MFN2 interacts with nuage-associated proteins and is essential for male germ cell development by controlling mRNA fate during spermatogenesis

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First decision letter

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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 from their reports, the referees recognise the potential of your work, but they also raise significant concerns about it. Given the nature of these concerns, I am afraid I have little choice other than to reject the paper at this stage.

However, having evaluated the paper, I do recognise the potential importance of this work. I would therefore be prepared to consider as a new submission an extension of this study that contains new experiments, data and discussions and that address fully the major concerns of the referees. The work required goes beyond a standard revision of the paper. Please bear in mind that the referees (who may be different from the present reviewers) will assess the novelty of your work in the context of all previous publications, including those published between now and the time of resubmission.

If you decide to resubmit, please go to BenchPress and click on the 'Submit a new manuscript' link within the Author Area.

Please ensure that you click the 'This is a resubmission' checkbox, and enter the manuscript identification number shown above. I would also ask you to provide in the cover letter an explanation of they key ways in which the manuscript differs from the current submission, followed by a point-by-point response to the referees' concerns.

Reviewer 1

Advance summary and potential significance to field

In this manuscript, Wang and colleagues begin by showing the expression pattern of MFN2 in the mouse germline, and then go on to characterize a conditional knockout of Mfn2 (and at times, Mfn1 and the double cKO). They show that Mfn2-cKO mice have reduced testis weight (this effect is earlier in Mfn1 and in the double mutant) and disrupted subcellular structures, including mitochondria, mitochondrial-associated ER membranes (MAMs), and contacts between mitochondria and ER. The authors also show that MFN2 co- immunoprecipitates with many of the proteins known to localize to the nuage in germ cells, including MIWI and DDX4. Several of these proteins show marked decrease in expression or mislocalization in the Mfn2 cKO.
The authors show that – consistent with disruptions in the nuage – the overall levels of pachytene piRNAs are reduced by 50% in the Mfn2 cKO, although transposon activity appears unchanged. RNA-seq data from purified pachytene spermatocytes and round spermatids is presented, and MFN2 is shown to associate with actively-translated (polysomal) fractions. Finally, there are some nice data showing that SPATA19 is expressed 3 days early in the Mfn2 cKO.

The authors have performed a substantial amount of work to characterize a complex phenotype, and they have some very intriguing data. The microscopy (both EM and fluorescence) is fantastic. The 'bones' of this paper are great, and already represent a significant advancement; if the authors can focus on the strengths of the paper and either solidify or discard the less convincing parts, it has the capacity to be truly excellent.

I do feel that the authors are over-interpreting their data in places (details in "major comments" section). They can either remove those bits, or re-write those sections so that the text is appropriately cautious, or they can do more analysis, or (possibly) 1-2 additional experiments. I tried not to ask for anything that seemed outrageous or beyond their capabilities. There are also places where promising leads almost seem under-pursued (details in "major comments," again) and might benefit from more computational analysis of existing data sets.

Quick note of explanation: I answered "no" on question #3 ("Do the title and abstract accurately represent the findings of the study?") because the title (and to a lesser extent, the abstract) draw attention to what I feel is shakiest part of the paper (the eEF1A part): "… and involves mRNA translational machinery…" That should be easy to fix.

Comments for the author

Major comments:

Nuage:
- The Mfn2 cKO clearly disrupts structures and protein localization in the nuage. The closest the authors get to showing a functional consequence of this is the panel in the SOM showing that total pachytene piRNAs are reduced by 50%. That's an important result!! I would move this to one of the main figures. Just because transposon activity doesn't seem to be affected, it doesn't mean that the 50% drop in piRNAs isn't having a very real effect on germ cell development, particularly on clearing meiotic transcripts (by cleavage) once their usefulness is over.

two relevant papers (this reviewer is not affiliated with either lab) -Goh, WS…. GJ Hannon. (2015) Genes & Dev. piRNA-directed cleavage of meiotic transcripts regulates spermatogenesis.
Larriba & Del Mazo (2018) Sci Rep. An integrative piRNA analysis of mouse gametes and zygotes

reveals new potential origins and gene regulatory roles.

• An analogy for what seems to be happening to the nuage: a tornado flattens a barn so that the only thing left is the foundation and maybe a couple of outside stone walls. Most of the horses won't recognize what's left as their barn (MIWI, DDX4, GASZ), but maybe one will recognize the foundation (TDRKH) and go back to where its stall once was. It seems from the literature as if TDRKH is instrumental at binding to mitochondria and recruiting MIWI; this could explain why TDRKH can still find the mitochondria in the Mfn2 cKO and why at least some (reduced) MIWI is still showing up (Figure 4). A very relevant paper (again, this reviewer has no affiliation):

Ding, D…. C Chen. (2019) Nuc Acids Res. Mitochondrial membrane-based initial separation of MIWI and MILI functions during pachytene piRNA biogenesis.
It would be nice if you could mention in the discussion how the Mfn2 cKO compares to the TDRKH lof. RNA-seq data:
The RNA-seq data is nice, but the extensive GO term analysis feels like an enormous distraction / tangent. I would take out the GO term panels completely (not even put them in SOM!) and move S7A and S7B (the scatter plots of the RNA-seq data, in spermatocytes and spermatids) to the beginning of Figure 5. Those panels should be easy for the reader to find.

I would use the extra space in Figure 5 to plot data from existing (published) RNA-seq data sets from any available KO or cKO of any of your major nuage proteins (ideally MIWI, but whatever you can find). For example: the RNAs that have been shown to be upregulated at least 2-fold in mutant (vs. wt) – where do they map on your scatter plots? Are they also more likely to be upregulated? (also plot a control set of RNAs that is not affected in the mutant).

Another way of looking at this: the RNAs that are upregulated in the Mfn2 cKO – could some of these be due to meiotic RNAs not getting cleared because pachytene piRNAs are down 50%? If they are, maybe there will be overlap with things upregulated in a MIWI lof.

I would like to see more of a nuanced discussion / acknowledgement of the limits of RNA-seq for investigating the function of a protein that is not a transcription factor, a chromatin regulator, or a predicted RNA-binding protein. I'm worried that most of the effects you see on the transcriptome in the cKO are indirect. Your two RNA-seq data sets will be most useful if you can show convincingly that mRNAs dramatically affected by MIWI (or its nuage compatriots) or MSY2 are statistically more likely to be mis- regulated in the Mfn2 cKO (ideally with no such enrichment for non-target mRNAs). Otherwise it's really hard to make sense of any of the changes in transcript levels.

Non-nuage part of the cytoplasm:
The possible connection to MSY2/YBX2 in the non-nuage part of the cytoplasm is interesting. I have some questions, though. I think this part of the paper needs a few more elements before it is convincing:

• Is the interaction between MSY2 and MFN2 RNA-independent? An RNA-independent interaction here would strengthen your argument that these two proteins might work together.

• Can you use ribosome profiling to show that MSY2 target RNAs (but not non-target RNAs) shift to the polysomal fraction in the Mfn2 cKO? Particularly at P25, where you're already noticing early expression of SPATA19 protein. It could provide compelling evidence that MFN2 has a global effect on translation (and who knows, you might find out that the RNAs that shift to the polysome in the cKO are independent of MSY2 –that would be an interesting result as well!). I know it can be a time-consuming experiment, but it also looks from the data included here (polysome fractionation and RNA-seq in separate figures) that you have the technical expertise to do it.

Given that MSY2 is known for repressing translation of its target RNAs and for protecting them from translation-dependent decay, I am puzzled by the fact that the MSY2 target RNAs that qualify as misregulated in your RNA-seq data set mostly appear to be expressed more *highly* in the Mfn2 cKO. I worry that this could be an indirect effect due to higher expression of MSY2 itself (in your own data) in both spermatocytes and round spermatids (a situation where MSY2 is over-active at repression and protecting RNAs from decay). I can't fit this together with the premature expression of SPATA19 protein (presumably due to premature translation), which would be consistent with a

reduction in function of MSY2 (not an increase in function). Hmm.* (this contradiction is something you might want to acknowledge in your discussion). On a related note to the MSY2 / RNA-seq analysis: if you're going to talk about the MSY2 target genes that are also upregulated in your RNA-seq data set, you should map all of the MSY2 target genes (and, in a different color, non-target genes) onto your RNA-seq scatter plots (currently S7A and B). (Targets/non-targets provided in Yang et al 2005a). Otherwise it looks like you are cherry-picking the data.

*the only explanation I can come up with, and maybe it doesn't even work: somehow the translational- repression function and the RNA-stabilizing function of MSY2 are getting separated in your cKO. There is extra MSY2, happily stabilizing RNAs, but somehow the loss of MFN2 means they are getting translated actively. Just not decayed. This argument would work on me, I think, but I would still want to see ribosome profiling data and ideally an RNA-independent interaction between MSY2 and MFN2.

