KATNB1 is a master regulator of multiple katanin enzymes in male meiosis and haploid germ cell development

Jessica E. M. Dunleavy, Anne E. O'Connor, Hidenobu Okuda, D. Jo Merriner and Moira K. O'Bryan
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MS TITLE: KATNB1 is a master regulator of multiple katanin enzymes in male meiosis and haploid germ cell development

AUTHORS: Jessica EM Dunleavy, Anne E O'Connor, Hidenobu Okuda, D Jo Merriner, and Moira K O'Bryan

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 BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will see, the referees express considerable interest in your work, but have some significant criticisms and recommend a substantial revision of your manuscript before we can consider publication. If you are able to revise the manuscript along the lines suggested, which may involve further experiments, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' major concerns. Please also note that Development will normally permit only one round of major revision.

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

Katanin is microtubule severing enzyme, that consists of KATNA and KATNB subunits. The authors examined the impact of loss of katanin regulatory B-subunit KATNB1 function in spermatogenesis. They generated mouse lines with a different combinations of Katnb1 allele using Katnb1Taily, that was previously established by the authors, and Katnb1GCKO, that was newly generated by germ cell specific Stra8-Cre mediated deletion. The authors compared the spermatogenic phenotypes in Katnb1Taily/ Taily Katnb1Taily/KO and Katnb1GCKO/GCKO testes. They demonstrated that KATNB1 is the master regulator of all katanin enzymatic A-subunits during mouse spermatogenesis and is required to maintain stability of katanin A-subunit by forming complex. They showed complete loss of KATNB1 from germ cells resulted in defects in sperm production, suggesting multiple spermatogenesis functions for KATNB1, including meiosis, acrosome formation, sperm tail assembly, sperm nuclear remodeling and seminiferous epithelium integrity. Their study highlights role of KATNB1 in key microtubule-based structures during spermatogenesis.
Overall, the manuscript provides new insights on KATNB1-mediated multiple functions in male germ cells with comprehensive data showing multiple phenotypes in the in Katnb1Taily/ Taily, Katnb1Taily/KO and Katnb1GCKO/GCKO testes. Especially, analyses of spermiogenesis are comprehensive and data rich, and well written. Although this study is interesting in the field, several minor concerns listed below should be properly addressed before it can be considered for publication.

Comments for the author

Katanin is microtubule severing enzyme, that consists of KATNA and KATNB subunits. The authors examined the impact of loss of katanin regulatory B-subunit KATNB1 function in spermatogenesis. They generated mouse lines with a different combinations of Katnb1 allele using Katnb1Taily, that was previously established by the authors, and Katnb1GCKO, that was newly generated by germ cell specific Stra8-Cre mediated deletion. The authors compared the spermatogenic phenotypes in Katnb1Taily/ Taily Katnb1Taily/KO and Katnb1GCKO/GCKO testes. They demonstrated that KATNB1 is the master regulator of all katanin enzymatic A-subunits during mouse spermatogenesis and is required to maintain stability of katanin A-subunit by forming complex. They showed complete loss of KATNB1 from germ cells resulted in defects in sperm production, suggesting multiple spermatogenesis functions for KATNB1, including meiosis, acrosome formation, sperm tail assembly, sperm nuclear remodeling and seminiferous epithelium integrity. Their study highlights role of KATNB1 in key microtubule-based structures during spermatogenesis.
Overall, the manuscript provides new insights on KATNB1-mediated multiple functions in male germ cells with comprehensive data showing multiple phenotypes in the in Katnb1Taily/ Taily, Katnb1Taily/KO and Katnb1GCKO/GCKO testes. Especially, analyses of spermiogenesis are comprehensive and data rich, and well written. Although this study is interesting in the field, several minor concerns listed below should be properly addressed before it can be considered for publication.

Comments

Fig1 Please indicate the age of male mice from which testes were isolated.
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Line128-129 Please label the meiotic prophase stage of spermatocytes and the seminiferous tubule stage in Fig2D and Fig S2B.
Line149-150,
The author state that KATNB1 is required to maintain the patency of cytoplasmic bridges between germ cells, germ-Sertoli cell adhesion and potentially the blood-testis-barrier. This is not supported from the data in Fig2 and highly speculative. They should show the evidence by electron microscopy or immunostaining.
Line 210 S4A should be S3A.

