Eukaryotic translation initiation factor eIF4E-5 is required for spermiogenesis in *Drosophila melanogaster*

Lisa Shao, Jaclyn M. Fingerhut, Brook L. Falk, Hong Han, Giovanna Maldonado, Yuemeng Qiao, Vincent Lee, Elizabeth Hall, Liang Chen, Gordon Polevoy, Greco Hernández, Paul Lasko and Julie A. Brill
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MS TITLE: Eukaryotic translation initiation factor eIF4E-5 is required for spermiogenesis in Drosophila melanogaster

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I am terribly sorry and apologize for the extremely long delay before being able to come back to you. This is due to the difficulty identifying three referees available to review your manuscript. I have now received all the referees reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPressand click on the 'Manuscripts with Decisions' queue in the Author Area.

The overall evaluation is positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript, though in my opinion comments 4 and 5 of Rev 3 need not be addressed for acceptance of the manuscript, and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so. If it would be helpful, you are welcome to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating your plans for addressing the referee's comments, and we will look over this and provide further guidance.

Reviewer 1

Advance summary and potential significance to field

This manuscript from Shao and colleagues reports the characterization of a testis-enriched Eukaryotic translation initiation factor eIF4E-5. eIF4E-5 is one of eight Drosophila paralogs of the cap-binding protein eIF4E. eIF4E together with the helicase eIF4A and the scaffolding protein eIF4G constitute the eukaryotic translation initiation factor eIF4F. Two of the eIF4E paralogs are essential

for viability, while a third, eIF4E-3 is needed for meiotic chromosome segregation and cytokinesis during spermatogenesis. Like eIF4E-3, eIF4E-4 eIF4E-5 and eIF4E-7 are highly expressed in testes and are expected to have specialized functions during spermatogenesis. Using CRISPR mutagenesis, authors generated three mutants that disrupt (in different ways) a conserved motif required for eIF4E binding to the scaffolding protein eIF4Gs. and other eIF4E-binding proteins. The deletions are predicted to produce a 4-amino acid in-frame deletion, a 1-amino acid in-frame deletion, and a frame-shift resulting in a 77-amino acid truncated protein. The two small deletions substantially reduce the level of eIF4E-5 protein, while it is not detected in the frame shift mutant.
All three mutants are viable but sterile. While the morphology of the testes appears normal, and there do not appear to be defects in the steps leading up to formation of the 64 cell cysts, there are defects in spermatid differentiation and the seminal vesicles lack mature sperm. The primary defect appears to be in the process of individualization. The mutant testes have abnormal shaped cystic bulges and the actin cones in the individualization complexes are not tightly aligned, but instead are scattered and disorganized. In wild type, activated caspase 3 accumulates in the cystic bulges. In the mutants, the levels of caspase-3 are elevated and the protein is distributed throughout much of elongating spermatid cyst. The authors show that the eIF4E-5 protein preferentially accumulates in close association with adducin near the distal end of the elongated spermatid cysts in wild type testes. A number of studies have shown that many mRNAs and also proteins show a similar enrichment near the distal tip of the elongated spermatid cyst. One of these is the Soti protein which encodes a testes specific E3 ubiquitin ligase inhibitor. While Soti and eIF4E-5 both localized to the distal end of the spermatid cysts, the relative levels of Soti and eiF4E-5 vary from one spermatid cyst to another. Though eif4e-5 mutants have no apparent effect on the localization of soti mRNA in elongating spermatid cysts, Soti protein accumulation is nevertheless substantially reduced. It is not clear how this might happen as spermatid cysts that have soti mRNA enriched at the distal tip, typically do not have eIF4E-5 protein at the distal tip. The authors also examined the expression and localization of the axonemal dynein kl-3. Unlike soti, eif4e-5 mutants have no effect on the accumulation of Kl-3. Nor was there any effect on kl-3 mRNA localization. Interestingly, kl-3 mRNA localization to the distal tip of the spermatid cysts appears separated in time from that of soti. The former being localized during the elongation phase, while later being localized towards the end of elongation. In addition to disrupting individualization, eif4e-5 mutants alleles have defects in spermatid cyst polarization. These polarization defects appear to be related to the functioning of the CPEB protein orb2, and the PAR protein apkc. The authors show that the frequency of polarization defects in eif4e 5 heterozygotes can be enhanced when the males also heterozygous for mutations in orb2 or apkc.
Overall this is a well-done manuscript on a problem of interest for researchers studying germline development, particularly spermatogenesis. More generally, it addresses a broader question, namely functional specialization of paralogs in this case within a single tissue, and nicely complements earlier work on eIF4E-3.

Comments for the author

Text:
a) The individualization defects, the lack of a properly formed cystic bulge the excess caspase 3 and the scattered actin cones point to role for eif4e-5 after spermatid tail elongation is complete. Supporting this view, expression of kl-3, which is needed earlier during elongation, is unaltered in eif4e-5 mutants. From what I could see, elongation also appears to be normal. (With a caveat—see b below) these observations suggest that the deployment (localization) of eif4e-5 is coordinated with the temporal progression of spermatid differentiation. This would also be consistent with the related but also discordant patterns of eIF4e-5 and Soti accumulation at the distal end of elongated spermatid tails. (In some cysts, the levels of both are high. In others, one is high and the other is low.) It seemed to me that these observations are both intriguing and important in thinking about the role eif4e-5 and the other testes paralogs in spermatogenesis. However, I think that the authors could improve the presentation of this idea. In the results section for example, they could reverse the order of their experiments—showing first that elongation appears to be relatively normal and then going on to Soti and individualization.

b) The fly in the ointment for focusing on a role relative late in spermatid differentiation (a) is that there is also an earlier defect in the polarization of the spermatid cyst. However, this would be another point for discussion—since an intermediate step, elongation, seems to be normal== and thus the activity of this factor is a two different steps/times.