Strengthen or cut from paper:
The bit at the end regarding eEF1A1 and 2 makes me nervous. These proteins have been shown to be involved in processes independent of translation – including as chaperones (see 2015 review by Abbas, Kumar, and Herbein), and it could be they are appearing at higher levels in the Mfn2 cKO just because the cells are starting to feel really stressed out / disrupted. I would be more convinced of a direct link if you could show that MFN2 bound eEF1A1/2 in an RNA-independent manner and actively blocked function in an in vitro assay.
As it stands right now, it's not convincing, and I think you have far more promising avenues to pursue.

minor comments:

p. 7 --
"While Mfn2-cKO mice were viable and appeared to be grossly normal, they displayed complete sterility after a 5 month-period fecundity test."
This could be explained a little better for readers who do not work with mice (5 months of age? 5 months with females?). Also, consider saying "0 out of n mice were fertile" just to get a # in there.

Fig 2G it might be helpful to use arrows / arrowheads to point out individual late-stage spermatocytes and spermatids that are showing signs of vacuole-ating

P 8, lines 12-14
"These data suggest that, upon Mfn2 deletion in postnatal testes, germ cells gradually lost from P35 by apoptosis, leading to infertility."

Replace with:
"These data suggest that, upon Mfn2 deletion in postnatal testes, most germ cells are gradually lost from P35 by apoptosis, and the remainder are morphologically defective."

P 9 lines 8-9
"In comparison, the first wave of spermatogenesis in Mfn2-cKO testes proceeds until P42."
Replace with "In comparison, the first wave of spermatogenesis in Mfn2-cKO testes proceeds normally until P42." (if I got your meaning right)

Fig S3A
Left panel: as laid out, it implies that top and bottom were run in the same lanes. Clearly they're not!
Redesign for more transparency… Right panel: some background binding to the IgG. :-/ See end of review for technical suggestions re: IPs.

top of page 10 you very nicely show the effect on mitochondrial distribution in Fig 2M, so point the reader to that (in addition to pointing them to the SOM). (right now you mention 2M only in the context of "we don't see this in the single mutants")

The disrupted distribution of mitochondria in the double cKO is very interesting! I wonder why that is happening.

Figure 3 –
- 3B supposedly has ER labeled, but I can't see it (and thus can't see the increased mito-ER distance).
- 3A&B – labels could be more visible (yellow? Maybe bigger? The images are great, and you want a reader who is printing out the paper to be able to see the labels)
- 3D – label for y axis is uninformative … "% of mitochondria in given AR range"?
- H – it's hard for me to see the tubular organization in the control. I think if you showed the two images to a random person (and didn't tell them it was an ER marker), they might say that the protein localization in the mutant was clumpy or mis-aggregating compared to wt. It could be that two-dimensional immunostaining images don't show off the normal tubular structure very well.

P 10 lines 25-27
Not sure you can conclude this! I would re-word to make it more cautious… (or just take that sentence out)

"These data indicate that developmental defects of male germ cells in both Mfn1- and Mfn2-cKO mice could be due to the fragmentation and swelling of mitochondria in germ cells."

P 11, line 28
Re-word, maybe… I'd save "inconsistent" for situations where your data are in direct conflict with a previous paper. Instead, say "in contrast to previous reports…" since you are looking at different tissue type. And maybe replace "The discrepancy" with "This difference" on line 30.
("discrepancy" means that one of these dissimilar results must be wrong… and as you point out, it could simply be the difference between germline and soma). [I have no horse in this race – I just want everyone to get along :)]

Figure 4
- F & G are missing the negative controls (samples without any Myc-tagged protein [or with a Myc-tagged random non-binding protein] but still containing Flag-MFN2)

P 13
lines 11-12: "Co-immunostaining of Mfn2-cKO and control testes revealed ectopic expression of MIWI, DDX4, and GASZ in Mfn2-cKO testes…" This could be a wording issue, but I don't see ectopic expression (Fig 4H) – if anything, I see reduced expression of these proteins. The figure legend (title) for S5 also uses the word "ectopic"…
Figure 4H: here's what my eyes tell me – MIWI is reduced in the cKO DDX4 may be reduced in the cKO but it's hard to tell, because it doesn't seem like the ATP5A staining worked as well as it does in other panels. If you have an image for DDX4 in the cKO where the ATP5A staining looks normal, I would use that.
GASZ looks very reduced
TDRKH looks about the same as in wt, or slightly reduced Line 16-19
"Of note, unlike the perinuclear granular mitochondrial localization showed in the control spermatocytes, DDX4 displayed a diffuse cytoplasmic expression pattern in Mfn2-cKO spermatocytes (Figure.S5D)" I have a hard time seeing this difference in localization in your images. It's subtle.

Figure S6A – more of a science question than a critique – do you know whether there is also a 50% reduction in the piRNA precursors? Or are the precursors normal but not getting processed as efficiently as usual? I think this could be really interesting!

P 16, lines 22-24:
this sentence seems unnecessary, given that the polysome profiling data alone show this:
"Combined with the RNA-Seq data, these results suggest that MFN2 could associate with cytoplasmic monosomes and polysomes."

P 18
"These data show that Spata19 was early activated and translated when a loss of MFN2 in male germ cells, suggesting MFN2 could regulate mRNA translation."

Replace with

“These data show that Spata19 is activated early and translated upon loss of MFN2 in male germ cells, suggesting MFN2 normally represses its translation.”

Minor writing / style comments

P 4 line 23 and line 26 : replace “secession” with “cessation” (they sound similar but mean very different things)
P 5 line 21: replace “Inspiringly,” with “Intriguingly,” P 8 line 4: no discernible abnormality was found
P 8 line 25, replace “Besides,” with “In contrast,”
P 10 line 12 “to fully develop” or “to develop fully” P 17 lines 3-6. This sentence needs a verb.

Technical advice for getting all of your IPs to a state where they’re publication-quality:

- separate the elution step from the boiling step. The streaky high-background you see in a couple of your IP lanes is likely due to a few beads (or bead material) getting loaded in well of the protein gel (we’ve had that problem before). Instead, elute in 40ul elution buffer (1% SDS, 10mM EDTA, 50mM Tris pH 8.0, 1x protease inhibitor) 30min at 70°C (vortex every 5-10min), transfer eluate to a fresh tube, then add 10ul 5xSDS loading buffer and boil 10min. It should look a lot cleaner on the western blot.

- to reduce the background binding of Mfn2(?) to the IgG control, consider blocking your beads (for all conditions) with BSA (10% for an hour is good). You may already be doing this; I just don’t see it mentioned in the methods.

- I realize that you have an issue staining for the two Mfns when they’re in the same lane because they’re so similar in size (and both antibodies are rabbit). If you can’t run them far enough to separate them, or have trouble stripping the blot* between antibodies, the next best thing is to load: input, IP, IgG, [empty lane], input, IP, IgG. Slice the membrane and treat with the two antibodies but make it clear from the figure layout that these are all separate lanes (and duplicate IPs run on the same day).

*mild stripping buffer is great for removing secondary but may not remove all primary antibody; you would want to check post-strip by incubating with anti-rabbit secondary antibody again (and hope it's blank) before incubating with the other primary Ab.

Reviewer 2

Advance summary and potential significance to field

In this manuscript, the authors have explored the phenotype of Mfn1 and Mfn2 single and double conditional KO mice. All 3 deletion combinations resulted in spermatogenesis defects, albeit to different levels of severity. It has been known for decades that spermatocytes and spermatids rely exclusively on OXPHOS (and thus, mitochondria) for ATP production, and thus providing a better understanding of mitochondrial function in these cells would be quite interesting. However, another group just published a highly similar paper (Chen et al., 2020), so this manuscript will need to be revised considerably to account for that.

Comments for the author

Overall, this is a well-written manuscript (with edits needed for Chinese-English grammar differences). However, this reviewer feels the authors tried to tackle too much, and didn't do an adequate job of convincingly demonstrating the mechanism of action in terms of translation. The authors should think about how to make a more focused story with more convincing evidence. Perhaps reporting the phenotype clearly in one manuscript, with a thorough analysis of translation in another? And mitochondria physiology in another?

Just this month, a new manuscript was published in Stem Cell Reports by Yuan Wang's group that reports a very similar story. In that manuscript, they used Ddx4-Cre (acts in fetal prospermatogonia) to inactivate Mfn1 and Mfn2, while the authors here use Stra8-Cre, which acts later in postnatal spermatogonia. Therefore, this reviewer suggests the authors focus their study on spermatocytes and spermatids and highlight differences from that study.

