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Glycolytic enzymes localize to ribonucleoprotein granules in Drosophila germ cells, bind Tudor and protect from transposable elements

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Editor: Esther Schnapp

1st Editorial Decision 24 September 2014

Thank you for the submission of your research manuscript to EMBO reports. It was sent to three referees, and so far we have received reports from two of them, which I copy below. Although the third referee has not returned his/her report, the other two referees are in fair agreement, so I am making a decision on your manuscript now in order to save you from any unnecessary loss of time.

As you will see, while both referees acknowledge that the findings are potentially very interesting, both of them also indicate that the current data are insufficient to support the main conclusions. Both referees point out that effects of the glycolytic enzyme mutants on siRNA and miRNA pathways need to be excluded in order to unambiguously conclude that these enzymes regulate the piRNA pathway. They also remark that the data are not sufficient to conclude that ATP availability in the vicinity of Tudor is required for piRNA biogenesis, that important controls and statistical analyses are missing, and that many results require verification.

Given these substantial concerns, the amount of work required to address them, the uncertain outcome of these experiments, and the fact that EMBO reports can only invite revision of papers that receive enthusiastic support from a majority of referees, I am sorry to say that we cannot offer to publish your manuscript at this point.

However, in case you feel that you can fully address the referee concerns (as mentioned above and
in their reports) in a timely manner and obtain data that would considerably strengthen the message of the study, then we would be happy to consider a new manuscript on the same topic in the near future. Please note that if you were to send a new manuscript this would be treated as a new submission rather than a revision and would be reviewed afresh, also with respect to the literature and the novelty of your findings at the time of resubmission.

At this stage of analysis, I am sorry to have to disappoint you. I nevertheless hope, that the referee comments will be helpful in your continued work in this area, and I thank you once more for your interest in our journal.

REFEREE REPORTS:

Referee #1:
This is a very interesting study that demonstrates association of glycolytic enzymes with Tudor in germline RNPs and proposes that glycolysis is important for germ cell specification/function and protection from transposable elements. This is a novel and exciting hypothesis. The interaction of PyK and GAPDH2 with Tudor and their localization in germ granules is solid. The purification and characterization of Tudor is also solid. Given the importance of glycolysis in all cellular functions, the use of clones to try and isolate their function in ovaries is appropriate. However this approach still cannot definitively address whether the ovarian defects in piRNA transposon silencing and fertility are secondary or primary (as the authors propose) to depletion of ATP in the vicinity of ATP-dependent RNA helicases required for piRNA biogenesis. A better experiment to prove this point would be to isolate PyK or GAPDH2 mutants that are catalytically active but do not interact with Tudor. However I realize that this is a difficult experiment beyond the scope of this initial report; and may still be inconclusive as PyK or GAPDH2 might interact with other Tdrds besides Tudor. Nevertheless, the authors should try and strengthen their findings. Experiment that would further probe the authors’ hypothesis are:
1. More detailed analyses of the piRNA pathway factors in PyK or GAPDH2 ovaries from mutant germine clones. For example, are the levels or localization of key piRNA factors (Aub, Ago3, Piwi, armi etc) altered in the ovaries? What about the miRNA or siRNA pathway? Is it affected? If the miRNA pathway is not affected then the observed effects would indeed be specific to the piRNA pathway.
2. In Figure 2, the average number of germ cells formed from ald and eno mutants is reported to be 5.4 and 8.9 per embryo, respectively. However the amount of embryos scored is small (10 and 24), followed by great a high standard deviation. Are these results statically significant?
Minor comments:
The term organelle used for the germ cell ribonucleoprotein complex is not appropriate as it is not separated with a membrane from the rest of the cellular materials; consider using particles, or granules.
Figure 1: The legend contains information (Tud full length/Δ3 expression levels and the Tud-Aub complex characterization) that is best listed in the results and discussion area. Tubulin peptide fragments are often found in protein immunoprecipitates as the result of co-precipitation of highly abundant proteins rather than actual interacting candidates. Are tubulins true interactors?