Results:
a) The authors show that eIF4e-5 and Soti protein accumulate in a related but somewhat discordant fashion at the distal tip of elongated spermatid cysts. I was not able to find a paper describing the relationship between soti mRNA localization and Soti protein localization. In thinking about their results and how they fit into the sequencing of events during spermatid differentiation, I believe it would be helpful to have images of soti mRNA and Soti protein (and ideally also eIF4e protein).

b) The authors show that Soti protein doesn't accumulate (to high levels) at the distal tip of eif4e-5 mutants. However, it wasn't clear to me from the images shown in the manuscript whether this was a failure in localization or an actual reduction in protein levels. Here a western might help.

c) The individualization defects presumably occur after the elongation of the spermatid cyst is mostly complete. I may have missed it, but I didn't see images of eIF4e-5 localization during individualization. Does eIF4e-5 remain localized at the tip as the actin cones are moving towards it?

d) It seems to me that the timing of appearance/localization of eIF4e-5 protein is important in trying to fit this translational regulator into the different steps in spermatogenesis. The section describing eIF4e-5 expression/localization doesn't provide much in the way of detail. From looking at the images, it would appear that localization to the distal tip might only occur as elongation is finishing. A more complete description of when this happens relative to elongation itself and to Soti mRNA and protein localization would help the readers.

Reviewer 2

Advance summary and potential significance to field

This work represents several significant advances. It reveals another testis-specific paralog of a gene required in spermatogenesis, thus adding to the growing list of genes that have spermatogenesis-specific "versions" needed for the exceptionally complicated development of the Drosophila male germline. By identifying this testis-specific translation initiation factor, it also uncovers another aspect of post-meiotic translational regulation during Drosophila spermatogenesis, which is a process that we do not fully understand at the molecular level. In particular, it focusses on translational regulation at the distal ends of elongated spermatid cysts, which not very well understood at all.
Finally, it ties this new molecular player to known molecular players in post-meiotic spermatogenesis, thus placing the findings into context. This paper is a significant achievement.

Comments for the author

Lines 150-151: The level of this protein was substantially reduced in testis extracts from eIF4E-5B8a or eIF4E-5B8b, and not detectable in eIF4E-5D19a homozygous mutants (Figs 1C and S2). In both cases a faint band is visible, so the protein is not "undetectable". This language should also be adjusted in the figure legends (lines 888 and 983).

Line 154- how "viable" are the homozygous mutants? Was a viability index calculated? Do all alleles show the same viability?

Figure 1D: Is a row missing? The fertility of 3xFLAG-eIF4E-5; eIF4E-5[B18a] is not indicated.

Lines 901-903 and Figure 2C- "Note that anti-eIF4E-5 antibodies non-specifically stain individualization complexes, as shown for the mispolarized cyst in eIF4E-5B8b (C-C'', cyan arrowheads)." Is this "non-specific staining consistent?
Because it looks very bright. How do we know it is non-specific?

Figure 3- There appears to be variation in the level of F-actin cone disorganization between the mutant alleles, with eIF4E-5[B8b] exhibiting the most severe phenotype. Is this observed consistently?

Figure S4 and line 211: “but not eIF4G”. Should “BD” and “GIGYF” be mentioned as well? Also, I do not see the empty vector negative controls in the figure.

The data in Figure 5 troubles me a little bit. A colocalization b4E-5etween Soti and eIF4E-5 was proposed based on overexpression of FLAG-tagged eIF4E-5.
Granted, back in figure 2, a convincing colocalization was shown between overexpressed eIF4E-5 and endogenous eIF4E-5. That said, has a colocalization been shown between endogenous eIF4E-5 and Soti? If not, why?

Reviewer 3

Advance summary and potential significance to field

Shao et al. reported a role of eIF4E-5 in Drosophila spermiogenesis. First using CRISPR/Cas9 genome editing, the authors generated a series of eIF4E-5 loss-of-function alleles for genetics experiments, where they show mutant phenotypes including spermatid cyst polarization, sperm individualization and male fertility. Second, by generating polyclonal antibodies and a Flag tagged genomic rescuing transgene, they showed eIF4E-5 expression pattern and localization. Third, they found these spermiogenesis roles of eIF4E-5 are through promoting local accumulation of Soti. Finally, the elf4E-5 mutants display mild spermatid cyst polarization defects, and have genetic interactions with Orb2 and aPKC. Overall, the experiments are presented in a logic manner from phenotypic analyses to more mechanistic insight. And the results should be of interest to developmental biologists, in particular germ cell biologists.

Comments for the author

However, many results were shown as single images, it would be a lot more convincing with quantification and proper statistical analyses. Here are the major comments:

1. The title may reflect that this is a non-canonical role of eIF, or based on the presented data, is it very sure that the phenotypes are not due to its translation initiation factor role?

2. Figure 1 and related S2: It is curious that a 4 or 1 amino acid deletion results in such a dramatic reduction in protein abundance. Is it already known that this motif (and binding to other proteins) is required for protein stability? Please cite if so.
Please indicate the predicted molecular weights of the mutated proteins on the western in FigS2A, specifically for the truncation mutants?
Because the polyclonal antibody was generated using the entire protein as an epitope it possible the antibody could recognize a truncated eIF4E5-d19a protein. As there is not much signal on the western below 24kDa, it is possible a smaller truncation transferred through the membrane. With modifying the transfer parameters, it would be interesting to test if a truncation is produced + stable.