Suggestions for improvement, roughly in order of appearance:

Overall

- Please use continuous line numbering, restarting every page is not helpful to the reviewers!
- Fig. 1: The seemingly unambiguous identification of stages (especially I-VI) on these tissues is suspect, without an acrosome marker – how
- when IPs and polysome gradients are done on whole testis, the authors seem to conclude that whatever they find is going on in germ cells.. this i
- They mention mitochondria physiology in the manuscript, but really don't examine it.
- translational mechanisms not convincing Introduction
- The authors, throughout the manuscript, use sentences containing "possibly...could", "might...suggest", "and/or" - make a stand based on your data, don't leave so much room for interpretation to your reader
- when IPs and polysome gradients are done on whole testis, the authors seem to conclude that whatever they find is going on in germ cells.. this is a dangerous assumption, as MFN1/2 are also in somatic cells, and things they stick to in a testis lysate may not represent physiologic relationships in germ cells - would suggest doing experiments using isolated germ cells
- They mention mitochondria physiology in the manuscript, but really don't examine it.
- translational mechanisms not convincing

Introduction

- P. 3: fusion and fission only in mammals? Please specify; mitochondria also critical for redox homeostasis; 6-7: aren't mito functions critical in ALL cells?; 8-13 are MAMs only in germ cells? If not, reword; 13 "increasingly was reported"?; 16 what part of testes, presumably germ cells? If so, what kind? Specify; 19 homolog to ortholog
- P. 4: 11-13 what is the relationship of these to the term "nuage"? Nuage is French for "cloud", and it was this reviewer's understanding that it referred to these structures in early germ cells (e.g. gonocytes) and not to the CB… please clarify; 22 "besides"… besides what?; 23 what are "transcriptional secessions" – is this cessation? And what about meiotic sex chromosome inactivation (MSCI), this is another very specific example affecting the X+Y chromosomes – please add; 24-5 histones are replaced by transition proteins, not protamines; 26-7 the authors seem to suggest these transcriptional and translational events are coincident and linked… is this true? What is the relationship?
- P. 5: 3-5 incomplete sentence; 5-7 what evidence supports this? Please provide; 8-9 actually, Norman Hecht's group taught us a lot about the mechanisms; 12 germ cells, or testes? 14 – what is meant by "the translational machinery"? Specify; 15 "the male germ cells" – which ones? Specify, reference; 16-7 is it "mitochondrial formation" that's affected? 18 "interoperates"? 19 testes or germ cells, please specify; 21 "phenotypes""? 24 what are "gamete mRNAs", and what are "gamete-specific mRNA fates"?
- P. 6: "storage/translational delay" – should introduce these concepts, would recommend adding a section in the introduction

Results

- P. 5: 11 "preferentially"?; 15 would not say the testes "mainly consist of spermatocytes", but that pach spermatocytes are the most advanced germ cell type – would guess germ cells may still be slightly outnumbered by somatic cells at this point; 17 there are no spermatogonia in the P0 testis, only prospermatogonia/gonocytes; 19 there aren't "late pachytene spermatocytes" at P14, they're just appearing; 17-19 also appear to be in Sertoli and PTMs at several ages, seem to be higher in spermatocytes than spermatogonia;would recommend co-immunostaining with a germ cell marker (e.g. TRA98) to ID germ cells.
- P. 6: 27 would say all pachs, not just late; and appears to be much lower in round spermatids than pachs; How are germ cell types identified so seemingly unambiguously? How were

stages determined – for example, how do the authors know that first image is IV-VI and not I-III – it's tough to tell without visualizing the acrosomes somehow… Also, the stage "IX-X" shown is definitely a IX, not a X;
- P. 7: 8 spermatocytes are not premeiotic; the magnification/resolution not high enough to conclude cytoplasmic granule staining (Fig. 1C); 13-14 – it is this reviewer's understanding the Stra8-cre is actually expressed earlier as well, in undifferentiated progenitors; 22, 28 – "complete sterility" is redundant, as is "severely atrophic abnormal";
- P. 8: 12-14 – your data does more than "suggest" this, it shows/indicates; 20-21 – not measuring "effects", just interaction; however, these IP studies don't assess germ cell interactions, which seem to be key to this study, but just that they interact in testis lysates
- P. 9: 8-9 – what does this sentence mean?
- P10: 5-7 – this is where "indicate" should be replaced with "suggest", as this is a speculation; 12 – "fail to full development"?; 17 – appeared to increase, or did increase? Can this be quantified?;
- P11: 4 – "by about" – be specific; 27 – did it appear to increase, or did it increase?
- P12: 1 – "imply a possible" is redundant; 3 – "physiological…evidence" – what evidence? 4 – "and/or" – do the authors want the readers to choose one?; 10 – "appeared" – again, was it or wasn't it?;20-21 – this is not a hypothesis "assumed…maybe"?;
- P13: 7 – "suggesting…may" not necessary together;
- P14: 4-5 "suggesting…might" and "tried to ask"? 14 – and/or – again, don't leave this for the reader to decide; 20-1 – this is not a fair premise – measuring the steady-state mRNA levels does not enable "understand(ing) the molecular mechanisms"; does up-regulation or downregulation presume changes in transcription, or mRNA stability? One important function ascribed to Nuage is RNA decay – an example is provided by Yumiko Saga's work on NANOS2 and its association in nuage with the CCR4-NOT deadenylase complex… were mRNAs that increased in abundance also show up in her dataset? 11 – what is "biological quality and localization"?? 16 – "genes were distributed in cell and intracellular part"- huh?! What meaning do these broad and seemingly meaningless titles provide to the reader? 18 – what is a "rich factor"? Would recommend removing this entire paragraph, as it adds little useful meaning to the paper
- P16: validation only of steady-state mRNA levels, not gene up or downregulation… would carefully rephrase; 8-11 – no, validation doesn't support that view, it just validates the fold-changes estimated by RNA-seq; 16 – what are "inactive translational mRNAs"?; 18-20 these are called polysome gradients; if these gradients were done on whole testis, how can conclusions be made for germ cells? 23 "suggest…could" – this is the results section, make a stand one way or the other;
- P17: 1-2 - is this sufficient data to make this overarching conclusion? 8 – could, or did? 12 – do the authors mean MSY2 instead of MFN2?
- How does MFN2 seemingly interact with everything you test? Are there proteins it does not interact with? 22-3 – but the processes indicated from RNA-seq GO analysis were broad and not germ cell specific, right? This reviewer is confused by this sentence

Mitochondria are a 3-D structures that are constantly undergoing fusion and fission - how is this altered in these mice?

Fig. 1B – step 7-8 are not elongating, they are round; step 14-16 are not elongating, they are condensing; Fig. 1C – no context is provided for these images – where is the basal lamina, what are they intending to show? This reviewer can see the PTMs and the spermatids and knows the polarity, but for a broader readership these need to be indicated;

Reviewer 3

Advance summary and potential significance to field

This manuscript describes (1) spermatogenesis failures and mitochodrial defects in Mfn1, Mfn2 and both Mfn1/2 conditional KO testes (stra8-cre), (2) interaction between MFN proteins and nuage proteins, and (3) a possible involvement of MFN2 in translational regulation during spermatogenesis.

About (1), Mfn1/2 conditional KO phenotypes during spermatogenesis have already been reported in detail in (Varuzhanyan et al., eLIFE, 2019). However and surprisingly, the authors of this

manuscript mentioned (Varuzhanyan et al, eLIFE, 2019) only once in the introduction section very briefly.

About (2), both physical and functional interaction between Mfn genes/proteins and GASZ, a nuage component, has already been reported in (Zhang et al. in EMBO reports, 2016). However, this important previous finding (i.e. MFN and GASZ interaction) is again only very briefly touched in this manuscript.

Novel conceptual findings described in this manuscript are mainly about (3). MFN2 is enriched in polysome fractions and it interacts with several translational regulators (MSY2 and eEF1As). This part is potentially intriguing, with regard to a possible involvement of a mitochondrial surface protein in mRNA translational regulation. However, there are no data or discussion about this important point. In all, in this reviewer's feel, this manuscript is still premature for publication in Development.

Comments for the author

Specific points

- The authors should properly cite other researchers' previous reports throughout this manuscript.

- page 10, line 17 : If the authors want to describe this finding ("the thickness of IMC appeared to increase"), actual data and statistical analyses are necessary.

- page 10, line 20 : lengths and ratios are different.

- page 11, line 3 : It is unclear how to define the distance between mitochondria and ER.

- page11 line 26 : Is it necessary to carry out RT for mtDNA PCR?

- page 16 line8 : About "Taken together, these RNA-Seq ... act in an integral manner," it is very difficult to understand how the authors draw this conclusion from the complex lists of many GO terms of various categories.

- p28 line 21 : Some of the dataset presented in this manuscript should be analyzed by non-parametric statistical methods.

- fig3d : it appears that the authors defined arbitrary bins for this histogram and then found a statistically significant difference in a certain bin (1.2-1.5). If this is not the case, the rationale for defining these uneven/specific bins needs to be explained.

- fig3j : how many replicates were examined for these PCR experiments?

- fig3k : it is difficult to see quantitative differences in staining intensities between the samples from these images.

- fig4fg : control IgGs must be used for IP.

- fig5ab: it is impossible to see "the purity and morphology of isolated PS and RS" from these images (left top).

- fig5gh : again, there are no indication about how many replicates were examined for these PCRs and stats for asterisks.

- fig6d: the MFN2 band in MSY2 IP is very weak. Also, the ratio between input and IP samples used should be indicated.

- fig6k : the MFN2 band in the eEF1a2 lane is clearly shifted. If this is MFN2, the reason for the shift needs to be explained.

- fig6f : this heatmap looks very uncommon. How were the data values scaled before making the heatmap (Z-score or other)?

- There are no fig1, fig2 ... labels on figures.