Referee #2:
In this study, the authors focused on glycolysis in Drosophila germ cells and found that glycolytic enzymes such as Pyruvate kinase (PyK) are components of large ribonucleoprotein granules residing in germ cells and that PyK and some other glycolytic enzymes and their mRNAs are enriched in germ cells. The authors also showed that these enzymes are specifically required for germ cell development and that they protect their genomes from transposable elements. The authors note that glycolytic enzymes associate with Tudor, a necessary factor in the piRNA-mediated transposon silencing pathway. Single-particle EM structural analysis of Tudor shows a flexible molecule, suggesting a mechanism in which Tudor recruits the glycolytic enzymes to the granules by binding to them. The authors finally note that their findings support a unifying concept for the crucial role of glycolysis in germ cells, as has been reported in stem cells and cancer.

I found this study potentially interesting. However, to come to the conclusion, much more convincing data are required. Many experiments require controls. Thus, in its current form, it is hard for me to recommend this manuscript be considered for publication in EMBO Reports, or any other
1) Figure 1A: The authors claim that PyK and GAPDH2 co-immunoprecipitated with Tudor from Drosophila ovarian extracts. What was the specificity of the co-immunoprecipitation? Because PyK and GAPDH2 are very abundant proteins, it is possible that they were just background-type signals of the experiments.

2) Figure 1B: pyK, gapdh2 and enolase should also be expressed in other cells in blastoderm stage embryos. However, the signals were merely detected in cells other than pole cells. Controls are missing. Does Enolase co-immunopirify with Tudor? This needs to be examined experimentally.

3) Figure 2A,B: The authors should perform similar experiments using antibodies for GAPDH2 and Enolase. If the antibodies are not available, GFP-tagged proteins can be alternatively used. The cellular localization of PyK is unclear in the figures. Clearer images are required.

4) Figure 2C,D: ImmnoEM for Tudor is required as a control. The authors noted "polar granules contain 5.5 times more PyK than would be expected from the same cytoplasmic area....". The data should be shown.

5) Figure 2D: Is PGK found in the Tudor complex in Figure 1A? Is PGK enriched in polar cells? These questions should be addressed experimentally.

6) Page 6: "ald mutant ovaries showed significant overexpression of many transposons". What types of transposons were overexpressed? Are those transposons overexpressed similar to those upregulated in piwi mutant ovaries? This should be examined.

7) Figure 3H: The authors should examine if the expression levels of miRNAs and endogenous siRNAs were affected in ald, pyk and eno mutants.

8) Page 6: "glycolytic mutations did not cause defects in the generation of secondary piRNAs by Ping-Pong cycle....". "We concluded that glycolytic mutants specifically affect primary piRNA biogenesis". I do not understand these sentences. Secondary piRNA production depends on primary piRNA production. So, if glycolytic mutants affect primary piRNA biogenesis, it would also affect secondary piRNA generation by Ping-Pong cycle. Primary piRNA production and population are different between ovarian somatic cells and germ cells because they require different factors and piRNA clusters expressed are different. Figure 3H shows that piRNAs arising from flamenco, a soma specific piRNA cluster, were not affected in ald, pyk and eno mutants, suggesting that these enzymes are not necessary for primary piRNA production in ovarian somatic cells. Clarification is required.

9) Page 7: "PyK is the major component of the crosslinked Tud complex". The authors should confirm this by performing IP/western using antibodies against Tudor and PyK or other means.

10) Figure 4B,C: No controls. The authors should show, for example, the N terminal part of Tudor (the part that is missing from Tud7-11) does not associate with PyK-GFP. The authors showed that Aub peptide methylated disrupts the interaction between Tudor and PyK-GFP. I wonder if the two Tudor mutants also associate with Aub. This also needs to be examined experimentally.

11) Does PyK contain sDMAs? This needs to be examined by western using antibodies recognizing sDMAs. Does PyK have RGs possibly modified as sDMAs?

12) The data shown in Figure 4H does not support the authors' claim that Tudor acts as a flexible scaffold. More solid data are required.