3. Figure 2: immunostaining shows that the anti- eIF4E-5 antibodies have quite some non-specific signals, could it be done by pre-incubation with null mutant then to wild-type samples to reduce the non-specific signals? The same with anti-Flag antibodies, could it be pre-incubated first with non-transgenic tissues and then apply to transgenic ones?

4. Figure 3 and S3: All phenotypic analyses were performed using homozygotes from the same CRISPR mutagenesis, better to use the trans-heterozygotes over deficiency, such as those combinations used in Figure 1.

5. Figure S4: for the Y2H candidate study: It is interesting that this experiment suggests a potential inhibitory mechanism for eIF4E-5 regulation.
However, this experiment would be more powerful if the CRISPR/Cas9 mutations were also tested to show that these inhibitory interactions are lost in their mutants.

6. Figure 4: It is hard to compare immunostaining signals among different genotypes, better to generate clones so that the signals can be directly compared within the same tissue. Or, at least, the experiments can be done with different genotyped tissues to be stained and imaged together,

carrying proper markers to distinguish them, and with information of Ns and statistics. The same comment for Figure 5 and 6 regarding the information of quantification, Ns and statistical analyses.

7. The major phenotype that likely contributes to sterility is the individualization defect. Therefore, it is curious the authors conclude with studies on spermatid cyst polarization. Particularly, when they state that this defect is likely not the cause of the fertility issues (Line 279). Despite this they continue with experiments on known cell polarity regulators Orb2 and aPKC using the mutant eIF4E-5 sensitized background. Again, the relationship between these two proteins (Orb2 ensures localized translation of aPKC) is not discussed in detail until the discussion.

Other suggestions:

Much of the background required to understand the logical flow of experiments was provided in the discussion section. If these details are described earlier in the manuscript, then many questions on the experimental premise would be removed. For example, the relationship between Soti and Caspase activity through the E3 Ub ligase complex CRL3, is explained in the discussion (Lines 325-329). Further, because the results discussing dysregulated caspase activity and Soti are separated by a Y2H experiment on eIF4E-5 binding proteins, this logical connection between the two activities is not immediately apparent. Here are some comments that could clarify background throughout the manuscript:

Some of the following line-questions are answered in the discussion section.
However, restructuring the manuscript could clarify many of these points early on.

Line 118: “Cup” and “comet” genes are named based on their mRNA distribution.
However, to those unfamiliar with spermiogenesis, it is not described in the manuscript what the “cup” or “comet” are.

Line 121: is it known how Soti regulates caspase activity?

Line 191: A sentence describing the role of activated caspase-3 during individualization would be helpful.

Line 194: What do the authors think is the cause of the activated caspase along the entire spermatid tail? Do they hypothesize that Soti is the regulator here?

-If so, clearly stating this logical progression will be helpful to the reader.

Line 198: Can the authors speculate on why they think the actin cones are disorganized in the sperm?

Lines 208-209: Additional context for the tested translational regulators and what their known role (activator, inhibitor) would help. i.e.: what are Cup and GIGYF, as this is the first time these proteins are mentioned.

Line 224: Here is a great place to clarify if Soti was tested to explain the activated caspase activity described in Figure 3.

Line 277: Do the authors have a reason for not quantifying cyst mispolarization in the D19a mutants?

First revision

Author response to reviewers' comments

Point-by-point response to the reviews (text in blue):

Reviewer 1 Advance Summary and Potential Significance to Field:
This manuscript from Shao and colleagues reports the characterization of a testis-enriched Eukaryotic translation initiation factor eIF4E-5. eIF4E-5 is one of eight Drosophila paralogs of the cap-binding protein eIF4E. eIF4E together with the helicase eIF4A and the scaffolding protein eIF4G constitute the eukaryotic translation initiation factor eIF4F. Two of the eIF4E paralogs are essential for viability, while a third, eIF4E-3 is needed for meiotic chromosome segregation and cytokinesis during spermatogenesis. Like eIF4E-3, eIF4E-4, eIF4E-5 and eIF4E-7 are highly expressed in testes and are expected to have specialized functions during spermatogenesis. Using CRISPR mutagenesis, authors generated three mutants that disrupt (in different ways) a conserved motif required for eIF4E binding to the scaffolding protein eIF4Gs. and other eIF4E- binding proteins. The deletions are predicted to produce a 4-amino acid in- frame deletion, a 1-amino acid in-frame deletion, and a frame-shift resulting in a 77- amino acid truncated protein. The two small deletions substantially reduce the level of eIF4E-5 protein, while it is not detected in the frame shift mutant. All three mutants are viable but sterile. While the morphology of the testes appears normal, and there do not appear to be defects in the steps leading up to formation of the 64 cell cysts, there are defects in spermatid differentiation and the seminal vesicles lack mature sperm. The primary defect appears to be in the process of individualization. The mutant testes have abnormal shaped cystic bulges and the actin cones in the individualization complexes are not tightly aligned, but instead are scattered and disorganized. In wild type, activated caspase 3 accumulates in the cystic bulges. In the mutants, the levels of caspase-3 are elevated and the protein is distributed throughout much of elongating spermatid cyst. The authors show that the eIF4E-5 protein preferentially accumulates in close association with adducin near the distal end of the elongated spermatid cysts in wild type testes. A number of studies have shown that many mRNAs and also proteins show a similar enrichment near the distal tip of the elongated spermatid cyst. One of these is the Soti protein, which encodes a testes specific E3 ubiquitin ligase inhibitor. While Soti and eIF4E-5 both localized to the distal end of the spermatid cysts, the relative levels of Soti and eiF4E-5 vary from one spermatid cyst to another. Though eif4e-5 mutants have no apparent effect on the localization of soti mRNA in elongating spermatid cysts, Soti protein accumulation is nevertheless substantially reduced. It is not clear how this might happen as spermatid cysts that have soti mRNA enriched at the distal tip, typically do not have eIF4E-5 protein at the distal tip. The authors also examined the expression and localization of the axonemal dynein kl-3.
Unlike soti, eif4e-5 mutants have no effect on the accumulation of Kl-3. Nor was there any effect on kl-3 mRNA localization. Interestingly, kl-3 mRNA localization to the distal tip of the spermatid cysts appears separated in time from that of soti. The former being localized during the elongation phase, while later being localized towards the end of elongation. In addition to disrupting individualization, eif4e-5 mutants alleles have defects in spermatid cyst polarization. These polarization defects appear to be related to the functioning of the CPEB protein orb2, and the PAR protein apkc. The authors show that the frequency of polarization defects in eif4e 5 heterozygotes can be enhanced when the males also heterozygous for mutations in orb2 or apkc.