Resubmission

First Submission

Author response to reviewers' comments

Reviewer #1 Advance Summary and Potential Significance to Field:

In this manuscript, Wang and colleagues begin by showing the expression pattern of MFN2 in the mouse germline, and then go on to characterize a conditional knockout of Mfn2 (and at times, Mfn1 and the double cKO). They show that Mfn2-cKO mice have reduced testis weight (this effect is earlier in Mfn1 and in the double mutant) and disrupted subcellular structures, including mitochondria, mitochondrial-associated ER membranes (MAMs), and contacts between mitochondria and ER. The authors also show that MFN2 co- immunoprecipitates with many of the proteins known to localize to the nuage in germ cells, including MIWI and DDX4. Several of these proteins show marked decrease in expression or mislocalization in the Mfn2 cKO.
The authors show that – consistent with disruptions in the nuage – the overall levels of pachytene piRNAs are reduced by 50% in the Mfn2 cKO, although transposon activity appears unchanged. RNA-seq data from purified pachytene spermatocytes and round spermatids is presented, and MFN2 is shown to associate with actively-translated (polysomal) fractions. Finally, there are some nice data showing that SPATA19 is expressed 3 days early in the Mfn2 cKO.

The authors have performed a substantial amount of work to characterize a complex phenotype, and they have some very intriguing data. The microscopy (both EM and fluorescence) is fantastic. The 'bones' of this paper are great, and already represent a significant advancement; if the authors can focus on the strengths of the paper and either solidify or discard the less convincing parts, it has the capacity to be truly excellent.

I do feel that the authors are over-interpreting their data in places (details in "major comments" section). They can either remove those bits, or re-write those sections so that the text is appropriately cautious, or they can do more analysis, or (possibly) 1-2 additional experiments. I tried not to ask for anything that seemed outrageous or beyond their capabilities. There are also places where promising leads almost seem under-pursued (details in "major comments," again) and might benefit from more computational analysis of existing data sets.

Quick note of explanation: I answered "no" on question #3 ("Do the title and abstract accurately represent the findings of the study?") because the title (and to a lesser extent, the abstract) draw attention to what I feel is shakiest part of the paper (the eEF1A part): "… and involves mRNA translational machinery…" That should be easy to fix.

Reply: We thank this Reviewer for his/her scrutiny of our work and the significance of our work, and we appreciate that this Reviewer has raised several critical points, which enabled us to improve the manuscript and highlight the importance of our study. At the same time, we apologize that several important aspects were not explicit in our previous version of this study. As we point-by- point response below, we have extensively revised the Title, Abstract, Introduction, Results, and Discussion sections following this Reviewer's suggestions.

Reviewer #1 Comments for the Author:
Major comments:
Nuage:

- The Mfn2 cKO clearly disrupts structures and protein localization in the nuage. The closest the

authors get to showing a functional consequence of this is the panel in the SOM showing that total pachytene piRNAs are reduced by 50%. That's an important result!! I would move this to one of the main figures. Just because transposon activity doesn't seem to be affected, it doesn't mean that the 50% drop in piRNAs isn't having a very real effect on germ cell development, particularly on clearing meiotic transcripts (by cleavage) once their usefulness is over.

two relevant papers (this reviewer is not affiliated with either lab) -Goh, WS…. GJ Hannon. (2015) Genes & Dev. piRNA-directed cleavage of meiotic transcripts regulates spermatogenesis.
Larriba & Del Mazo (2018) Sci Rep. An integrative piRNA analysis of mouse gametes and zygotes reveals new potential origins and gene regulatory roles.

Reply: This is a great suggestion. We moved the piRNA reduction results to the new **Figure.4I** in the revision, as suggested. We also cited the above two critical papers into the discussion section to interpret the importance of the finding of reduced piRNAs in *Mfn2*-cKO mice (see the discussion part).

- An analogy for what seems to be happening to the nuage: a tornado flattens a barn so that the only thing left is the foundation and maybe a couple of outside stone walls. Most of the horses won't recognize what's left as their barn (MIWI, DDX4, GASZ), but maybe one will recognize the foundation (TDRKH) and go back to where its stall once was. It seems from the literature as if TDRKH is instrumental at binding to mitochondria and recruiting MIWI; this could explain why TDRKH can still find the mitochondria in the Mfn2 cKO and why at least some (reduced) MIWI is still showing up (Figure 4). A very relevant paper (again, this reviewer has no affiliation):
Ding, D…. C Chen. (2019) Nuc Acids Res. Mitochondrial membrane-based initial separation of MIWI and MILI functions during pachytene piRNA biogenesis.
It would be nice if you could mention in the discussion how the Mfn2 cKO compares to the TDRKH lof.

Reply: Thank you for making such a vivid and appropriate analogy. Our data showed that both MIWI and DDX4 are absent in the CB of *Mfn2*-cKO round spermatids, resembling MIWI absence in the CB of *Tdrkh*-cKO (Stra8-Cre induced) spermatids (Ding et al., 2019). Given the feature that TDRKH is a mitochondrial membrane-anchored protein, and MFN2 is a mitochondrial fusion protein (Hales and Fuller, 1997; Saxe et al., 2013), it is raising a possibility that the recruitment function of TDRKH requires the coordinated regulation of MFN2. We mentioned and discussed this comparison of *Mfn2*-cKO and *Tdrkh*-cKO in the discussion section (see lines 653-666).

RNA-seq data:
The RNA-seq data is nice, but the extensive GO term analysis feels like an enormous distraction / tangent. I would take out the GO term panels completely (not even put them in SOM!) and move S7A and S7B (the scatter plots of the RNA-seq data, in spermatocytes and spermatids) to the beginning of Figure 5. Those panels should be easy for the reader to find.

Reply: Done as suggested.

I would use the extra space in Figure 5 to plot data from existing (published) RNA-seq data sets from any available KO or cKO of any of your major nuage proteins (ideally MIWI, but whatever you can find). For example: the RNAs that have been shown to be upregulated at least 2-fold in mutant (vs. wt) – where do they map on your scatter plots? Are they also more likely to be upregulated? (also plot a control set of RNAs that is not affected in the mutant).

Reply: This is great point to identify the possible relationship of MFN2 and Nuage proteins (like MIWI) in regulating meiotic transcripts during spermatogenesis. Based on the reviewer's suggestion, we conducted a bioinformatics analysis to compare whether the differentially expressed genes identified from our RNA-seq data (*Mfn2*-cKO) were related to the differentially expressed genes from the published RNA-seq data (we chose *Miwi*-KO)(GEO: GSE64138) (Goh et al., 2015). The results showed that 74 genes were up- regulated in both *Mfn2*-cKO and *Miwi*-KO pachytene spermatocytes (**new Figure.5E-G**), and 152 genes were up-regulated in both *Mfn2*-cKO and *Miwi*-KO round spermatids (**new Figure.5H-J**), indicating that ~12% and ~8% up- regulated genes in *Miwi*-KO pachytene spermatocytes and round spermatids, respectively, are likely to be up-regulated in that

of *Mfn2*-cKO. Interestingly, GO term analyses showed that the commonly up-regulated genes in *Mfn2*-cKO and *Miwi*-KO mainly enriched in cytosolic ribosome cellular component, rRNA processing, RNA binding and translation factor activity, etc. (**new Figure. 5K-L**).
In addition, 304 and 106 genes were found to be commonly down-regulated in *Mfn2*-cKO and *Miwi*-KO pachytene spermatids (**new Figure.S6E-G**) and round spermatids respectively (**new Figure.S6H-J**), which account for ~20% and ~6% of down-regulated genes in *Miwi*-KO pachytene spermatocytes and round spermatids, respectively. Importantly, the down-regulated genes are mostly related to germ cell development, spermatogenesis, and meiotic division (new **Figure. S6K-L**).

Given our data showing MFN2 interact with Nuage proteins (like MIWI) (**Figure. 4**) and the functions of MIWI reported in meiotic mRNA cleavage, the new bioinformatics data suggested that MFN2 and MIWI probably share some common pathways or cooperate in such a particular way to regulate meiotic mRNA in spermatogenesis. The new data also suggested that MFN2 could cooperate with MIWI (a Nuage protein) to control mRNA fates during spermatogenesis.

Another way of looking at this: the RNAs that are upregulated in the Mfn2 cKO– could some of these be due to meiotic RNAs not getting cleared because pachytene piRNAs are down 50%? If they are, maybe there will be overlap with things upregulated in a MIWI lof.

Reply: Indeed, there are overlapped up-regulated genes between *Mfn2*-cKO and *Miwi*-KO pachytene spermatocytes and round spermatids as this reviewer's expect. Importantly, to address the possible effects of decreased piRNA in *Mfn2*- cKO, we compared the up-regulated genes identified from *Mfn2*-cKO pachytene spermatocytes and round spermatids with the 330 putative piRNA targeting mRNAs reported by Goh et al. (Goh et al., 2015). We found that ~10% (34/330 in pachytene spermatocytes and 32/330 in round spermatids) piRNA targeting mRNAs up-regulated in *Mfn2*-cKO spermatogenic cells (**new Figure.5C-D**). In comparison, ~20% (72/330) piRNA targeting mRNAs were reported to upregulated in *Miwi*-KO round spermatids(Goh et al., 2015). Therefore, there are ~50% overlapped up-regulated putative piRNA targeting mRNAs between in *Mfn2*-cKO and *Miwi*-KO spermatogenic cells, suggesting the 50% decrease of piRNA observed in *Mfn2*-cKO could influence the expression of piRNA targeting mRNAs.