Glycolytic enzymes such PyK and PGK may accumulate at the high dense structures in germ cells (Figure 2C,D). However, no solid evidence to conclude that the structures are indeed "large ribonucleoprotein granules" was provided. Even though those are the large ribonucleoprotein granules in which piRNA biogenesis takes place, the authors failed to provide evidence to support the idea that ATPs are indeed produced through glycolysis in the granules. Transposons were upregulated in the glycolytic enzyme mutants. However, the authors failed to determine if those transposons are targets of piRNAs or endogenous siRNAs. This is very important considering the fact that endogenous siRNAs in flies are known to silence transposons. piRNAs arising from piRNA clusters decreased in the glycolytic enzyme mutants. However, it is not sufficient to suggest a direct link between piRNA pathway and glycolysis because it could be just an indirect effect.

I think that this study is still immature and too far to be published in any journal.
We appreciate your attention to our manuscript. We have carefully considered the referees’ comments. In response to these comments, this revised manuscript contains new data including a new Figure 4D, two new supplementary Figures S2(A-C) and S4(A-F) and a new supplementary Table S1. These new data clearly show that contrary to microRNAs and small interfering RNAs, glycolytic enzyme mutants specifically decrease piRNA levels. Also, our new data strengthen our conclusion that all the ATP-generating glycolytic enzymes are located in the same granules and molecular complexes as ATP-dependent RNA helicases. Furthermore, to address the comments, we have performed statistical analyses that show that our results are statistically significant and we have addressed referee #2’s comments on control experiments.

Our specific responses follow.

Referee #1:

Introductory Comment: ‘Given the importance of glycolysis in all cellular functions, the use of clones to try and isolate their function in ovaries is appropriate. However this approach still cannot definitively address whether the ovarian defects in piRNA transposon silencing and fertility are secondary or primary (as the authors propose) to depletion of ATP in the vicinity of ATP-dependent RNA helicases required for piRNA biogenesis. A better experiment to prove this point would be to isolate PyK or GAPDH2 mutants that are catalytically active but do not interact with Tudor. However I realize that this is a difficult experiment beyond the scope of this initial report; and may still be inconclusive as PyK or GAPDH2 might interact with other TdRds besides Tudor.’

We have revised the manuscript to clarify that all ATP-producing glycolytic enzymes, namely PGK and PyK, are found to localize to germ granules (Figure 2C,D). In addition, we have strengthened our data by including a new supplementary Figure S2 that shows the results of immunoEM experiments which confirm that the germ granules, presented in Figure 2C,D, contain the ATP-dependent RNA helicases, Vasa and eIF4A. In addition, the new Figure S2C shows directly that the same granule contains both Vasa and PyK. Furthermore, we have emphasized the fact that we show in this study that Tudor is in the same molecular complex with ATP-producing PyK and eIF4A helicase. These findings are consistent with the hypothesis that glycolytic ATP is provided to ATP-dependent helicases in germ granules.

‘More detailed analyses of the piRNA pathway factors in PyK or GAPDH2 ovaries from mutant germline clones. For example, are the levels or localization of key piRNA factors (Aub, Ago3, Piwi, armi etc) altered in the ovaries?’

We tested whether Aub and Ago3, are affected in the germline of glycolytic mutants that show defects in piRNA biogenesis. However, we found that these Piwi
proteins showed normal distribution in mutant germline clones thereby ruling out
the effect of the glycolytic enzymes on the localization of Aub and Ago3 to germ
granules (these data are not shown in the manuscript).

‘What about the miRNA or siRNA pathway? Is it affected? If the miRNA pathway is not
affected then the observed effects would indeed be specific to the piRNA pathway.’

We examined the possible role of glycolysis in the miRNA and siRNA
pathways. Contrary to piRNA biogenesis, the miRNA pathway was not affected in
glycolytic mutants (new Figures S4A, S4C and S4E). Furthermore, we observed that
siRNA levels actually increase in all glycolytic mutants (new Figures S4B, S4D and
S4F). Therefore, effects on miRNAs or siRNAs cannot explain the defects in silencing
of transposable elements observed in our study. Interestingly, an increase in siRNA
levels has been described previously in piwi mutants, suggesting that primary defect
in piRNA biogenesis may indirectly trigger an siRNA response [1]. Therefore, our
extended analysis has considerably strengthened our conclusion on the specific
effect of glycolysis on piRNA pathway.