Overall this is a well-done manuscript on a problem of interest for researchers studying germline development, particularly spermatogenesis. More generally, it addresses a broader question, namely functional specialization of paralogs in this case within a single tissue, and nicely complements earlier work on eIF4E-3.

We agree and thank the reviewer for their favorable assessment of our manuscript.

Reviewer 1 Comments for the
Author: Text:
a) The individualization defects, the lack of a properly formed cystic bulge, the excess caspase 3

and the scattered actin cones point to role for eif4e-5 after spermatid tail elongation is complete. Supporting this view, expression of kl-3, which is needed earlier during elongation, is unaltered in eif4e-5 mutants. From what I could see, elongation also appears to be normal. (With a caveat—see b below) these observations suggest that the deployment (localization) of eif4e-5 is coordinated with the temporal progression of spermatid differentiation. This would also be consistent with the related but also discordant patterns of eIF4e-5 and Soti accumulation at the distal end of elongated spermatid tails. (In some cysts, the levels of both are high. In others, one is high and the other is low.) It seemed to me that these observations are both intriguing and important in thinking about the role eif4e-5 and the other testes paralogs in spermatogenesis. However, I think that the authors could improve the presentation of this idea. In the results section, for example, they could reverse the order of their experiments—showing first that elongation appears to be relatively normal and then going on to Soti and individualization.
In response to this comment, as well as comment 7 of reviewer 3, we reorganized the manuscript to discuss the eIF4E-5 mutant phenotypes in order of the stages of spermiogenesis: (1) polarization of spermatid cysts relative to the long axis of the testis (we observe defects here; Fig. 3), (2) translation of axonemal dynein (Kl-3) (no obvious defects; Fig 4), (3) elongation of spermatid cysts (no obvious defects; Fig. 5), and (4) individualization of spermatids to form mature sperm (we observe defects here; Fig. 5).

b) The fly in the ointment for focusing on a role relative late in spermatid differentiation (a) is that there is also an earlier defect in the polarization of the spermatid cyst. However, this would be another point for discussion—since an intermediate step, elongation, seems to be normal== and thus the activity of this factor is a two different steps/times.
The reviewer makes an excellent point that the earlier polarization defect is unlikely to cause the later defects, especially since apkc and orb2 heterozygotes, which show a similar polarization defect, are fertile. Moreover, "the effects of eIF4E-5 mutations on cyst polarity are not fully penetrant" (lines 240-241). Thus, the simplest interpretation, as suggested by the reviewer, is that eIF4E-5 acts at two different stages. We have added a discussion point to this effect, "Our data indicate that eIF4E-5 is essential for Drosophila male fertility and is needed during at least two different stages of spermatogenesis for faithful polarization of spermatid cysts and individualization of spermatids to form mature sperm" (lines 309-311).

Results:
a) The authors show that eIF4e-5 and Soti protein accumulate in a related but somewhat discordant fashion at the distal tip of elongated spermatid cysts. I was not able to find a paper describing the relationship between soti mRNA localization and Soti protein localization. In thinking about their results and how they fit into the sequencing of events during spermatid differentiation, I believe it would be helpful to have images of soti mRNA and Soti protein (and ideally also eIF4e protein).

This is an excellent point, which we have addressed in triple-staining experiments examining the distribution of soti mRNA, Soti protein and eIF4E-5 protein in wild-type testes (Fig. 7B-D).

b) The authors show that Soti protein doesn't accumulate (to high levels) at the distal tip of eif4e-5 mutants. However, it wasn't clear to me from the images shown in the manuscript whether this was a failure in localization or an actual reduction in protein levels. Here a western might help.

We too were not convinced about the effect of eIF4E-5 mutants on Soti accumulation based on our immunofluorescence results (new images now shown in Fig. 6A-D''). In immunoblotting experiments, we examined Soti protein levels and were able to show that the overall levels of Soti protein appear reduced in eIF4E-5 mutants (Fig. S6).

c) The individualization defects presumably occur after the elongation of the spermatid cyst is mostly complete. I may have missed it, but I didn't see images of eIF4e-5 localization during individualization. Does eIF4e-5 remain localized at the tip as the actin cones are moving towards it?