Fig4 The authors demonstrated KATNB1-KATNA, -KATNAL1, -KATNAL2 interactions by PLA and assessed proximity of those complexes to MT. The authors state that those complexes are abundant in prophase and declined gradually in the following Meta I and Ana I. However, this data lacks quantification. The authors should show quantification of PLA dots at each stage.
Line 233-235 The authors state that during prometaphase, metaphase and anaphase, KATNB1 localised to kinetochore astral and interpolar spindle fibres. I agree KATNB1 localized along spindle fibres , but it is not clear whether KATNB1 well localized to kinetochores. Please show colocalization of KATNB1 with kinetochore protein marker by IF.
Line 241 The authors state that KATNB1 foci remained localised to the midbody MTs (Fig4D). However, it is not clear whether this KATNB1 immunostaining is significant, since high background is observed. Please show co-immunostaining of KATNB1 and midbody marker.
FigS7 Please label which bands are KATNB, KATNA1 and KATNAL1.

Reviewer 2

Advance summary and potential significance to field

This study investigated the fucntion of KATNB1 in male germ cell development using three different mouse models with a graded series of loss-of-function (LOF) mutations. The results demonstrated that KATNB1 played multiple function in germ cell development. This study provide the information for better understanding microtubule severing in germ cell development.

Comments for the author

In this manuscript, Jessica EM Dunleavy and colleagues investigated the functions of KATNB1 in mouse spermatogenesis. They used three different mouse models with a graded series of loss-of-function (LOF) mutations. They found that complete loss of KATNB1 in germ cells is incompatible with sperm production.
This study revealed that loss of KATNB1 caused multiple defects in spermatogenesis, including defect in meiosis, acrosome formation, sperm tail assembly, and regulating both the Sertoli and germ cell cytoskeletons during sperm nuclear remodelling and in maintaining seminiferous epithelium integrity.
They concluded that katanins are able to differentially regulate almost all key microtubule-based structures during mammalian male germ cell development.
Generally, the results in this study are very preliminary. The authors only described the phenotype of KATNB1 ko mice and lack of mechanism study. Some conclusions are overstated.

Specific comments:
1, In this study, the authors used three different mouse models.
Katnb1Taily/Taily, Katnb1 Taily/KO, and Katnb1KO/KO mice. It is very confusing.
2, This study only described the phenotype of KATNB1 ko mice. More detailed mechanistic studies are necessary to strengthen the conclusion.
3, Multiple defects of germ cell development were observed in KATNB1 ko mice.
However, whether the defects of spermiogenesis is a consequence of abnormal development of germ cell at early stage. This need to clarify.
4, The author claimed that KATNB1 also played role in Sertoli cells. However, no direct evidence to support this conclusion. Sertoli cell specific cre mice need to be used to investigate the function of KATNB1 in Sertoli cells.
5, The number of sperm was decreased in all three mouse models, whether these mice are infertile. The results of fertility test are not presented in this study.

Reviewer 3

Advance summary and potential significance to field

The paper by Dunleavy et al., entitled ‘KATNB1 is a master regulator of multiple katanin enzymes to male meiosis and haploid germ cell development’ clarifies the phenotypes of KATNB1 deficiency that include reduced spermatogenic output, meiotic abnormalities such as misaligned metaphase

chromosomes and abnormal spindle architecture, and spermiogenesis phenotypes with sperm tail defects and manchette-related failures.
KATNs are composed of catalytic (A subunit, p60) and regulatory (B subunit, p80) subunits and play a critical role in the remodeling of microtubule structures. There are three different A subunits, Katna1, Katnal1, and Katnal2, and two B subunits, Katnb1 and Katnbl1. The authors' group previously reported the phenotypes of the mutant mice of Katnb1 and Katnal2. The Katnb1 mutant mice carry an ENU-induced point mutation, called "the Taily mutation". The Taily mice are sterile due to abnormal morphology, poor motility, and low numbers of sperm. In this manuscript, they generated a mouse model exhibiting conditional knockout of Katnb1 in the male germline (Katnb1GCKO/GCKO).
They analyzed not only Katnb1 GCKO/GCKO but also Katnb1Taily/KO mice to observe the gene dosage-dependent phenotype. The authors deeply analyzed and described the phenotype of the mice at the light microscopic and the electron microscopic levels. The quality of their immunostaining, in situ PLA, and electron microscopic analyses is very high and the data are convincing. Novelty-wise, this study extends the knowledge of the Katnb1 function because the Taily mutant mice only showed partial effects of Katnb1 depletion, and all the current analyses have been done in vivo so that they are supposed to be observing close to what is happening in mice. Because the KATN family is one of the key microtubules severing proteins, this study is an important work and will attract broad interest.