‘In Figure 2, the average number of germ cells formed from ald and eno mutants is
reported to be 5.4 and 8.9 per embryo, respectively. However the amount of embryos
scored is small (10 and 24), followed by great a high standard deviation. Are these
results statistically significant?’

Our text has been revised to include the results of statistical analyses, which
confirmed that the reductions of germ cells in both ald and eno mutants compared
with the wild-type control are statistically very significant (P<0.0001). ald mutant
flies can lay only very few embryos and stop laying eggs in several days (Figure
S3A). Therefore, it is technically difficult to obtain a large collection of ald embryos
for immunostaining.

Minor comments:
1. ‘The term organelle used for the germ cell ribonucleoprotein complex is not
   appropriate as it is not separated with a membrane from the rest of the cellular
   materials; consider using particles, or granules.’

   We have changed the term ‘organelle’ to ‘granule’ or ‘particle’ in the title and
   throughout our manuscript.

2. ‘Tubulin peptide fragments are often found in protein immunoprecipitates as the
   result of co-precipitation of highly abundant proteins rather than actual interacting
   candidates. Are tubulins true interactors?’

   We carefully controlled our immunoprecipitations to ensure the specificity of
   interactions with Tud and have added the following statement in the section of
   manuscript describing the identification of Tud complex components. ‘Importantly,
   all the proteins of Tud complex were recovered repeatedly from independent
complex isolations and were never found in control ovarian GFP immunoprecipitations performed under the same conditions as Tud immunoprecipitations as analyzed by mass spectrometry (Supplemental Experimental Procedures).

Referee #2:

1. ‘Figure 1A: The authors claim that PyK and GAPDH2 co-immunoprecipitated with Tudor from Drosophila ovarian extracts. What was the specificity of the co-immunoprecipitation? Because PyK and GAPDH2 are very abundant proteins, it is possible that they were just background-type signals of the experiments.’

As stated above, we carefully controlled our immunoprecipitations to ensure the specificity of interactions with Tud and to eliminate the background. Accordingly, we have added the following statement in the section of manuscript describing the identification of Tud complex components. ‘Importantly, all the proteins of Tud complex were recovered repeatedly from independent complex isolations and were never found in control ovarian GFP immunoprecipitations performed under the same conditions as Tud immunoprecipitations as analyzed by mass spectrometry (Supplemental Experimental Procedures).’ Furthermore, consistent with the specificity of the observed interactions, we found that 1) PyK binds specifically to Tud domains of Tud (Fig. 4B); 2) PyK contains a symmetrically dimethylated arginine (new Fig 4D) which is a bona fide molecular determinant of interactions with Tud domains.

2. ‘Figure 1B: pyK, gapdh2 and enolase should also be expressed in other cells in blastoderm stage embryos. However, the signals were merely detected in cells other than pole cells. Controls are missing. Does Enolase co-immunopurify with Tudor? This needs to be examined experimentally.’

Figure 1B illustrates that pyk, gapdh2, and eno mRNAs accumulate at higher levels in blastoderm-stage pole cells than in somatic cells, but these data do not exclude that these mRNAs are present in the soma. In the earlier pre-blastoderm stages of embryogenesis, these mRNAs are more uniformly distributed throughout the embryo. We have made these points in the text. We conclude that sufficient levels of these mRNAs are present in somatic cells to produce the amount of protein that is required for cellular metabolism, or that these enzymes are synthesized in early development and are quite stable.

We have done multiple co-immunoprecipitation experiments and have not detected Enolase in Tud complexes.

3. ‘Figure 2A,B: The authors should perform similar experiments using antibodies for GAPDH2 and Enolase. If the antibodies are not available, GFP-tagged proteins can be alternatively used.’
We have tested a commercially available anti-Enolase antibody, however, it failed to recognize Drosophila endogenous Enolase. Constructing GFP-tagged enzymes with wild-type expression and localization patterns and testing their functionality is a lengthy process and as we are in a competitive situation with regard to this work we are unwilling to delay publication for so long.

4. ‘Figure 2C,D: ImmunoEM for Tudor is required as a control. The authors noted “polar granules contain 5.5 times more PyK than would be expected from the same cytoplasmic area...”. The data should be shown.’