This is an excellent point. To address the timing of eIF4E-5 localization at the distal ends of

elongated spermatid cysts, we co-stained for Soti, eIF4E-5 and polyglycylated tubulin, which marks the sperm tails at the onset of individualization (Fig. 7A). We discovered that endogenous eIF4E-5 is present at the distal ends of cysts marked with Soti and is also present at the distal ends of cysts marked with polyglycylated tubulin. In contrast, Soti is absent from the distal ends of polyglycylated tubulin-positive cysts. Thus, we conclude that Soti appears before eIF4E-5 and that eIF4E-5 persists at the distal ends of elongated spermatid cysts after Soti is degraded during individualization.

d) It seems to me that the timing of appearance/localization of eIF4e-5 protein is important in trying to fit this translational regulator into the different steps in spermatogenesis. The section describing eIF4e-5 expression/localization doesn't provide much in the way of detail. From looking at the images, it would appear that localization to the distal tip might only occur as elongation is finishing. A more complete description of when this happens relative to elongation itself and to Soti mRNA and protein localization would help the readers.

The experiments examining the relative distribution of soti mRNA, Soti protein, eIF4E-5 protein and polyglycylated tubulin, described in response to comments a and c above, address this timeline for the stages involved in spermatid elongation and individualization. Based on our results (Fig. 7), we agree with the reviewer that eIF4E-5 localizes to the distal tip as elongation is finishing, and our data further show that eIF4E-5 persists at the distal end of spermatid cysts undergoing individualization.

Reviewer 2 Advance Summary and Potential Significance to Field:
This work represents several significant advances. It reveals another testis-specific paralog of a gene required in spermatogenesis, thus adding to the growing list of genes that have spermatogenesis-specific "versions" needed for the exceptionally complicated development of the Drosophila male germline. By identifying this testis-specific translation initiation factor, it also uncovers another aspect of post-meiotic translational regulation during Drosophila spermatogenesis, which is a process that we do not fully understand at the molecular level. In particular, it focusses on translational regulation at the distal ends of elongated spermatid cysts, which not very well understood at all. Finally, it ties this new molecular player to known molecular players in post-meiotic spermatogenesis, thus placing the findings into context. This paper is a significant achievement.

We agree and thank the reviewer for their enthusiasm regarding our manuscript.

Reviewer 2 Comments for the Author:
Lines 150-151: The level of this protein was substantially reduced in testis extracts from eIF4E-5B8a or eIF4E-5B8b, and not detectable in eIF4E-5D19a homozygous mutants (Figs 1C and S2). In both cases a faint band is visible, so the protein is not "undetectable". This language should also be adjusted in the figure legends (lines 888 and 983).

We agree that residual protein is detectible in extracts from B8a and B8b but not D19a homozygous mutants and thank the reviewer for pointing this out, as we had intended to correct the wording prior to submission. The revised text now reads, "eIF4E-5 was substantially reduced in testis extracts from eIF4E-5^{B8a} or eIF4E-5^{B8b} and undetectable in eIF4E-5^{D19a} homozygous mutants (Figs 1D and S2A-B)." (lines 173-175); also described in the figure legend, "Immunoblot of total testis extracts probed with the indicated antibodies reveals reduced (eIF4E-5^{B8a}, eIF4E-5^{B8b}) or undetectable (eIF4E-5^{D19}) levels of eIF4E-5 protein." (lines 942-944).

Line 154- how "viable" are the homozygous mutants? Was a viability index calculated? Do all alleles show the same viability?

We have now examined viability of the homozygous eIF4E-5 mutants and find no significant differences in adult eclosion rates compared to the expected Mendelian ratios (Fig. S2C; see Materials and Methods, lines 436-442).

Figure 1D: Is a row missing? The fertility of 3xFLAG-eIF4E-5; eIF4E-5[B18a] is not indicated.

For unknown reasons, we initially had trouble crossing the transgene into the B8a mutant background using double-balanced stocks. We have now done this and demonstrated that fertility is restored in B8a mutant flies carrying the 3xFLAG-eIF4E-5 transgene (Fig. 1E).

Lines 901-903 and Figure 2C- "Note that anti-eIF4E-5 antibodies non-specifically stain individualization complexes, as shown for the mispolarized cyst in eIF4E- 5B8b (C-C'', cyan arrowheads)." Is this "non-specific staining consistent? Because it looks very bright. How do we know it is non-specific?
We believe the individualization complex staining is non-specific, as this staining (as well as the nuclear staining) was present in testes from males that were homozygous mutant for any of the eIF4E5 mutant alleles. Moreover, this staining was not observed in testes from males expressing 3xFLAG-eIF4E-5 and stained with anti-FLAG antibodies.

Figure 3- There appears to be variation in the level of F-actin cone disorganization between the mutant alleles, with eIF4E-5[B8b] exhibiting the most severe phenotype. Is this observed consistently?

We did not observe consistent differences in the severity of the F-actin cone disorganization among the different alleles. However, we showed that the defects in actin cone organization become more severe as the cones progress down the length of the spermatid cysts (Fig. 5I-L'''). We now state, "Morphology of nascent actin cones at the basal end of the testis appeared unaffected by loss of eIF4E-5, forming organized individualization complexes similar to the control (Fig. 5Ii-Li). However, in contrast to the synchronous movement of groups of 64 actin cones towards the testis tip in the control (Fig. 5I-Iiii), actin cones in eIF4E-5 mutants appeared to scatter and become disorganized as they progressed (Fig. 5J-Liii)." (lines 264-269).

Figure S4 and line 211: "but not eIF4G". Should "BD" and "GIGYF" be mentioned as well? Also, I do not see the empty vector negative controls in the figure.

We apologize for neglecting to describe aspects of our yeast two-hybrid experiments. BD is the empty GAL4 DNA binding domain vector and AD is the empty activator domain vector. No growth is detected with the empty vectors. We have now moved the yeast two-hybrid results to the beginning of the Results (Fig. 1A) and have defined GYGIF as Grb10-interacting GYF (line 145), and we defined BD and AD in the legend (lines 928-929).