I would like to see more of a nuanced discussion / acknowledgement of the limits of RNA-seq for investigating the function of a protein that is not a transcription factor, a chromatin regulator, or a predicted RNA-binding protein. I'm worried that most of the effects you see on the transcriptome in the cKO are indirect. Your two RNA-seq data sets will be most useful if you can show convincingly that mRNAs dramatically affected by MIWI (or its nuage compatriots) or MSY2 are statistically more likely to be mis-regulated in the Mfn2 cKO (ideally with no such enrichment for non-target mRNAs). Otherwise it's really hard to make sense of any of the changes in transcript levels.

Reply: This is a good point. Following this reviewer's suggestion, we reanalyzed our RNA-data and compared them with the piRNA targeting mRNAs and MSY2- nontarget mRNAs. Our new results showed ~50% piRNA targeting mRNAs commonly upregulated in *Mfn2*-cKO and *Miwi*-KO, and ~ 46% MSY2-nontarget mRNAs are misregulated in *Mfn2*-cKO. In other words, ~50% of the effects we observed on the transcript in *Mfn2*-cKO are directly caused by MFN2 depletion. However, we have made a nuanced discussion of the limits of RNA-seq data in this revised manuscript, as suggested (see lines 686-706).

Non-nuage part of the cytoplasm:
The possible connection to MSY2/YBX2 in the non-nuage part of the cytoplasm is interesting. I have some questions, though. I think this part of the paper needs a few more elements before it is convincing:

- Is the interaction between MSY2 and MFN2 RNA-independent? An RNA- independent interaction here would strengthen your argument that these two proteins might work together.

Reply: Based on the new results obtained from the RNase A treatment experiments, the interaction between MFN2 and MSY2 does not require RNA because digestion of testicular extracts with RNase A did not affect the ability of MFN2 to co-immunoprecipitate MSY2. This data suggesting the interaction of MFN2 and MSY2 is an RNA-independent manner, and MFN2 is direct interacts with MSY2, and we showed the data in the new **Figure. 6C**.

• Can you use ribosome profiling to show that MSY2 target RNAs (but not non- target RNAs) shift to the polysomal fraction in the Mfn2 cKO? Particularly at P25, where you're already noticing early expression of SPATA19 protein. It could provide compelling evidence that MFN2 has a global effect on translation (and who knows, you might find out that the RNAs that shift to the polysome in the cKO are independent of MSY2 – that would be an interesting result as well!). I know it can be a time-consuming experiment, but it also looks from the data included here (polysome fractionation and RNA-seq in separate figures) that you have the technical expertise to do it.

Reply: As suggested, we redid the polysome profiling assays using WT and *Mfn2*-cKO testes at P25. We found the MSY2 target mRNAs (*Spata19*) indeed exhibited a distribution shift toward heavier polysome fractions in *Mfn2*-cKO testes (new **Figure.6I-J**). This new data revealed an increased translation activity of MSY2-target mRNA in the cytoplasm of *Mfn2*-cKO testicular cells.

Given that MSY2 is known for repressing translation of its target RNAs and for protecting them from translation-dependent decay, I am puzzled by the fact that the MSY2 target RNAs that qualify as misregulated in your RNA-seq data set mostly appear to be expressed more *highly* in the Mfn2 cKO. I worry that this could be an indirect effect due to higher expression of MSY2 itself (in your own data) in both spermatocytes and round spermatids (a situation where MSY2 is over-active at repression and protecting RNAs from decay). I can't fit this together with the premature expression of SPATA19 protein (presumably due to premature translation), which would be consistent with a reduction in function of MSY2 (not an increase in function). Hmm.* (this contradiction is something you might want to acknowledge in your discussion). On a related note to the MSY2 / RNA-seq analysis: if you're going to talk about the MSY2 target genes that are also upregulated in your RNA-seq data set, you should map all of the MSY2 target genes (and, in a different color, non-target genes) onto your RNA-seq scatter plots (currently S7A and B). (Targets/non-targets provided in Yang et al 2005a). Otherwise it looks like you are cherry-picking the data.

Reply: We appreciate this reviewer's comments to improve our manuscript quality again. As suggested, we map all of the MSY2 related genes (both MSY2- bound and non-bound genes) onto our RNA-seq data. The results showed that, for MSY2-bound gamete-specific mRNAs, of which ~57% (26/46) is upregulated in *Mfn2*-cKO round spermatids, including *Msy2* itself, ~9% (4/46) down-regulated and the remaining 34% (16/46) mRNAs are unaffected (**Figure.6E** and **Table S6**). In contrast, for MSY2-nonbound mRNAs in *Mfn2*-cKO round spermatids, most of them are down-regulated (33%, 16/48) and unaffected (54%, 26/48), only 12% MSY2-nobound mRNA is up-regulated (6/48) (**Figure.6F** and **Table S7**). The situation in *Mfn2*-cKO pachytene spermatocytes is almost the same as in round spermatids (**Table S8-9**).
Therefore, most MSY2-bound mRNAs upregulated in *Mfn2*-cKO germ cells, which might be explained by the upregulation of *Msy2*. Since MSY2 is known to be able to link transcription and mRNA storage/stabilization in male germ cells, its function might be disturbed by the deletion of its interaction protein MFN2. However, more research is needed to elucidate the mechanism due to the complexities of expression of MSY2 binding/nonbinding mRNAs as germ cell differentiation. We also discussed this possibility of MSY2 effects in *Mfn2*-cKO in the revision (see lines 686-706).

*the only explanation I can come up with, and maybe it doesn't even work: somehow the translational-repression function and the RNA-stabilizing function of MSY2 are getting separated in your cKO. There is extra MSY2, happily stabilizing RNAs, but somehow the loss of MFN2 means they are getting translated actively. Just not decayed. This argument would work on me, I think, but I would still want to see ribosome profiling data and ideally an RNA independent interaction between MSY2 and MFN2.

Reply: We agreed with this reviewer's explanation and also added this explanation in the discussion part of the revision (see line 694-706).

Strengthen or cut from paper:
The bit at the end regarding eEF1A1 and 2 makes me nervous. These proteins have been shown to be involved in processes independent of translation – including as chaperones (see 2015 review by Abbas, Kumar, and Herbein), and it could be they are appearing at higher levels in the Mfn2 cKO just because the cells are starting to feel really stressed out / disrupted. I would be more

convinced of a direct link if you could show that MFN2 bound eEF1A1/2 in an RNA-independent manner and actively blocked function in an in vitro assay.
As it stands right now, it's not convincing, and I think you have far more promising avenues to pursue.

Reply: Thank you for pointing out this issue. We removed this part of the data (eEF1A1/2) from the revision to avoid the misunderstanding for the readers.

minor comments:

P. 7 --
"While Mfn2-cKO mice were viable and appeared to be grossly normal, they displayed complete sterility after a 5 month-period fecundity test."
This could be explained a little better for readers who do not work with mice (5 months of age? 5 months with females?). Also, consider saying "0 out of n mice were fertile" just to get a # in there.

Reply: Done as suggested.

Fig 2G it might be helpful to use arrows / arrowheads to point out individual late- stage spermatocytes and spermatids that are showing signs of vacuole-ating.

Reply: Done as suggested.

P 8, lines 12-14
"These data suggest that, upon Mfn2 deletion in postnatal testes, germ cells gradually lost from P35 by apoptosis, leading to infertility."
Replace with:
"These data suggest that, upon Mfn2 deletion in postnatal testes, most germ cells are gradually lost from P35 by apoptosis, and the remainder are morphologically defective."

Reply: Done as suggested. Thank you.

P 9 lines 8-9
"In comparison, the first wave of spermatogenesis in Mfn2-cKO testes proceeds until P42."
Replace with
"In comparison, the first wave of spermatogenesis in Mfn2-cKO testes proceeds normally until P42."
(if I got your meaning right)

Reply: Done as suggested. Thank you for your careful review.

Fig S3A
Left panel: as laid out, it implies that top and bottom were run in the same lanes.
Clearly they're not!
Redesign for more transparency… Right panel: some background binding to the IgG. :-/ See end of review for technical suggestions re: IPs.

Reply: Yes, these two WB blots were not run in the same lanes. We redid the IPs of MFN1 and MFN2 again following this reviewer's technical advice, and now the results are much better than before. Thank you so much for your constructive help.

top of page 10
you very nicely show the effect on mitochondrial distribution in Fig 2M, so point the reader to that (in addition to pointing them to the SOM). (right now you mention 2M only in the context of "we don't see this in the single mutants")
The disrupted distribution of mitochondria in the double cKO is very interesting! I wonder why that is happening.

Reply: We have now corrected the improper reference to **Figure 2M** (in line 280). The observation of the abnormal distribution of mitochondria in spermatocytes of MFN1/2 double knockout mice is indeed exciting, which may further indicate that MFN1 and MFN2 have a complex coordinating role

in the regulation of the mitochondria distribution. However, how MFN1/2 regulates the localization and distribution of mitochondria in spermatocytes remains to be further studied. This result stimulates our enthusiasm for exploring the functions of MFN1/2 in regulating the mitochondrial distribution in the future.