We have included a new Figure S2 as a control to show that the same granules shown in Fig. 2C,D contain the RNA helicases Vasa and eIF4A, which are known components of polar granules. Also, in the manuscript we have the data concerning the number of gold particles counted to determine the enrichment of glycolytic enzymes in the granules, namely 1079 gold particles for PyK and 603 gold particles for PGK.

5. ‘Figure 2D: Is PGK found in the Tudor complex in Figure 1A? Is PGK enriched in polar cells? These questions should be addressed experimentally.’

We have not detected PGK in Tud complex. The list of Tud complex components shown in Figure 1A is a complete list of proteins repeatedly detected in independent Tud complex isolations. However, we found that similarly to PyK, PGK localizes to polar granules (Figure 2D).

6. ‘Page 6: "ald mutant ovaries showed significant overexpression of many transposons". What types of transposons were overexpressed? Are those transposons overexpressed similar to those upregulated in piwi mutant ovaries? This should be examined.’

We have included a new table (Table S1) which shows transposons upregulated in ald mutants. While some transposons are overexpressed in ald mutant ovaries and in piwi mutants [2], we see no significant correlation between their overexpression values in these mutants.

7. ‘Figure 3H: The authors should examine if the expression levels of miRNAs and endogenous siRNAs were affected in ald, pyk and eno mutants.’

This was addressed in our response to reviewer 1. We examined the possible role of glycolysis in the miRNA and siRNA pathways. Contrary to piRNA biogenesis, the miRNA pathway was not affected in glycolytic mutants (new Figures S4A, S4C and S4E). Furthermore, we observed that siRNA levels actually increase in all glycolytic mutants (new Figures S4B, S4D and S4F). Therefore, effects on miRNAs or siRNAs cannot explain the defects in silencing of transposable elements observed in our study. Interestingly, an increase in siRNA levels has been described previously in piwi mutants, suggesting that primary defect in piRNA biogenesis may indirectly
trigger an siRNA response [1]. Therefore, our extended analysis has considerably strengthened our conclusion on the specific effect of glycolysis on piRNA pathway.

8a. 'Page 6: "glycolytic mutations did not cause defects in the generation of secondary piRNAs by Ping-Pong cycle......". "We concluded that glycolytic mutants specifically affect primary piRNA biogenesis". I do not understand these sentences. Secondary piRNA production depends on primary piRNA production. So, if glycolytic mutants affect primary piRNA biogenesis, it would also affect secondary piRNA generation by Ping-Pong cycle.'

We believe that glycolytic components may be similar to described known factors which affect primary piRNA biogenesis without affecting Ping-Pong cycle, for example, Vreteno and Zucchini (see, for example, [3]).

8b. 'Primary piRNA production and population are different between ovarian somatic cells and germ cells because they require different factors and piRNA clusters expressed are different. Figure 3H shows that piRNAs arising from flamenco, a soma specific piRNA cluster, were not affected in ald, pyk and eno mutants, suggesting that these enzymes are not necessary for primary piRNA production in ovarian somatic cells. Clarification is required.'

The glycolytic mutants we examined are mutant germline clones, and thus the enzymes are lacking only in germline cells. Ovarian somatic cells have wild-type copies of glycolytic genes.

9. 'Page 7: "PyK is the major component of the crosslinked Tud complex". The authors should confirm this by performing IP/western using antibodies against Tudor and PyK or other means.'

Figure 1A shows that mass spec identification of PyK in Tud complexes has consistently revealed more PyK peptides sequenced and more successful PyK identifications in independently isolated Tud complexes compared to the rest of the identified components. Therefore, these data suggest that PyK is the major component of the Tud complex. In response to the referee’s comment we changed our original text to consider the possibility that other proteins may also be abundant in Tud complex, perhaps at other developmental stages. Now it reads: 'The biochemical screen and mass spectrometry data reported here (Figure 1A) indicated that PyK is an abundant component of the crosslinked Tud complex, suggesting that Tud may bind directly to PyK within germ granules.'

10a. 'Figure 4B,C: No controls. The authors should show, for example, the N terminal part of Tudor (the part that is missing from Tud7-11) does not associate with PyK-GFP.'