Comments for the author

Concerns and suggestions

The KATNB1 reduction causes the loss of all three katanin A-subunits and there is little information about the knockout phenotype of Katna1 and Katnbl1 genes in the male germline. Therefore, the interpretation of the phenotype at the molecular aspect would be affected by the expression of the three catalytic subunits and the two regulatory subunits at each stage of male germ cell differentiation, and whether or not they can complement each other.

1. Please show expression profiles of all katanin A-subunits in germ cells from spermatogonia until elongated spermatids as well as in Sertoli and Leydig cells in supplemental information by using a graph or a table, which will help readers to understand the results better.

2. Katnal1 was shown to be dominantly expressed in Sertoli cells (Smith et al. 2012, PLoS genetics). Katnb1GCKO/GCKO mice should lose their alleles only in the germ cell lineage because the authors used Stra8-Cre. Why was almost all the KATNAL1 expression lost in the Katnb1 GCKO/GCKO mice? Is the expression of Katnal1 downregulated in Sertoli cells?
A similar question applies to KATNA1. Katna1 seems to be expressed ubiquitously in many cell types. Was the expression of KATNA1 in somatic cells, such as interstitial cells, blood and lymphatic cells, lost in the Katnb1 GCKO/GCKO mouse testis?

3. Are all three katanin A-subunits ubiquitinated if Katnb1 is abrogated in mice? It may be that the possible ubiquitinated protein can be detected in the Katnb1 GCKO/GCKO testes.

4. Katnb1KO/KO mice die in utero. Are there any phenotypes in tissues such as brain heart, lung or oviduct, in Katnb1Taily/KO mice, where microtubules participate in development and function?

First revision

Author response to reviewers' comments

Response to reviewers
We thank the reviewers for the time and effort they have put into reviewing our manuscript. We appreciate their thoughtful insights enormously. We have addressed each comment below and where possible have added new data and additional analyses of existing data. We note however, that extensive experimental revisions were not possible due to the strict Melbourne COVID-19

lockdown that has been in place since August 5th, 2021. This lockdown has severely restricted our laboratory access. Moreover, over the past 18 months, such lockdowns (this is our sixth lockdown in Melbourne, with a total of 264 days in lockdown to date) have meant we have been forced to reduce the number and size of our mouse colonies. As such, experiments were restricted to those that involved the use of existing samples. Despite these restrictions, we feel the additions we have made have addressed the requests and significantly improved the manuscript. We trust that is now acceptable for publication in Development.

Reviewer 1

Advance Summary, Potential Significance to Field and Comments for the Author:
Katanin is microtubule severing enzyme, that consists of KATNA and KATNB subunits. The authors examined the impact of loss of katanin regulatory B-subunit KATNB1 function in spermatogenesis.They generated mouse lines with different combinations of *Katnb1* allele using *Katnb1*^{Taily}, that was previously established by the authors, and *Katnb1*^{GCKO}, that was newly generated by germ cell specific Stra8-Cre mediated deletion. The authors compared the spermatogenic phenotypes in *Katnb1*$^{Taily/Taily}$, *Katnb1*$^{Taily/KO}$ and *Katnb1*$^{GCKO/GCKO}$ testes. They demonstrated that KATNB1 is the master regulator of all katanin enzymatic A-subunits during mouse spermatogenesis and is required to maintain stability of katanin A-subunit by forming complex. They showed complete loss of KATNB1 from germ cells resulted in defects in sperm production, suggesting multiple spermatogenesis functions for KATNB1, including meiosis, acrosome formation, sperm tail assembly, sperm nuclear remodelling and seminiferous epithelium integrity. Their study highlights roles of KATNB1 in key microtubule-based structures during spermatogenesis. Overall, the manuscript provides new insights on KATNB1-mediated multiple functions in male germ cells with comprehensive data showing multiple phenotypes in the in *Katnb1*$^{Taily/Taily}$, *Katnb1*$^{Taily/KO}$ and *Katnb1*$^{GCKO/GCKO}$ testes. Especially, analyses of spermiogenesis are comprehensive and data rich and well written. Although this study is interesting in the field, several minor concerns listed below should be properly addressed before it can be considered for publication.