Figure 3 –

- 3B supposedly has ER labeled, but I can't see it (and thus can't see the increased mito-ER distance).

Reply: We have now labeled the ER using green color in the revision.

- 3A&B – labels could be more visible (yellow? Maybe bigger? The images are great, and you want a reader who is printing out the paper to be able to see the labels)

Reply: We relabeled all ultrastructure, including ER, IMC, MAM, and mitochondria. ER labeled as green, IMC labeled as blue, MAM labeled as red, and mitochondria labeled as cyan.

- 3D – label for y axis is uninformative … "% of mitochondria in given AR range"?

Reply: We changed the label of the Y-axis to "% of mitochondria in given AR range," as suggested.

- H – it's hard for me to see the tubular organization in the control. I think if you showed the two images to a random person (and didn't tell them it was an ER marker), they might say that the protein localization in the mutant was clumpy or mis-aggregating compared to wt. It could be that two-dimensional immunostaining images don't show off the normal tubular structure very well.

Reply: To better show the tubular structure, we circled the seminiferous tubule with dotted lines in the control and mutant group.

P 10 lines 25-27
Not sure you can conclude this! I would re-word to make it more cautious… (or just take that sentence out)
"These data indicate that developmental defects of male germ cells in both Mfn1- and Mfn2-cKO mice could be due to the fragmentation and swelling of mitochondria in germ cells."

Reply: Done as suggested. Thank you for your reword.

P 11, line 28
Re-word, maybe… I'd save "inconsistent" for situations where your data are in direct conflict with a previous paper. Instead, say "in contrast to previous reports…" since you are looking at different tissue type. And maybe replace "The discrepancy" with "This difference" on line 30.
("discrepancy" means that one of these dissimilar results must be wrong… and as you point out, it could simply be the difference between germline and soma). [I have no horse in this race – I just want everyone to get along:)]

Reply: Done as suggested. Thank you again for your careful review.

Figure 4

- F & G are missing the negative controls (samples without any Myc-tagged protein [or with a Myc-tagged random non-binding protein] but still containing Flag-MFN2)

Reply: We performed the IP assays again and added IgG as negative controls in **Figure.4F-G**. We also conducted additional IP assays using Myc-tagged hnRNPA2, an MFN2 non-binding protein, as negative controls following the reviewer's suggestion. The data were added to the **Figure. S5C**.

P 13
lines 11-12: "Co-immunostaining of Mfn2-cKO and control testes revealed ectopic expression of MIWI, DDX4, and GASZ in Mfn2-cKO testes…" This could be a wording issue, but I don't see ectopic expression (Fig 4H) – if anything, I see reduced expression of these proteins. The figure legend

(title) for S5 also uses the word “ectopic”…

Reply: We changed the word “ectopic” as “reduced”.

Figure 4H: here’s what my eyes tell me –MIWI is reduced in the cKO DDX4 may be reduced in the cKO but it’s hard to tell, because it doesn’t seem like the ATP5A staining worked as well as it does in other panels. If you have an image for DDX4 in the cKO where the ATP5A staining looks normal, I would use that.

GASZ looks very reduced
TDRKH looks about the same as in wt, or slightly reduced

Reply: Done as suggested. We now replaced the image for DDX4 staining with a new one that DDX4 in the cKO where the ATP5A staining looks normal.

Line 16-19
“Of note, unlike the perinuclear granular mitochondrial localization showed in the control spermatocytes, DDX4 displayed a diffuse cytoplasmic expression pattern in Mfn2-cKO spermatocytes (Figure.S5D)” I have a hard time seeing this difference in localization in your images. It’s subtle.

Reply: We deleted this sentence to avoid confusion for the readers.

Figure S6A – more of a science question than a critique – do you know whether there is also a 50% reduction in the piRNA precursors? Or are the precursors normal but not getting processed as efficiently as usual? I think this could be really interesting!

Reply: This is very good scientific question. To determine whether the abundance of piRNA precursors is affected in *Mfn2*-cKO, we randomly chose 10 piRNA precursors from 214 piRNA clusters(Zhou et al., 2017) to analyze. The data showed that piRNA precursor levels in *Mfn2*-cKO testes at P25 were not different from the wild type, suggesting the precursors are normal but not processed as efficiently as usual in MFN2 deficient testes. We added this part of the data into **Figure.4J** in the revision.

P 16, lines 22-24:
this sentence seems unnecessary, given that the polysome profiling data alone show this:
“Combined with the RNA-Seq data, these results suggest that MFN2 could associate with cytoplasmic monosomes and polysomes.”

Reply: We changed this sentence to “These data suggest that MFN2 associate with cytoplasmic monosomes and polysomes”.

P 18
“These data show that Spata19 was early activated and translated when a loss of MFN2 in male germ cells, suggesting MFN2 could regulate mRNA translation.”
Replace with
“These data show that Spata19 is activated early and translated upon loss of MFN2 in male germ cells, suggesting MFN2 normally represses its translation.”

Reply: Done as suggested.

Minor writing / style comments

P 4 line 23 and line 26 : replace “secession” with “cessation” (they sound similar but mean very different things)
P 5 line 21: replace “Inspiringly,” with “Intriguingly,” P 8 line 4: no discernible abnormality was found
P 8 line 25, replace “Besides,” with “In contrast,” P 10 line 12 “to fully develop” or “to develop fully” P 17 lines 3-6. This sentence needs a verb.

Reply: The above typos are all corrected. Thank you very much for pointing out them.

Technical advice for getting all of your IPs to a state where they're publication- quality:

- separate the elution step from the boiling step. The streaky high-background you see in a couple of your IP lanes is likely due to a few beads (or bead material) getting loaded in well of the protein gel (we've had that problem before). Instead, elute in 40ul elution buffer (1% SDS, 10mM EDTA, 50mM Tris pH 8.0, 1x protease inhibitor) 30min at 70°C (vortex every 5-10min), transfer eluate to a fresh tube, then add 10ul 5xSDS loading buffer and boil 10min. It should look a lot cleaner on the western blot.
- to reduce the background binding of Mfn2(?) to the IgG control, consider blocking your beads (for all conditions) with BSA (10% for an hour is good). You may already be doing this; I just don't see it mentioned in the methods.
- I realize that you have an issue staining for the two Mfns when they're in the same lane because they're so similar in size (and both antibodies are rabbit). If you can't run them far enough to separate them, or have trouble stripping the blot* between antibodies, the next best thing is to load: input, IP, IgG, [empty lane], input, IP, IgG. Slice the membrane and treat with the two antibodies but make it clear from the figure layout that these are all separate lanes (and duplicate IPs run on the same day).

*mild stripping buffer is great for removing secondary but may not remove all primary antibody; you would want to check post-strip by incubating with anti- rabbit secondary antibody again (and hope it's blank) before incubating with the other primary Ab.

Reply: We are very grateful to this reviewer for his/her valuable technical advice to help us improve the quality of our IPs results. We also included this advice into the revised IP methods.

Reviewer #2 Advance Summary and Potential Significance to Field:
In this manuscript, the authors have explored the phenotype of Mfn1 and Mfn2 single and double conditional KO mice. All 3 deletion combinations resulted in spermatogenesis defects, albeit to different levels of severity. It has been known for decades that spermatocytes and spermatids rely exclusively on OXPHOS (and thus, mitochondria) for ATP production, and thus providing a better understanding of mitochondrial function in these cells would be quite interesting. However, another group just published a highly similar paper (Chen et al., 2020), so this manuscript will need to be revised considerably to account for that.

Reply: Thank you so much for the evaluation of our work. Understanding the function of mitochondria and its interaction with other organelles in male germ cell development is indeed the fundamental pursuit in the reproductive biology field. We have been studying the role of MFN1/2 in male germ cell development for about 4 years, and we do not know that other research groups are doing similar work until they published the papers(Varuzhanyan et al., 2019; Chen et al., 2020). However, the overall conclusion and underlying mechanism identified are different between our current study and the published papers. Moreover, the published papers are mainly focused on the spermatogonial differentiation and meiotic processes, while our research concentrated on late spermatogenesis and the function of Nuage structure. Thus, we believe that our study provides a new conceptual framework for mitochondrial fusion proteins (MFN1/2) regulation in the male germline, which differs from the published work and worthy of publishing. We highlighted these differences in the revised manuscript according to this reviewer's suggestion (see lines 557-601).

Reviewer #2 Comments for the Author:
Overall, this is a well-written manuscript (with edits needed for Chinese-English grammar differences). However, this reviewer feels the authors tried to tackle too much, and didn't do an adequate job of convincingly demonstrating the mechanism of action in terms of translation. The authors should think about how to make a more focused story with more convincing evidence. Perhaps reporting the phenotype clearly in one manuscript, with a thorough analysis of translation in another? And mitochondria physiology in another?