Figure 4B,C shows a negative control (GFP) that does not bind to Tudor. In addition, this Figure presents another negative control, namely, Aub-peptide
preincubated Tudor protein that does not bind to PyK. A further control presented in the Figure, shows that if Tudor is preincubated with non-Aub control peptide, there is a binding between PyK and Tudor.

10b. ‘I wonder if the two Tudor mutants also associate with Aub. This also needs to be examined experimentally.’

These mini-Tudor constructs in fact associate with Aub and these results have been previously published (see, for example, [4, 5]).

11. ‘Does PyK contain sDMAs? This needs to be examined by western using antibodies recognizing sDMAs.’

We now include a new Figure 4D which shows that PyK reacts with an anti-sDMA antibody in a western-blot experiment, indicating that PyK contains sDMA.

12. ‘The data shown in Figure 4H does not support the authors’ claim that Tudor acts as a flexible scaffold. More solid data are required.’

The data presented in the original Figure 4H (Figure 4I in the resubmitted manuscript) did not intend to support our claim that Tudor is a flexible scaffold as reflected in our original text. The single-particle EM data that support this conclusion are presented in Figures 4E-G (Figures 4F-H in the resubmitted manuscript) and supplementary Figure S4 (Figure S6 in the resubmitted manuscript). These data show variably shaped Tudor molecules, confirmed to be functionally active in the binding assay with Tudor’s target Aubergine, consistent with Tudor’s structural flexibility. Since Tudor binds to multiple partners in germ granules ([4] and this submission) and it is required for polar granule assembly, our results support its scaffold role in the granules.

Final comment (a): ‘Glycolytic enzymes such PyK and PGK may accumulate at the high dense structures in germ cells (Figure 2C,D). However, no solid evidence to conclude that the structures are indeed “large ribonucleoprotein granules” was provided. Even though those are the large ribonucleoprotein granules in which piRNA biogenesis takes place, the authors failed to provide evidence to support the idea that ATPs are indeed produced through glycolysis in the granules.’

Ribonucleoprotein germ granules such as those shown in Figures 2C,D have well-known characteristic morphology under EM which has been described in multiple studies during more than 50 years of research (see, for example, [6, 7]). Also, to address this comment, we have included a new supplementary Figure S2 which labels the granules shown in Figures 2C,D, with known markers for these granules, the ATP-dependent RNA helicases, Vasa and eIF4A. Also, we included in this Figure an immunoEM image that shows that the same granule is co-labeled with both Vasa and PyK. In the manuscript we propose that the presence of all ATP-producing glycolytic enzymes, PyK and PGK, and ATP-dependent helicases in the
same structure as evidenced by our EM data (Figure 2C,D and Figure S2) and identification of both PyK and eIF4A in the same Tud complex (Figure 1A), is consistent with our hypothesis that glycolytic ATP is used by ATP-dependent helicases in the granules.

Final comment (b): ‘Transposons were upregulated in the glycolytic enzyme mutants. However, the authors failed to determine if those transposons are targets of piRNAs or endogenous siRNAs. This is very important considering the fact that endogenous siRNAs in flies are known to silence transposons. piRNAs arising from piRNA clusters decreased in the glycolytic enzyme mutants. However, it is not sufficient to suggest a direct link between piRNA pathway and glycolysis because it could be just an indirect effect.’

As described above in response to comment 7, we believe we have addressed this point with the addition of the new Figure S4 that rules out decreases in miRNAs and siRNAs as contributing to the observed effects on transposon activity we observe in glycolytic pathway mutants.

We are grateful for your re-consideration of our work. Since the original submission of this manuscript we have become aware that an abstract from another group entitled ‘Role of glycolysis in primordial-germ-cell development in Drosophila embryos’ was submitted to Germ cells meeting at Cold Spring Harbor, NY which will take place on Oct. 7-11th, this year. While we have not seen an actual publication of these results, this title indicates that another group have data that are similar to some of our results submitted here. We therefore are hopeful of a timely response.

References:


On behalf of all authors, I would like to thank you for your attention to our resubmission.
Thank you for the submission of your revised study as a new manuscript. We have now received the comments from both referees who acknowledge that you have successfully addressed most of the previous concerns.

However, referee 1 still has a few comments that all need to be addressed before we can offer publication of the manuscript by EMBO reports.