The data in Figure 5 troubles me a little bit. A colocalization between Soti and eIF4E-5 was proposed based on overexpression of FLAG-tagged eIF4E-5. Granted, back in figure 2, a convincing colocalization was shown between overexpressed eIF4E-5 and endogenous eIF4E-5. That said, has a colocalization been shown between endogenous eIF4E-5 and Soti? If not, why?

As described in our response to comment c of Reviewer 1 above, we co-stained for Soti, eIF4E5 and polyglycylated tubulin, which marks the sperm tails at the onset of individualization (Fig. 7A). This experiment revealed that, similar to 3xFLAG-eIF4E-5, endogenous eIF4E-5 is present at the distal ends of cysts marked with Soti.

Reviewer 3 Advance Summary and Potential Significance to Field:
Shao et al. reported a role of eIF4E-5 in Drosophila spermiogenesis. First, using CRISPR/Cas9 genome editing, the authors generated a series of eIF4E-5 loss-of-function alleles for genetics experiments, where they show mutant phenotypes including spermatid cyst polarization, sperm individualization and male fertility. Second, by generating polyclonal antibodies and a Flag tagged genomic rescuing transgene, they showed eIF4E-5 expression pattern and localization. Third, they found these spermiogenesis roles of eIF4E-5 are through promoting local accumulation of Soti. Finally, the elf4E-5 mutants display mild spermatid cyst polarization defects and have genetic interactions with Orb2 and aPKC. Overall, the experiments are presented in a logic manner from phenotypic analyses to more mechanistic insight. And the results should be of interest to developmental biologists, in particular germ cell biologists.

We agree and thank the reviewer for their favorable assessment of our manuscript.

Reviewer 3 Comments for the Author:
However, many results were shown as single images, it would be a lot more convincing with quantification and proper statistical analyses. Here are the major comments:

1. The title may reflect that this is a non-canonical role of eIF, or based on the presented data, is it very sure that the phenotypes are not due to its translation initiation factor role?

Although the idea of eIF4E-5 carrying out a non-canonical role is one we find intriguing, our experiments to date cannot distinguish between a non-canonical role vs. a canonical role for eIF4E-5. Future experiments (beyond the scope of the current manuscript) will be required to discover the mechanism of action of eIF4E-5. Thus, it seems prudent to keep the title as is.

2. Figure 1 and related S2: It is curious that a 4 or 1 amino acid deletion results in such a dramatic reduction in protein abundance. Is it already known that this motif (and binding to other proteins) is required for protein stability? Please cite if so.

The residues affected in these small deletions are predicted (by homology to the canonical eIF4E1) to be directly involved in binding of eIF4E-5 to eIF4G and 4EBPs and thus affect the structure of a crucial part of the protein. To better explain the nature of these mutations, we added, "All three mutations affect the His-Pro-Leu motif that is required for binding to eIF4G and other 4E-BPs (His55, Pro56, Leu57; Grüner et al., 2016; Kinkelin et al., 2012; Peter et al., 2015): eIF4E-5^{B8a} removes Pro56, Leu57, Glu58, His59; eIF4E-5^{B8b} removes His55; and eIF4E-5^{D19a} has a frameshift after His55 that removes all subsequent amino acids, replacing them with 22 amino acids from an alternate reading frame. Thus, all three eIF4E-5 mutations are predicted to interfere with eIF4G/4E-BP binding and eIF4F complex formation." (lines 164-169).

Please indicate the predicted molecular weights of the mutated proteins on the western in FigS2A, specifically for the truncation mutants?

The predicted molecular weights of the proteins produced by the B8a (4 aa deletion) and B8b (1 aa deletion) alleles should be very similar to that of the full-length protein (~26.9 kDa). Thus, these proteins would be expected to run at a similar position on the blot. A truncated peptide with a predicted molecular weight of approximately 8.4 kDa was not detectable in the eIF4E-5^{D19a} lane. We did not indicate the size of this predicted peptide because the molecular weight markers used on the blot were a bit larger in size (smallest marker = 12 kDa), so it was difficult to identify the precise position where the protein should have migrated.

Because the polyclonal antibody was generated using the entire protein as an epitope it possible the antibody could recognize a truncated eIF4E5-d19a protein. As there is not much signal on the western below 24kDa, it is possible a smaller truncation transferred through the membrane. With modifying the transfer parameters, it would be interesting to test if a truncation is produced + stable.

In addition to the blot shown in Fig. S2A, we ran other blots with lower molecular weight markers that transferred well to the filter, and these blots also did not reveal a truncated peptide of approximately 8.4 kDa in eIF4E-5^{D19a}. Thus, it appears likely that either the truncated peptide is unstable, or the anti-eIF4E-5 antibodies cannot detect this peptide on a blot. We note that because this allele behaves genetically as a null allele and lacks any obvious dominant or semi-dominant phenotypes, we feel it is unlikely that the predicted truncated peptide, if produced, contributes to the phenotype of the eIF4E-5^{D19a} allele.

3. Figure 2: immunostaining shows that the anti- eIF4E-5 antibodies have quite some non-specific signals, could it be done by pre-incubation with null mutant then to wild-type samples to reduce the non-specific signals?

Preincubating anti-eIF4E-5 antibodies with mutant extracts to see if we can clean up the immunofluorescence signal is a good idea. However, we do not feel this is necessary, as we have been able to confirm localization of eIF4E-5 at the distal ends of elongated spermatid cysts using anti-FLAG antibody staining of 3xFLAG-eIF4E-5.

