments is sub-optimal. Nevertheless, the authors have undertaken additional efforts to add more regions to the analysis and the obtained data is consistent with a model in which 42AB RNA levels are decreased in kumo mutants.
The data on the link to HP1 biology is still weak in my opinion. I appreciate that the authors provide a reciprocal IP-experiment to show that Kumo and HP1a physically interact. However, I want to state again, that the stoichiometry of the interaction shown in panel 7E right is at odds with the reality in the ovary. HP1a is an abundant nuclear protein, present in all cells of the ovary. In contrast, a small fraction of Kumo is present in the nucleus only during early stages. Considering the entire pool of Kumo, this is a tiny portion. Nevertheless, in this experiment, the authors show that an IP with tagged Kumo brings down levels of HP1 that are comparable with those of the bait itself.
I am also puzzled about the ChIP experiment. For region 42AB-2, the data suggest that the ChIP of HP1a brings down ~10% of the input material. How would that be possible if we consider that in the later stages of oogenesis, where the authors do not see any changes in HP1 levels, the cells are massively poly-ploid, but that the earlier cells (those where Kumo is nuclear) are only diploid. So of the total chromatin input material, only a small raction will come from the early cells.

In my opinion, the data on the nuclear role of Kumo is a bit pre-mature and one might consider removing it. Parts 1 and 2 are novel and do warrant publication in EMBO in my opinion.

additional comments:
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Please state explicitly what the orientation refers to. Does it refer to the sense and antisense strands of transposable elements or the sense/antisense strands of piRNA clusters?

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Referee #2:

Anand and Kai: revised manuscript

In their revised manuscript, the authors have addressed my major concerns in a satisfactory manner. In particular, the normalization of the libraries now makes sense, the added statistics for HP1 occupancy as well as the more accurate description of the Gurken localization defects have all helped to improve the quality of the results.

As pointed out in my previous review, the authors present an interesting story on a novel Tudor domain containing factor that has functions both in the nucleus as well as the nuage. With the revisions the authors have satisfied my previous criticisms and I judge the manuscript ready for publication, with the exception of a number of grammatical problems, some of which I am pointing out below - I would urge the authors again to have a native English speaker read through the manuscript before final submission.

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2nd Revision - authors' response 14 November 2011

General Response: We thank the reviewers for their positive recommendation on our work. We deeply appreciate their thoughtful and constructive comments. We have made changes to the manuscript according to the comments and we hope that this improved manuscript is now acceptable for publication in *EMBO journal*. Please find our response to the comments below.

Referee #1:

In their revised manuscript, the authors have attempted to address most of the points raised by the two reviewers.

considering my previous split of the manuscript into three parts:

1. characterization of Kumo as an oogenesis gene and as an integral nuage component
2. evidence that Kumo is required for the piRNA pathway (transposon de-repression and small RNA analysis in Kumo mutants)
3. elucidation of a nuclear function of Kumo in early oogenesis.

Significant improvements have been made on section 1 (mostly based on comments from reviewer #2) and section 2. I still think, that the computational small RNA analysis has a few irregularities. For example in Figure 4A: The legend says, that this plot represents piRNAs. But the plot ranges from 20nt to 30nt, so it does include also siRNAs. In addition, the 21mer population is essentially exclusively antisense. Given the distributions of small RNAs mapping to individual elements (Figure

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5), this comes as a surprise as many elements do contain sizable sense populations as well, as would be expected from siRNAs

Response: We truly appreciate the reviewer for pointing this irregularity. Indeed, there was a small mistake in our calculation. Now we included all the small RNAs those match to piRNA clusters and transposons. The equal numbers of sense and antisense 21-mer endo-siRNA are discernible in both the control and the mutant libraries in the amended plots. We also amended the figure legend as below;

> "Length histogram of sense and antisense small RNAs produced in the *kumo* mutant and the control ovaries."

Though endo-siRNAs matching to plus strand are present (3-fold less than the antisense population), they are masked in the plots as the majority of the small RNAs in the plots are piRNAs. However, the overall endo-siRNAs population matching to the entire genome has almost equal numbers of sense and antisense matching reads.

For the calculations of changes in piRNA levels, the authors use the 24-30nt pool, although 23mers should be included as bona fide piRNAs as well.