Please submit the revised manuscripts as soon as possible. It should not exceed 35,000 characters (including spaces and references) and 5 main plus 5 supplementary figures. Please let me know in case you would like to include more than 5 main figures, as we recently decided that we will also publish longer articles from now on.

Regarding data quantification, can you 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 is currently incomplete and must be provided in all relevant figure legends, including the ones for expanded view figures. Please also add scale bars to all microscopy images.

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 effort of the authors' to improve the manuscript should be highly appreciated. However, I still have several concerns, which should be addressed before this manuscript is recommend for publication in EMBO Reports.

1) "different glycolytic genes cause defects in germ cell development and transposon silencing mechanisms, suggesting that the entire glycolytic pathway, rather than individual components, plays a special role in germ cell specification and contributes to the protection of germline DNA against transposons". No solid data supporting the ideas that the glycolytic activity indeed takes place in the granules and that the glycolytic activity is directly involved in the primary piRNA pathway particularly in the germline cells was provided. Thus, rewriting of this part is required.

2) Secondary piRNA production depends on primary piRNA production. So, if glycolytic mutants affect primary piRNA biogenesis, it would also affect secondary piRNA generation by Ping-Pong cycle. However, the authors still claim that glycolytic mutations did not cause defects in the generation of secondary piRNAs by Ping-Pong cycle. Rewriting of this part is required.

3) "ald mutant ovaries showed significant overexpression of many transposons". Are these transposons targeted specifically by piRNAs? The question needs to be addressed before consideration for the publication.

4) The authors showed experimentally that PyK contains sDMAs. Does PyK have RGs in its peptide sequence that can be sDMA-modified? The question also needs to be addressed before consideration for the publication.

Referee #2:

The authors addressed most of my comments in this revised version. The strength of the paper is its novelty; eventually more work needs to be done to solidify the authors findings but this first report is quite exciting and suitable for publication.
We thank you for your attention to our manuscript. We have revised our manuscript according to your suggestions and referees’ comments.

Our specific responses follow.

Referee #1:
1. "different glycolytic genes cause defects in germ cell development and transposon silencing mechanisms, suggesting that the entire glycolytic pathway, rather than individual components, plays a special role in germ cell specification and contributes to the protection of germline DNA against transposons". No solid data supporting the ideas that the glycolytic activity indeed takes place in the granules and that the glycolytic activity is directly involved in the primary piRNA pathway particularly in the germline cells was provided. Thus, rewriting of this part is required.

We have rewritten the above-mentioned part according to the referee’s comment as follows. ‘Our data indicate that mutations in different glycolytic genes cause defects in germ cell development and transposon silencing mechanisms. Therefore, the entire glycolytic pathway, rather than individual components, might play a special role in germ cell specification and contribute to the protection of germline DNA against transposons’.

2. ‘Secondary piRNA production depends on primary piRNA production. So, if glycolytic mutants affect primary piRNA biogenesis, it would also affect secondary piRNA generation by Ping-Pong cycle. However, the authors still claim that glycolytic mutations did not cause defects in the generation of secondary piRNAs by Ping-Pong cycle. Rewriting of this part is required.’

To address this comment, we have included the following statement and additional reference in the manuscript. ‘We propose that glycolytic components may be similar to described known factors which affect primary piRNA biogenesis without affecting Ping-Pong cycle, for example, Vreteno and Zucchini [ref. [28]: 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].’

3. "ald mutant ovaries showed significant overexpression of many transposons". Are these transposons targeted specifically by piRNAs? The question needs to be addressed before consideration for the publication.

We suggest that these transposons are targeted by piRNAs since the same transposons overexpressed in ald mutants are also upregulated in other bona fide piRNA pathway mutants, notably rhino mutants [Klattenhoff C et al (2009) The Drosophila HP1 homolog Rhino is required for transposon silencing and piRNA production by dual-strand clusters. Cell 138: 1137-1149].

4. ‘The authors showed experimentally that PyK contains sDMAs. Does PyK have RGs in its peptide sequence that can be sDMA-modified? The question also needs to be addressed before consideration for the publication.’

Indeed, we detected such an RG motif in PyK sequence consistent with our data that PyK has sDMAs.