Reply: This is a good point. Our original idea is a plan to report the phenotype of *Mfn1/2*-cKO mice and the mitochondrial physiological function during spermatogenesis first, and to publish the underlying mechanism of MFN1/2 to regulate male germ cell development separately. However, when we were preparing the manuscript, we suddenly noticed that a research group published an article about Mitofusions regulating the mitochondrial biological function of spermatogonia and

meiosis(Varuzhanyan et al., 2019). Therefore, we decided to combine the phenotypic study and mechanism study of the loss function of MFN1/2 in male germ cells for publication. To make a more focused story with more convincing evidence for the readers, we carefully revised our manuscript, including Introduction, Results, and Discussion sections. We also reanalyzed the RNA-seq data and ribosome profiling to mainly focus on the mechanism action in Nuage-associated protein in piRNAs regulation and MSY2 mediated translational processes.

Just this month, a new manuscript was published in Stem Cell Reports by Yuan Wang's group that reports a very similar story. In that manuscript, they used Ddx4-Cre (acts in fetal prospermatogonia) to inactivate Mfn1 and Mfn2, while the authors here use Stra8-Cre, which acts later in postnatal spermatogonia. Therefore, this reviewer suggests the authors focus their study on spermatocytes and spermatids and highlight differences from that study.

Reply: Dr. Yuan Wang's article was published online when our manuscript was being under review in DEVELOPMENT. Although her group did similar work, the CRE mice used by our two groups were different, as this reviewer said, and our conclusions were also different. We highlighted the differences in our study from Dr. Wang's research in our revised manuscript, as suggested by this reviewer (see lines 569-596).

Suggestions for improvement, roughly in order of appearance: Overall
-Please use continuous line numbering, restarting every page is not helpful to the reviewers!

Reply: Done as suggested.

-Fig. 1: The seemingly unambiguous identification of stages (especially I-VI) on these tissues is suspect, without an acrosome marker – how, in Fig. 1B, were they able to distinguish I-III vs IV-V with such confidence?; also, how was modest, high, highest levels of detection determined for MFN2?

Reply: Thank you for pointing out this question. We roughly distinguished the stages of spermatogenesis based on the γ-H2AX (a marker of meiotic DNA damage response) staining and the DAPI (cell nuclear marker) staining in testicular sections. Although this identification method is not as accurate as acrosome marker staining, it is also feasible, and many research groups have used this method to publish articles(Di Giacomo et al., 2013; Dong et al., 2019). Besides, we carefully revised the text of the manuscript to describe the expression level of MFN2 in male germ cells more accurately.

- The authors, throughout the manuscript, use sentences containing "possibly...could", "might...suggest", "and/or" - make a stand based on your data, don't leave so much room for interpretation to your reader.

Reply: We corrected all the uncertain words to make a stand based on our data interpretation, as suggested.

- when IPs and polysome gradients are done on whole testis, the authors seem to conclude that whatever they find is going on in germ cells.. this is a dangerous assumption, as MFN1/2 are also in somatic cells, and things they stick to in a testis lysate may not represent physiologic relationships in germ cells - would suggest doing experiments using isolated germ cells.

Reply: We appreciate the reviewer's comments that using isolated germ cells to do IPs and polysome gradients might be better. However, isolating germ cells and redid all IPs and polysome gradients might cause a significant delay in publishing the results. Moreover, we would like to point out that the interacted proteins of MFN2 were studied in our study by IP experiments are germ cell-specific proteins, such as MIWI, DDX4, GASZ, TDRKH, MSY2, et.al. Our data also showing MFN2 is predominantly expressed in germ cells, but very low expressed in somatic cells, like Sertoli cells (Figure S1). We also checked the papers that presented polysome gradients results by using whole testes(Grivna et al., 2006; Unhavaithaya et al., 2009; Castaneda et al., 2014; Gao et al., 2016), suggesting that using total testis lysate to do polysome gradients is widely accepted in the field.

- They mention mitochondria physiology in the manuscript, but really don't examine it.

Reply: Thank you for pointing out this concern. We now delete the description of mitochondria physiology detection in the revised manuscript since we only examine respiratory complexes' function.

- translational mechanisms not convincing.

Reply: For this part, we redid the polysome profiling assays using age-matched control and *Mfn2*-cKO testicular extracts at P25 when both pachytene spermatocytes and round spermatids enriched. Interestingly, the polysome profiles of control and *Mfn2*-cKO testes are identical (**new Figure.6I**), indicating the global translation was not affected in *Mfn2*-cKO testes at P25. To further reveal the mechanism underlying of MFN2 in the regulation of translational delay for MSY2-bound gamete specific mRNA, we measured cytoplasmic translation by quantifying the polysome distribution of *Spata19* (an MSY2-bound mRNA) mRNAs at P25 testes. The new result showed that Spata19 mRNA exhibited a distribution shift toward heavier polysome fractions (**new Figure.6J**), indicating the observed early-translated SPATA19 protein in *Mfn2*-cKO testes is caused by translational activity. Together, combined with our previous data, we believe that in this revision, our new data provide a piece of relatively convincing evidence for the function of MFN2 in translational machinery during spermatogenesis.

Introduction
-P. 3: fusion and fission only in mammals? Please specify; mitochondria also critical for redox homeostasis; 6-7: aren't mito functions critical in ALL cells?; 8- 13 are MAMs only in germ cells? If not, reword; 13 "increasingly was reported"?; 16 what part of testes, presumably germ cells? If so, what kind? Specify; 19 homolog to ortholog.

Reply: All the above questions have been corrected. Thank you.

-P. 4: 11-13 what is the relationship of these to the term "nuage"? Nuage is French for "cloud", and it was this reviewer's understanding that it referred to these structures in early germ cells (e.g. gonocytes) and not to the CB… please clarify; 22 "besides"… besides what?; 23 what are "transcriptional secessions" – is this cessation? And what about meiotic sex chromosome inactivation (MSCI), this is another very specific example affecting the X+Y chromosomes – please add; 24-5 histones are replaced by transition proteins, not protamines; 26-7 the authors seem to suggest these transcriptional and translational events are coincident and linked… is this true? What is the relationship?

Reply: All the above questions have been clarified and we revised the text accordingly. Thank you for your careful review.

-P. 5: 3-5 incomplete sentence; 5-7 what evidence supports this? Please provide; 8-9 actually, Norman Hecht's group taught us a lot about the mechanisms; 12 germ cells, or testes? 14 – what is meant by "the translational machinery"? Specify; 15 "the male germ cells" – which ones? Specify, reference; 16-7 is it "mitochondrial formation" that's affected? 18 "interoperates"? 19 testes or germ cells, please specify; 21 "phenotypes""? 24 what are "gamete mRNAs", and what are "gamete-specific mRNA fates"?

Reply: All the above questions have been corrected and specified. We also cited Dr. Norman Hecht's papers in the revised text.

-P. 6: "storage/translational delay" – should introduce these concepts, would recommend adding a section in the introduction

Reply: We added several sentences into the introduction to introduce mRNA storage/translational delay (see lines 123-137), as suggested.

Results
-P. 5: 11 "preferentially"?; 15 would not say the testes "mainly consist of spermatocytes", but that

pach spermatocytes are the most advanced germ cell type – would guess germ cells may still be slightly outnumbered by somatic cells at this point; 17 there are no spermatogonia in the P0 testis, only prospermatogonia/gonocytes; 19 there aren't "late pachytene spermatocytes" at P14, they're just appearing; 17-19 also appear to be in Sertoli and PTMs at several ages, seem to be higher in spermatocytes than spermatogonia;would recommend co-immunostaining with a germ cell marker (e.g. TRA98) to ID germ cells.

Reply: We revised the above questions accordingly. We also performed co- immunostaining with a TRA98 (a germ cell marker, also called GCNA1) and MFN2 to further confirm the expression pattern of MFN2 in testicular cells, as suggested. Similar to **Figure 1** and **Figure.S1E-G**, the new data shown MFN2 was highly expressed in pro-spermatogonia, spermatogonia, spermatocytes, and round spermatids, but lower expressed in Sertoli cells. We included the new TRA98 data as **Figure.S1F** in the revision.

-P. 6: 27 would say all pachs, not just late; and appears to be much lower in round spermatids than pachs; How are germ cell types identified so seemingly unambiguously? How were stages determined – for example, how do the authors know that first image is IV-VI and not I-III – it's tough to tell without visualizing the acrosomes somehow… Also, the stage "IX-X" shown is definitely a IX, not a X;

Reply: We changed "late pachytene" to "pachytene" and we also deleted "with the highest expression level" to make the description more accurate. For the spermatogenesis stage determination, we referred the method from the literature(Di Giacomo et al., 2013) by co-staining with γ-H2AX and roughly identified the stages. We also changed the stage "IX-X" to "IX", as suggested. Thank you again for pointing out this question.

-P. 7: 8 spermatocytes are not premeiotic; the magnification/resolution not high enough to conclude cytoplasmic granule staining (Fig. 1C); 13-14 – it is this reviewer's understanding the Stra8-cre is actually expressed earlier as well, in undifferentiated progenitors; 22, 28 – "complete sterility" is redundant, as is "severely atrophic abnormal";

Reply: We carefully revised the text accordingly. Thank you for pointing out these questions again.

-P. 8: 12-14 – your data does more than "suggest" this, it shows/indicates; 20-21 – not measuring "effects", just interaction; however, these IP studies don't assess germ cell interactions, which seem to be key to this study, but just that they interact in testis lysates.