The same with anti-Flag antibodies, could it be pre-incubated first with non-transgenic tissues and then apply to transgenic ones?

We previously tried pre-incubation of the anti-FLAG antibodies and found that this did not remove the background signal from the sperm tails.

4. Figure 3 and S3: All phenotypic analyses were performed using homozygotes from the same CRISPR mutagenesis, better to use the trans- heterozygotes over deficiency, such as those combinations used in Figure 1.
The editor felt it was unnecessary for us to address this.

5. Figure S4: for the Y2H candidate study: It is interesting that this experiment suggests a potential inhibitory mechanism for eIF4E-5 regulation. However, this experiment would be more powerful if the CRISPR/Cas9 mutations were also tested to show that these inhibitory interactions are lost in their mutants.

The editor felt it was unnecessary for us to address this.

6. Figure 4: It is hard to compare immunostaining signals among different genotypes, better to generate clones so that the signals can be directly compared within the same tissue. Or, at least, the experiments can be done with different genotyped tissues to be stained and imaged together, carrying proper markers to distinguish them, and with information of Ns and statistics. The same comment for Figure 5 and 6 regarding the information of quantification, Ns and statistical analyses.

We agree that is hard to compare immunostaining signals among different genotypes, especially since we were unsuccessful in quantifying relative differences in Soti abundance in our immunofluorescence experiments due to variability in the Hts signal we were trying to normalize to. Although clonal analysis is often a powerful approach, in our experience, it is challenging to identify GFP-negative germline clones at the stages of interest, as the GFP signal is typically quite dilute by late stages of spermatid elongation, making it difficult to identify the mutant cysts. However, our immunoblot analysis suggests that Soti levels are indeed reduced in all three eIF4E-5 mutants (Fig. S6), supporting the idea that eIF4E-5 directly or indirectly affects accumulation or stability of Soti protein.

7. The major phenotype that likely contributes to sterility is the individualization defect. Therefore, it is curious the authors conclude with studies on spermatid cyst polarization. Particularly, when they state that this defect is likely not the cause of the fertility issues (Line 279). Despite this, they continue with experiments on known cell polarity regulators Orb2 and aPKC using the mutant eIF4E-5 sensitized background. Again, the relationship between these two proteins (Orb2 ensures localized translation of aPKC) is not discussed in detail until the discussion.

In response to this comment, as well as Text comments a and b of reviewer 1, we reorganized the manuscript to discuss the eIF4E-5 mutant phenotypes in order of the stages of spermiogenesis: (1) polarization of spermatid cysts relative to the long axis of the testis (we observe defects here; Fig. 3), (2) translation of axonemal dynein (Kl-3) (no obvious defects; Fig 4), (3) elongation of spermatid cysts (no obvious defects (Fig. 5), and (4) individualization (we observe defects here; Fig. 5).

Other suggestions:
Much of the background required to understand the logical flow of experiments was provided in the discussion section. If these details are described earlier in the manuscript, then many questions on the experimental premise would be removed. For example, the relationship between Soti and Caspase activity, through the E3 Ub ligase complex CRL3, is explained in the discussion (Lines 325-

329).
We apologize for neglecting to provide sufficient background regarding the relationship, roles and regulation of Soti, the E3 Ub ligase CRL3 and activated caspase in the introduction. We have now added to the Introduction, "By binding the testis-specific Cullin-3 ring E3 ubiquitin ligase, Soti inhibits ubiquitination and degradation of the inhibitor of apoptosis-like protein dBruce, thus preventing caspase activation (Kaplan et al., 2010)." (lines 119-121).

Further, because the results discussing dysregulated caspase activity and Soti are separated by a Y2H experiment on eIF4E-5 binding proteins, this logical connection between the two activities is not immediately apparent.
We have changed the order of presentation in the revised manuscript to address this. In particular, we moved the Y2H results to the beginning of the Results, and we now introduced the individualization defects, including dysregulated caspase activity, immediately before we describe the effect of eIF4E-5 mutants on Soti protein distribution and levels.

Here are some comments that could clarify background throughout the manuscript:

Some of the following line-questions are answered in the discussion section. However, restructuring the manuscript could clarify many of these points early on.

We have restructured the manuscript to clarify these points early on.

Line 118: "Cup" and "comet" genes are named based on their mRNA distribution. However, to those unfamiliar with spermiogenesis, it is not described in the manuscript what the "cup" or "comet" are.

We now describe the "cups and comets" in the introduction, " a group of "cup" and "comet" genes – named based on their mRNA distribution at the growing ends of elongating spermatid cysts – is transcribed post-meiotically (Barreau et al., 2008a,b)." (lines 115-117).

Line 121: is it known how Soti regulates caspase activity?

As described above, we have now added to the Introduction, "By binding the testis-specific Cullin-3 ring E3 ubiquitin ligase, Soti inhibits ubiquitination and degradation of the inhibitor of apoptosis-like protein dBruce, thus preventing caspase activation (Kaplan et al., 2010)." (lines 119-121).

Line 191: A sentence describing the role of activated caspase-3 during individualization would be helpful.

We have now added to the Discussion, "Non-apoptotic caspase activity is needed for progression of actin cones and degradation of unneeded organelles during individualization (Arama et al., 2003, 2007; Huh et al., 2004; Muro et al., 2006)." (lines 355-357)

Line 194: What do the authors think is the cause of the activated caspase along the entire spermatid tail? Do they hypothesize that Soti is the regulator here? -If so, clearly stating this logical progression will be helpful to the reader.