In addition to the comments addressed above, we have modified the title and abstract according to your suggestions. Also, we have included a detailed description of statistical analysis of germ cell formation phenotypes, added scale bars to all microscopy images and are submitting a completed author checklist. Also, a short summary (‘Synopsis’) along with bullet points has been included in the manuscript file after the abstract and we are submitting a synopsis image.

Furthermore, we are submitting a cover image motif for your consideration that highlights a fundamental metabolic aspect (special role of glycolysis) in cells capable of producing multiple generations: germ cells, stem cells and tumor cells.

On behalf of all authors, I would like to thank you for your attention to our revised manuscript and hope that it can now be accepted for publication in EMBO Reports.

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Thank you for submitting your revised manuscript to EMBO reports. I still have a few questions. I cannot find the answers you provide to the referee's concerns points 3 and 4 in the main manuscript text. Can you please add them to the main text?

Regarding figure 3, you mention p-values and a statistical test in the figure legend, but you do not show the data. Please remove the sentence from the legend and instead include it in the main manuscript text, or add the data to the figure. Can you please also explain on how many experiments (n=?) the data shown in panels 3D-H is based? If the data are based on a single experiment, this needs to be mentioned.

For supplementary figure 3C, please also specify "n" and the error bars.

Given that you have 4 main and 6 supplementary figures, we can move one more figure to the main manuscript file. Please let me know whether you agree. If you agree, you can move one figure and modify the relevant files and send them to us by reply to this email.

Thank you for your prompt response to our revised manuscript. According to your comments, we have modified manuscript as follows.

1. Our answers to the referee's concerns #3 and #4 have been added in the main text (page 7 and 10 respectively) with references included.

2. Fig 3 legend: statistical sentences were removed from the legend and put in the main text (page 7).

Also, in the Fig3D-H legend, the following sentence was added. 'In panels D-H, data are based on the sequencing results from RNA library (n=1), either transcriptome library (D-G) or small RNA library (H) for a given mutant or wild-type control.'

3. Supplementary Fig 3C (became Suppl. Fig 2C since Suppl. Fig 1 was transferred to the main text as Fig 5) : the following statement has been added. 'For a given mutant or wild-type control box-and-whiskers plot, the line inside the box is the median of Ping-Pong scores for 112 transposons. The bars (whiskers) represent the highest and lowest Ping-Pong score. The data are based on sequencing results from RNA library (n=1) for a given mutant or wild-type control.'

4. As indicated above, we have moved Supplementary Fig.1 into the main text as Fig. 5 and revised the text to reflect that change.

I have attached the modified main text, Fig. 5, and modified supplementary information file.

Please let me know if you would like additional information.
Thank you for sending the revised files so promptly. I have one more question regarding the RNA libraries. As far as I understand, you have 2 libraries, a transcriptome library and a small RNA library, is this correct? If yes, I suggest to modify the legend for Fig.3D-H to:

'In panels D-H, data are based on the sequencing results from two RNA libraries:
the transcriptome library (D-G, n=1) and the small RNA library (H, n=1) for a given mutant or wild-type control.'

Have any of the data been confirmed by qPCR, in addition to the blood transposon? Sorry, I do not remember now, but I think it would be important to confirm at least some of the data if n=1. If the 2 libraries have been generated independently, this is some kind of validation too.

Can you please specify on which of the two libraries the data in Supplementary Fig 2C is based?

Thank you for clarifying.

Regarding your question on the RNA libraries, you are correct, we have 2 libraries.
I have modified the Fig. 3D-H legend according to your suggestion (please see attached manuscript file that includes that change).

In addition to blood transposon, we detected consistent overexpression of other transposons by qPCR specifically, Burdock and copia. Therefore, independent qPCR experiments showed increases in several transposons' levels confirming that RNA-seq data reported in our manuscript are valid. Accordingly, the following statement has been included in 3D legend.
'Consistent with the RNA-seq data, independent qPCR experiments showed overexpression of blood, Burdock and copia transposons.'

Supplementary Fig. 2C legend has been modified to specify that the data are based on the small RNA libraries (please see attached modified supplementary info file).

Please let me know if you would like further information.

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.