Specific comments

1. Fig 1: Please indicate the age of male mice from which testes were isolated.

Response: we have updated the figure legend text accordingly.

2. Line112-116, The author state that loss of KATNB1 resulted in destabilization of all the A-subunits, leading to the idea that KATNB1 is required for stability of different A-subunits KATNA, -KATNAL1, -KATNAL2. The authors should show by immunoprecipitation of KATNB1 from testes extracts to see whether KATNB1 forms different complexes with KATNA, -KATNAL1, -KATNAL2.

Response: Respectfully, we feel that these additional experiments are unnecessary. KATNB1 has been shown to form complexes with each of KATNA1, KATNAL1 and KATNAL2 via immunoprecipitation across multiple cells types in multiple publications (e.g. (Cheung et al., 2016; Hartman et al., 1998; McNally et al., 2000; McNally & McNally, 2011; Mishra-Gorur et al., 2014; Rezabkova et al., 2017; Srayko et al., 2000), including for KATNB1-KATNAL2 in our own (Dunleavy et al., 2017)). Further, herein we show KATNB1 forms complexes with each of these subunits in spermatocytes via Duolink *in situ* proximity ligation assays, and that these complexes disappear in *Katnb1*$^{GCKO/GCKO}$ spermatocytes.

We have added text detailing these previous characterisations on lines 115-120 of the manuscript.

3. Line128-129, Please label the meiotic prophase stage of spermatocytes and the seminiferous tubule stage in Fig2D and Fig S2B.

Response: we have updated these figures accordingly (note Fig. S2B is now S3B). We note for the *Katnb1*$^{Taily/KO}$ and *Katnb1*$^{GCKO/GCKO}$ models due to the severity of the seminiferous tubule

phenotype these stage numbers are our best estimation based on the morphology of spermatogonia and spermatocytes, and in Fig. 2D, on the acrosome.

4. Line149-150, The author state that KATNB1 is required to maintain the patency of cytoplasmic bridges between germ cells, germ-Sertoli cell adhesion and potentially the blood-testis-barrier. This is not supported from the data in Fig 2 and highly speculative. They should show the evidence by electron microscopy or immunostaining.

Response: The presence of prematurely sloughed germ cells in the epididymis is clear evidence for a failure in Sertoli-germ cell adhesion and is illustrated in Fig. 2E. We have now, however, also included additional higher magnification images of espin staining of wild type versus mutant testes in Fig. S7C to show close examination of the process. We note that the pattern suggests junctions form and consistent with our EM analysis these junctions initially appear normal. Ectopic tracts of espin then accumulate in disorganised areas of seminiferous epithelium and at sites of germ cell loss, suggesting dysregulation of apical ES function. We have updated the text on lines 426-432 to indicate this.

Similarly, the presence of multinucleated syncytia of germ cells and Sertoli cells is clear evidence of a loss of integrity in the intercellular germ-germ and Sertoli-Sertoli connections. 'Catching' this brief period of dysfunction prior to cell aggregation is extremely difficult (unlikely). As such we were not able to add additional information to the manuscript regarding this point.

We have, however, provided additional EM analysis of the blood-testis barrier in Fig. S7D. Unfortunately, we no longer have mice available to directly assess the integrity of the BTB via biotin assays or via staining for immune infiltrates. As we predict the collapse of Sertoli cells into symplasts is a secondary effect, notably in testes wherein KATNB1 is only removed from germ cells, we did not feel this was an essential experiment. Accordingly, we have modified the text on lines 164 -168 to clarify that this hypothesis was not directly tested.

5. Line 210 S4A should be S3A

Response: We have updated the text accordingly.

6. Fig4 The authors demonstrated KATNB1-KATNA, -KATNAL1, -KATNAL2 interactions by PLA and assessed proximity of those complexes to MT. The authors state that those complexes are abundant in prophase and declined gradually in the following Meta I and Ana I. However, this data lacks quantification. The authors should show quantification of PLA dots at each stage.