Response: We thank the reviewer for pointing this out. We now have included 23-nt piRNAs as well, while describing the reduction in the overall piRNA levels.

Finally, as stated in my previous report, I continue to emphasize that the ping-pong analysis should be approached in a more statistically controlled manner. At the moment, the manuscript suggests that some elements are participating in ping-pong independently of Kumo. But as pointed out by the authors, an increase in sense piRNAs might increase the 10nt overlap population. However, if that sense piRNA increase is random, it will also increase the 9mer, 8mer, etc overlap population. A properly controlled ping-pong analysis will in this case still report a reduced ping-pong signal.

Response: In the revised manuscript, as suggested by the reviewer, we listed all the transposons for those ping-pong cycle does not seem to be affected due to a general increase sense piRNAs (Fig S9). All these transposons belong to a class that is targeted by both germline and somatic piRNAs (Fig S9) (Malone et al 2009).

Indeed, as the reviewer anticipated, we observed an increase in the level of piRNAs those overlap other than 10 nt for *blood*, *mdg3* and *mclintock* (Fig S8, in the density plots). An increase in the sense-antisense pairsthose overlap other than 10 nt reduces the ratio of those with10-nt overlap versus those with other than 10 nt overlap. The ratio of *blood* piRNAs harbouring 10-nt overlap versus those other than 10-nt overlap in the control heterozygous is 0.197, while in the *kumo* mutant, the ratio is 0.06, indicating that *kumo* is required at least for the production of a subset of ping-pong derived *blood* piRNAs. For *mdg3* and *mclintock*, however, these ratios did not differ significantly (in the control and in the mutant, 0.089 vs. 0.093, and 0.147 vs. 0.135, for *mdg3* and *mclintock*, respectively). This indicates that generation of overlapping piRNAs for *mdg3* and *mclintock* are independent of *kumo.* To make this point clearer, we have now included the following statement in Results of the revised manuscript.

> "We also observed an overall increase in the piRNAs those match to sense strand of *blood*, *mgd3* and *mclintock* in the *kumo* mutant ovaries. Such an increase of sence piRNAs results in a significant loss of the 10-nt overlap bias of *blood* piRNAs in the *kumo* mutant, indicating a requirement of *kumo* for the production of a subset of the ping-pong derived *blood* piRNAs. However, no significant change in the 10-nt bias was observed for *mdg3* and *mclintock*, suggesting that the production of the ping-pong piRNAs for these transposons may be independent of *kumo*."

The third part of the manuscript (concerning the nuclear role of Kumo) is still rather speculative in my opinion.

The newly added data supports a nuclear localization of Kumo in early oogenesis. Furthermore, no

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co-localization and interaction with Rhino, a known regulator of germline piRNA clusters has been observed. As pointed out in my previous report, PCR analysis of cluster transcripts or chromatin-IP experiments is sub-optimal. Nevertheless, the authors have undertaken additional efforts to add more regions to the analysis and the obtained data is consistent with a model in which 42AB RNA levels are decreased in kumo mutants.
The data on the link to HP1 biology is still weak in my opinion. I appreciate that the authors provide a reciprocal IP-experiment to show that Kumo and HP1a physically interact. However, I want to state again, that the stoichiometry of the interaction shown in panel 7E right is at odds with the reality in the ovary. HP1a is an abundant nuclear protein, present in all cells of the ovary. In contrast, a small fraction of Kumo is present in the nucleus only during early stages. Considering the entire pool of Kumo, this is a tiny portion. Nevertheless, in this experiment, the authors show that an IP with tagged Kumo brings down levels of HP1 that are comparable with those of the bait itself.

Response: We appreciate the reviewer's concern about the measurement of cluster transcription. We would like to highlight that method we used has been widely accepted in many peer-reviewed studies (Klattenhoff et al 2009; Rangan et al 2011; Pane et al 2011,). In addition to the primers reported in those credible articles, we also performed ChIP with the additional sets of primers those we designed in this study, which also supported our observation. As pointed by the reviewer, in Figure 7E right panel, a significant amount of HP1 was pulled down with Myc-Kumo. We cannot absolutely exclude a possibility that it may include artifactual signal. Alternatively, IgG signal that is very close to HP1may partially contribute to the strong HP1 signal. However, we would like to highlight that we confirmed the interaction of Kumo and HP1 in S2 cells, and the reciprocal IP using HP1 pulled down a small amount of Myc-kumo, which appears to be reflecting such a small fraction of Kumo in the nucleus. We strongly believe that those experiments complement and support our observation for the interaction of HP1 and nuclear Kumo.