This phenotype is observed in soti and other mutants where caspase activity is not properly regulated. We have now added to the Discussion, "Graded distribution of Soti and dBruce along the length of elongated spermatid cysts, highest at the distal end and lowest at the nuclear end, is required for this localized caspase activation (Arama et al., 2007; Kaplan et al., 2020). In this study, we show that localized caspase activation is disrupted, and actin cones are scattered in individualizing spermatids of eIF4E-5 mutants, resembling soti mutants (Kaplan et al., 2020). We also show that localized accumulation of Soti protein is reduced at the distal end of elongated spermatids in eIF4E-5 mutants, where soti mRNA is typically found in wild-type testes. Collectively, these results suggest that eIF4E-5 post-transcriptionally regulates non-apoptotic caspase activity during spermiogenesis." (lines 361-369).

Line 198: Can the authors speculate on why they think the actin cones are disorganized in the sperm?
This phenotype is observed in soti and other mutants where caspase activity is not properly regulated. We have now added to the Discussion, "In elongated spermatid cysts, caspases are initially activated at the nuclear end, where actin cones form, and repressed along the spermatid tails. As the actin cones move away from the nuclei, the peak of caspase activity remains associated with the cystic bulge. This localized caspase activity allows for controlled degradation of organelles, and disruption of this activity leads to failure of individualization, characterized by scattered actin cones." (lines 357-361).

Lines 208-209: Additional context for the tested translational regulators and what their known role (activator, inhibitor) would help. i.e.: what are Cup and GIGYF, as this is the first time these proteins are mentioned.

We have now added to the first section of the Results, "To determine if eIF4E-5 can interact with known translational regulators, we used yeast two-hybrid (Y2H) assays to examine the ability of eIF4E-5 ("bait") to bind potential interactors ("prey"): the ubiquitous translation initiation factor eIF4G and testis-specific eIF4G-2, which have roles in sperm development and male fertility (Hernández et al. 1998; Baker and Fuller 2007; Franklin-Dumont et al., 2007; Ghosh and Lasko 2015); and the 4E-BPs Thor (also known as 4E-BP; Miron et al. 2001), 4E transporter (4E-T) (Kamenska et al. 2014), Cup, (Nelson et al, 2004; Zappavigna, et al., 2004), and GRB10- interacting GYF (GIGYF) (Russica et al., 2019) (Fig. 1A)." (lines 139-146).

Line 224: Here is a great place to clarify if Soti was tested to explain the activated caspase activity described in Figure 3.

We have now added to the Results, "Because eIF4E-5 and soti mutants show similar defects in individualization, we investigated whether Soti mRNA or protein was affected in eIF4E-5 mutants." (lines 276-277).

Line 277: Do the authors have a reason for not quantifying cyst mispolarization in the D19a mutants?

The D19a allele was isolated six months after the other alleles, and we had already completed the cyst mispolarization experiments by the time we finished our initial characterization of D19a. Because D19a has the same phenotypes as the other alleles, and because it appears unlikely that the cyst mispolarization defects cause the male sterility in eIF4E-5 mutants, we decided not to conduct these imaging- and time-intensive experiments on D19a. We have now added to the appropriate section of the Materials and Methods, "Note that eIF4E-5^{D19a} was isolated six months after eIF4E-5^{B8a} and eIF4E-5^{B8b} and was not included in these experiments." (lines 560-561).

Second decision letter

MS ID#: DEVELOP/2021/200477

MS TITLE: Eukaryotic translation initiation factor eIF4E-5 is required for spermiogenesis in Drosophila melanogaster

AUTHORS: Lisa Shao, Jaclyn M. Fingerhut, Brook L. Falk, Hong Han, Giovanna Maldonado, Yuemeng Qiao, Vincent Lee, Elizabeth Hall, Liang Chen, Gordon Polevoy, Greco Hernández, Paul Lasko, and Julie A Brill
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.

Reviewer 2

Advance summary and potential significance to field

This work represents several significant advances. It reveals another testis-specific paralog of a gene required in spermatogenesis, thus adding to the growing list of genes that have spermatogenesis-specific “versions” needed for the exceptionally complicated development of the Drosophila male germline. By identifying this testis-specific translation initiation factor, it also uncovers another aspect of post-meiotic translational regulation during Drosophila spermatogenesis, which is a process that we do not fully understand at the molecular level. In particular, it focusses on translational regulation at the distal ends of elongated spermatid cysts, which not very well understood at all.
Finally, it ties this new molecular player to known molecular players in post-meiotic spermatogenesis, thus placing the findings into context. This paper is a significant achievement.

Comments for the author

All of my concerns have been addressed. I have no further comments. I think it's a fine paper.

Reviewer 3

Advance summary and potential significance to field

Shao et al. reported a role of eIF4E-5 in Drosophila spermiogenesis. First using CRISPR/Cas9 genome editing, the authors generated a series of eIF4E-5 loss-of-function alleles for genetics experiments, where they show mutant phenotypes including spermatid cyst polarization, sperm individualization and male fertility. Second, by generating polyclonal antibodies and a Flag tagged genomic rescuing transgene, they showed eIF4E-5 expression pattern and localization. Third, they found these spermiogenesis roles of eIF4E-5 are through promoting local accumulation of Soti. Finally, the elf4E-5 mutants display mild spermatid cyst polarization defects, and have genetic interactions with Orb2 and aPKC. Overall, the experiments are presented in a logic manner from phenotypic analyses to more mechanistic insight. And the results should be of interest to developmental biologists, in particular germ cell biologists.

Comments for the author

The authors have done a great job to revise the manuscript, which has a much better flow now and is easier to read and appreciate the findings. The additional results and data analyses also enhance the paper and make the conclusions more solid. With these, acceptance is recommended.