Response: In the absence of being able to repeat these experiments for this purpose, we have removed the comments related to the relative abundance of complexes in prophase relative to later stages.

7. Line 233-235 The authors state that during prometaphase, metaphase and anaphase, KATNB1 localised to kinetochore, astral and interpolar spindle fibres. I agree KATNB1 localized along spindle fibres, but it is not clear whether KATNB1 well localized to kinetochores. Please show colocalization of KATNB1 with kinetochore protein marker by IF.

Response: The wording of this comment suggests the reviewer has confused kinetochores with kinetochore spindle fibres. As such, we have updated the wording on lines 248-249 to clarify that we mean colocalization along the kinetochore spindle fibres (K-fibres) not to the kinetochores. We have already demonstrated co-localization of KATNB1 and KATNB1 based complexes with spindle fibre microtubules (Figure 4).

8. Line 241 The authors state that KATNB1 foci remained localised to the midbody MTs (Fig4D). However, it is not clear whether this KATNB1 immunostaining is significant, since high background is observed. Please show co-immunostaining of KATNB1 and midbody marker.

Response: We were unfortunately unable to find a midbody marker that was compatible with the Bouin's fixed paraffin testis sections that we had available. Respectfully, however we feel that a midbody marker is not necessary, as the midbody microtubules are clear in the current figure and distinct KATNB1 puncta can be seen localised to these. We have clarified the figure with arrowheads to indicate the midbody microtubules and these puncta.

9. Fig S7 Please label which bands are KATNB, KATNA1 and KATNAL1.

Response: we have updated the figure accordingly (note this is now Fig. S8).

Reviewer 2

Advance Summary and Potential Significance to Field:
This study investigated the function of KATNB1 in male germ cell development using three different mouse models with a graded series of loss-of-function (LOF) mutations. The results demonstrated that KATNB1 played multiple function in germ cell development. This study provide the information for better understanding microtubule severing in germ cell development.

Comments for the Author:
In this manuscript, Jessica EM Dunleavy and colleagues investigated the functions of KATNB1 in mouse spermatogenesis. They used three different mouse models with a graded series of loss-of-function (LOF) mutations. They found that complete loss of KATNB1 in germ cells is incompatible with sperm production. This study revealed that loss of KATNB1 caused multiple defects in spermatogenesis, including defect in meiosis, acrosome formation, sperm tail assembly, and regulating both the Sertoli and germ cell cytoskeletons during sperm nuclear remodelling and in maintaining seminiferous epithelium integrity. They concluded that katanins are able to differentially regulate almost all key microtubule-based structures during mammalian male germ cell development. Generally, the results in this study are very preliminary. The authors only described the phenotype of KATNB1 ko mice and lack of mechanism study. Some conclusions are overstated.

Specific comments:

1. In this study, the authors used three different mouse models. in *Katnb1*$^{Taily/Taily}$, *Katnb1*$^{Taily/KO}$ and *Katnb1*$^{GCKO/GCKO}$ testes. It is very confusing.

Response: In the absence of specific examples, we are unable to address this comment. We note that these are the correct names for the mouse models.

2. This study only described the phenotype of KATNB1 ko mice. More detailed mechanistic studies are necessary to strengthen the conclusion.

Response: We respectfully disagree. The phenotypes within genetically modified mice are a direct indication of the processes within which individual genes/proteins play a role. We have defined a mechanistic role for KATNB1 in each of male meiosis (metaphase spindle organisation, metaphase chromosome alignment, chromosome segregation and cytokinesis), acrosome biogenesis, sperm tail formation, sperm head shaping, and the release of sperm form the seminiferous epithelium.

3. Multiple defects of germ cell development were observed in KATNB1 ko mice. However, whether the defects of spermiogenesis is a consequence of abnormal development of germ cell at early stage. This need to clarify.

Response: This is a question that we have already gone to great length to address. The power of an allelic series is that it allowed the dissection of the function of a gene/protein in each phase of a developmental process. Our results are summarized in Table 1.

4. The author claimed that KATNB1 also played role in Sertoli cells. However, no direct evidence to support this conclusion. Sertoli cell specific cre mice need to be used to investigate the function of KATNB1 in Sertoli cells.