I am also puzzled about the ChIP experiment. For region 42AB-2, the data suggest that the ChIP of HP1a brings down ~10% of the input material. How would that be possible if we consider that in the later stages of oogenesis, where the authors do not see any changes in HP1 levels, the cells are massively poly-ploid, but that the earlier cells (those where Kumo is nuclear) are only diploid. So of the total chromatin input material, only a small raction will come from the early cells.

Response: For region 42AB-2, we observed around 8-fold increase in HP1 occupancy in the *kumo* mutant, which was highest among ones observed for any region at the *42AB* cluster. However, the change in HP1 binding to this particular region may not reflect as the overall trend of the HP1 levels. It may also be possible that nuclear Kumo in early stages functions to set the epigenetic state of piRNA clusters via HP1, and facilitates transcription even in the later stages. Hence in the absence *kumo*, HP1 binding to cluster may remain high during all stages of oogenesis, though the upregulation of total HP1 is not really discernible.

In my opinion, the data on the nuclear role of Kumo is a bit pre-mature and one might consider removing it. Parts 1 and 2 are novel and do warrant publication in EMBO in my opinion.

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both reviewers have pointed out that the manuscript contains several errors in terms of spelling and grammar. This is, unfortunately and sadly still the case. One wonders, why not more effort is undertaken to submit a more mature manuscript at the revision stage. Here are some examples:

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Response: We have corrected size markers and stated that the small arrows indicate the specific bands.

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the ZAM primers, etc.

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Response: As the reviewer suggested, we now clearly state the decrease in piRNAs those match to sense and antisense strand of the cluster.

why does the data in Figure 7C suddenly look different (region 42AB 1 plus and minus)? compared to the old manuscript, there is a strong change in the relative values.

Response: In the previous manuscript, we re-plotted the cluster transcription levels with respect to the control as suggested by the reviewer. Hence the plots look different from the previous version.. However, the fold difference in expression levels between the control and mutants remains the same to what we presented in the first manuscript.

Referee #2:

Anand and Kai: revised manuscript

In their revised manuscript, the authors have addressed my major concerns in a satisfactory manner. In particular, the normalization of the libraries now makes sense, the added statistics for HP1 occupancy as well as the more accurate description of the Gurken localization defects have all helped to improve the quality of the results.

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Response: We thank the reviewer for the appreciation of our study. We amended all the points raised by the reviewer stated below. Furthermore we corrected many other grammatical errors pointed by native English speakers.

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Malone CD, Brennecke J, Dus M, Stark A, McCombie WR, Sachidanandam R, Hannon GJ (2009) Specialized piRNA pathways act in germline and somatic tissues of the Drosophila ovary. *Cell* **137:** 522-535

Klattenhoff C, Xi H, Li C, Lee S, Xu J, Khurana JS, Zhang F, Schultz N, Koppetsch BS, Nowosielska A, Seitz H, Zamore PD, Weng Z, Theurkauf WE (2009) The Drosophila HP1 homolog Rhino is required for transposon silencing and piRNA production by dual-strand clusters. *Cell* **138:** 1137-1149

Rangan P, Malone CD, Navarro C, Newbold SP, Hayes PS, Sachidanandam R, Hannon GJ, Lehmann R (2011). piRNA Production Requires Heterochromatin Formation in Drosophila. **Curr Biol** 21:1373-9

Attilio Pane, Peng Jiang, Dorothy Yanling Zhao, Mona Singh and Trudi Schüpbach (2011). The Cutoff protein regulates piRNA cluster expression and piRNA production in the Drosophila germline. The EMBO Journal doi:10.1038/emboj.2011.334.

3rd Editorial Decision 16 November 2011

Thank you for submitting a revised version of your manuscript, I have looked through it and your response to the remaining concerns and I am happy to accept it for publication in The EMBO Journal. You will receive the official acceptance letter in the next day or so.

Yours sincerely,

Editor
The EMBO Journal