Response: We respectfully disagree. Sertoli cell specific functions can be inferred by comparing the differences between the *Katnb1*$^{Taily/KO}$ mice, wherein both germ and Sertoli cells are KATNB1 deficient, and the *Katnb1*$^{GCKO/GCKO}$ mice, wherein only the germ cells are KATNB1 deficient but KATNB1 remains intact within the Sertoli cells. Given that the manuscript already contains 3 mouse models a Sertoli cell specific mouse is outside the scope of this study.

5. The number of sperm was decreased in all three mouse models, whether these mice are infertile. The results of fertility test are not presented in this study.

Response: We have previously shown that the *Katnb1*$^{Taily/Taily}$ are male sterile (O'Donnell et al., 2012), and as such that even partial loss of KATNB1 results in male infertility. This is stated on line 73 of the manuscript. Given that the loss of KATNB1 in the *Katnb1*$^{GCKO/GCKO}$ and *Katnb1*$^{Taily/KO}$ is even more severe, sterility can be assumed. This conclusion is strongly supported by the histology of the seminiferous epithelium and epididymal sperm counts shown in Figure 2D and 2C, respectively.

Reviewer 3

Advance Summary and Potential Significance to Field:
The paper by Dunleavy et al., entitled 'KATNB1 is a master regulator of multiple katanin enzymes to male meiosis and haploid germ cell development' clarifies the phenotypes of KATNB1 deficiency that include reduced spermatogenic output, meiotic abnormalities such as misaligned metaphase chromosomes and abnormal spindle architecture, and spermiogenesis phenotypes with sperm tail defects and manchette-related failures. KATNs are composed of catalytic (A subunit, p60) and regulatory (B subunit, p80) subunits and play a critical role in the remodeling of microtubule structures. There are three different A subunits, Katna1, Katnal1, and Katnal2, and two B subunits, Katnb1 and Katnbl1. The authors' group previously reported the phenotypes of the mutant mice of Katnb1 and Katnal2. The Katnb1 mutant mice carry an ENU-induced point mutation, called "the Taily mutation". The Taily mice are sterile due to abnormal morphology, poor motility, and low numbers of sperm. In this manuscript, they generated a mouse model exhibiting conditional knockout of Katnb1 in the male germline (Katnb1GCKO/GCKO). They analyzed not only Katnb1 GCKO/GCKO but also Katnb1Taily/KO mice to observe the gene dosage-dependent phenotype. The authors deeply analyzed and described the phenotype of the mice at the light microscopic and the electron microscopic levels. The quality of their immunostaining, in situ PLA, and electron microscopic analyses is very high and the data are convincing. Novelty-wise, this study extends the knowledge of the Katnb1 function because the Taily mutant mice only showed partial effects of Katnb1 depletion, and all the current analyses have been done in vivo so that they are supposed to be observing close to what is happening in mice. Because the KATN family is one of the key microtubules severing proteins, this study is an important work and will attract broad interest.

Concerns and suggestions
The KATNB1 reduction causes the loss of all three katanin A-subunits and there is little information about the knockout phenotype of Katna1 and Katnbl1 genes in the male germline. Therefore, the interpretation of the phenotype at the molecular aspect would be affected by the expression of the three catalytic subunits and the two regulatory subunits at each stage of male germ cell differentiation, and whether or not they can complement each other.

Specific comments
1. Please show expression profiles of all katanin A-subunits in germ cells from spermatogonia until elongated spermatids as well as in Sertoli and Leydig cells in supplemental information by using a graph or a table, which will help readers to understand the results better.

Response: This data has been included as new Fig. S1.

2. Katnal1 was shown to be dominantly expressed in Sertoli cells (Smith et al. 2012, PLoS genetics). *Katnb1*$^{GCKO/GCKO}$ mice should lose their alleles only in the germ cell lineage because the authors used Stra8-Cre. Why was almost all the KATNAL1 expression lost in the

Katnb1$^{GCKO/GCKO}$ mice? Is the expression of Katnal1 downregulated in Sertoli cells? A similar question applies to KATNA1. Katna1 seems to be expressed ubiquitously in many cell types. Was the expression of KATNA1 in somatic cells, such as interstitial cells, blood and lymphatic cells, lost in the Katnb1 GCKO/GCKO mouse testis?

Response: While the antibody staining in the Smith et al study suggested KATNAL1 was dominantly expressed in Sertoli cells, single cell analyses of the testis RNA transcriptome have since then revealed that *Katnal1* is in fact much more enriched in the germ cell population of the testis relative to the somatic cells. This has been shown in both mice and humans across multiple independent studies (Ernst et al., 2019; Guo et al., 2018; Jung et al., 2019; Wang et al., 2018). We cannot explain this discordance. Likewise, the published single cell RNAseq datasets have revealed *Katna1* is highly enriched in the germ cells relative to the somatic cell population of the testis. The expression profile data included as the new Fig. S1 to address Reviewer 3's first comment show this.

We note the antibody used in the current study is different to the one in the Smith et al study and was tested for specificity as shown in Fig. S7 (now Fig. S8).

As an aside, and not included in this manuscript, the interpretation described above is consistent with the preliminary phenotype generated from *Katnal1* germ cell KO mice. This analysis is ongoing, and thus, not suitable for inclusion in the current manuscript.

We thus believe the dramatic reductions in KATNA1 and KATNAL1 expression seen in the *Katnb1*$^{GCKO/GCKO}$ testis lysates is due to the fact they are both most enriched in the germ line, which comprises the bulk of the testis. We would anticipate that with a longer exposure of the western blot, we would see faint bands corresponding to Sertoli cell expression. Unfortunately, due to the rolling COVID-19 lockdowns in Melbourne over the past 18 months, we have had to reduce our mouse colonies and are not currently maintaining a *Katnb1*$^{GCKO/GCKO}$ line. To repeat this experiment would thus necessitate the generation of new animals which would take 6 months due to the 2-step breeding strategy from the *Katnb1*$^{Flox/Flox}$ line. As such, this experiment was not possible in the revision timeframe.

In addition to the new Fig. S1, we have added text on lines 126-131 addressing this comment.

3. Are all three katanin A-subunits ubiquitinated if Katnb1 is abrogated in mice? It may be that the possible ubiquitinated protein can be detected in the *Katnb1*$^{GCKO/GCKO}$ testes.

Response: This is an interesting question, but we posit, it is beyond the scope of the current study.

4. *Katnb1*$^{KO/KO}$ mice die in utero. Are there any phenotypes in tissues such as brain, heart, lung or oviduct, in *Katnb1*$^{Taily/KO}$ mice, where microtubules participate in development and function?

Response: To do this properly would require a huge amount of work and is beyond the scope of the current (already very large) study. As stated in the manuscript, we have previously established KATNB1 has roles in nodal cilia and in the establishment of left-right signalling during embryonic development, and that its loss resulted in heart looping defects in *Katnb1*$^{KO/KO}$ mice (Furtado et al., 2017). Defects beyond the heart were not assessed. KATNB1 has however, been shown to play essential roles in brain development in humans, Zebrafish and *Drosophila*, with KATNB1 deficiency resulting in microcephaly (Hu et al., 2014; Mishra- Gorur et al., 2014). Based on this, we would anticipate extra-testicular defects in *Katnb1*$^{Taily/KO}$ mice. We have included additional text on lines 98-101 to address these predictions.

Reference list

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Second decision letter

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MS TITLE: KATNB1 is a master regulator of multiple katanin enzymes in male meiosis and haploid germ cell development

AUTHORS: Jessica EM Dunleavy, Anne E O'Connor, Hidenobu Okuda, D Jo Merriner, and Moira K O'Bryan
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 1

Advance summary and potential significance to field

The authors responded to all the concerns I made, and the newly provided data and descriptions satisfied every question. They also seemed to do a reasonable job with Reviewer#2 and Reviewer#3's concerns.
Now revised manuscript has been well written. I felt some of the concerns might be rather too tough for the authors for revision. I think the authors overall satisfied the reviewrs's comments.Now revised manuscript has been well written.Thus, the manuscript should be open for the fields and are supposed to deserve publication. I think this work is significant enough to open to the field.
Kei-ichiro Ishiguro, Kumamoto university.

Comments for the author

I do not think more revision is required.

Reviewer 2

Advance summary and potential significance to field

No

Comments for the author

The reviewer's comments were not appropriately responded.

Reviewer 3

Advance summary and potential significance to field

The revision, new analyses and additional data had addressed many of my concerns.

Comments for the author

The revised paper is considerably improved. I believe that the revised paper is acceptable for publication in Development