Reply: We deleted the "effects" and revised the manuscript as suggested.

-P. 9: 8-9 – what does this sentence mean?

Reply: We would like to emphasize the severe phenotype in *Mfn1*-cKO compared to *Mfn2*-cKO mice in this sentence. Now we changed this sentence accordingly to make a clear description for the readers.

-P10: 5-7 – this is where "indicate" should be replaced with "suggest", as this is a speculation; 12 – "fail to full development"?; 17 – appeared to increase, or did increase? Can this be quantified?;

Reply: We revised the manuscript, as suggested. For the thickness of IMC, it did increase based on the images we observed. We did not quantify the thickness because we can easily see the differences by naked eyes.

-P11: 4 – "by about" – be specific; 27 – did it appear to increase, or did it increase?

Reply: We reworded this sentence to better describe our data in the revised manuscript. The mtDNA copy number was increased significantly in both *Mfn1*- cKO and *Mfn2*-cKO adult testes. We have changed "appear to increase" to "was increased significantly."

-P12: 1 – "imply a possible" is redundant; 3 – "physiological…evidence" – what evidence? 4 – "and/or" – do the authors want the readers to choose one?; 10 – "appeared" – again, was it or

wasn't it?;20-21 – this is not a hypothesis "assumed…maybe"?;

Reply: We have corrected and revised these questions accordingly.

-P13: 7 – "suggesting…may" not necessary together;

Reply: Done as suggested.

-P14: 4-5 "suggesting…might" and "tried to ask"? 14 – and/or – again, don't leave this for the reader to decide; 20-1 – this is not a fair premise – measuring the steady-state mRNA levels does not enable "understand(ing) the molecular mechanisms"; does up-regulation or downregulation presume changes in transcription, or mRNA stability? One important function ascribed to Nuage is RNA decay – an example is provided by Yumiko Saga's work on NANOS2 and its association in nuage with the CCR4-NOT deadenylase complex… were mRNAs that increased in abundance also show up in her dataset? 11 – what is "biological quality and localization"?? 16 – "genes were distributed in cell and intracellular part"- huh?! What meaning do these broad and seemingly meaningless titles provide to the reader? 18 – what is a "rich factor"? Would recommend removing this entire paragraph, as it adds little useful meaning to the paper

Reply: We revised the text to address the above concerns accordingly. We changed "understand the underlying molecular mechanisms" to "investigate the molecular consequences" to avoid the over-interpretation of our RNA-seq data. In addition, since Dr. Yumiko Saga's work used E15.5 male gonads to conduct GeneChip analyses, there is no reason to make a comparison of our RNA-seq dataset with her dataset because of the different subjects. We also removed the entire paragraph of GO term analyses, as this reviewer suggested.

-P16: validation only of steady-state mRNA levels, not gene up or downregulation… would carefully rephrase; 8-11 – no, validation doesn't support that view, it just validates the fold-changes estimated by RNA-seq; 16 – what are "inactive translational mRNAs"?; 18-20 these are called polysome gradients; if these gradients were done on whole testis, how can conclusions be made for germ cells? 23 "suggest…could" – this is the results section, make a stand one way or the other;

Reply: We revised the manuscript to address the above questions accordingly.

-P17: 1-2 - is this sufficient data to make this overarching conclusion? 8 – could, or did? 12 – do the authors mean MSY2 instead of MFN2?

Reply: We revised the manuscript accordingly. We also redid the Co-IP experiments for MFN2 and MSY2 and found MFN2 interacts with MSY2 with an RNA-independent manner.

-How does MFN2 seemingly interact with everything you test? Are there proteins it does not interact with? 22-3 – but the processes indicated from RNA-seq GO analysis were broad and not germ cell specific, right? This reviewer is confused by this sentence.

Reply: Thank you for pointing out these questions. We only chose the interaction proteins to show in the previous version, now we added the non-interaction protein (hnRNPA2) as a negative control (see **new Figure.S4C**) in the revision to avoid the misunderstanding. Also, we found that MFN2 does not interact with MAEL in testes (see **Figure.S3B**). For the RNA-seq GO analysis description, we deleted this sentence since those analyses do not adequately support our conclusion (Reviewer #1 also suggested we remove the GO term analysis).

Mitochondria are a 3-D structures that are constantly undergoing fusion and fission - how is this altered in these mice?

Reply: Although mitochondria are 3-D structures, their structural, morphological alterations can be visualized under an electron microscope. Indeed, we observed mitochondria exhibit swelling and fragmentation with fewer cristae in germ cells in adult *Mfn2*-cKO testes (**Figure 3A-B**) using a transmission electron microscope.

Fig. 1B – step 7-8 are not elongating, they are round; step 14-16 are not elongating, they are

condensing; Fig. 1C – no context is provided for these images – where is the basal lamina, what are they intending to show? This reviewer can see the PTMs and the spermatids and knows the polarity, but for a broader readership these need to be indicated;

Reply: We relabeled the Figure legend in **Figure.1B** and cited **Figure.1C** in the revised context (in line 194). We used a white dotted line to separate the basal lamina and germ cells in **Figure.1C** for a broader readership.

Reviewer #3 Advance Summary and Potential Significance to Field:
This manuscript describes (1) spermatogenesis failures and mitochodrial defects in Mfn1, Mfn2 and both Mfn1/2 conditional KO testes (stra8-cre), (2) interaction between MFN proteins and nuage proteins, and (3) a possible involvement of MFN2 in translational regulation during spermatogenesis.
About (1), Mfn1/2 conditional KO phenotypes during spermatogenesis have already been reported in detail in (Varuzhanyan et al., eLIFE, 2019). However and surprisingly, the authors of this manuscript mentioned (Varuzhanyan et al, eLIFE, 2019) only once in the introduction section very briefly.
About (2), both physical and functional interaction between Mfn genes/proteins and GASZ, a nuage component, has already been reported in (Zhang et al. in EMBO reports, 2016). However, this important previous finding (i.e. MFN and GASZ interaction) is again only very briefly touched in this manuscript.
Novel conceptual findings described in this manuscript are mainly about (3). MFN2 is enriched in polysome fractions and it interacts with several translational regulators (MSY2 and eEF1As). This part is potentially intriguing, with regard to a possible involvement of a mitochondrial surface protein in mRNA translational regulation. However, there are no data or discussion about this important point. In all, in this reviewer's feel, this manuscript is still premature for publication in Development.

Reply: We appreciate this reviewer for his/her evaluation of our work. We have been studying the role of MFN1/2 in male germ cell development for about 4 years, and we do not know that other research groups are doing similar work until they published the papers(Varuzhanyan et al., 2019; Chen et al., 2020). However, the overall conclusion and underlying mechanism identified are different between our current study and the published papers. Moreover, the published papers are mainly focused on the spermatogonial differentiation and meiotic processes, while our research concentrated on late spermatogenesis and the function of Nuage structure. Thus, we believe that our study provides a new conceptual framework for mitochondrial fusion proteins (MFN1/2) regulation in the male germline, which differs from the published work and worthy of publishing. We highlighted these differences and discussed the previous works in the revised manuscript (see lines 557-601).
For the translational regulation part, we reanalyzed the RNA-seq data and redid polysome profiling assays to provide a piece of relatively convincing evidence for the function of MFN2 in translational machinery during spermatogenesis. We also discussed this important point in the revised discussion section.

Reviewer #3 Comments for the Author:

Specific points
- The authors should properly cite other researchers' previous reports throughout this manuscript.

Reply: Thanks to this reviewer for pointing out this issue. We now properly cited other researches' previous reports(Zhang et al., 2016; Varuzhanyan et al., 2019; Chen et al., 2020) in the revised manuscript.

- page 10, line 17 : If the authors want to describe this finding ("the thickness of IMC appeared to increase"), actual data and statistical analyses are necessary.

Reply: This is a good suggestion. But based on the TEM images, we can see the increased thickness of IMC by naked eyes. We now labeled the IMC as blue color in the TEM images of **Figure. 3A-B** to avoid the misjudgment.

- page 10, line 20 : lengths and ratios are different.

Reply: Corrected in the revision.

- page 11, line 3 : It is unclear how to define the distance between mitochondria and ER.

Reply: We referred to the previous literature(Naon et al., 2016) to define ERMICC and the distance between and ER, which is shown in the following cartoon. We also cited this literature in the revised manuscript.

We have removed unpublished data provided for the referees in confidence.

- page11 line 26 : Is it necessary to carry out RT for mtDNA PCR?

Reply: The goal of the experiment is to determine the mtDNA copy number. RT- PCR is an effective method to measure it based on the published literature (Chen et al., 2010).

- page 16 line8 : About "Taken together, these RNA-Seq ... act in an integral manner," it is very difficult to understand how the authors draw this conclusion from the complex lists of many GO terms of various categories.

Reply: We now revised this conclusion and removed the GO term analyses (Reviewer #1 and 2 have the same suggestion).

- p28 line 21 : Some of the dataset presented in this manuscript should be analyzed by non-parametric statistical methods.

Reply: We agreed with this point. Non-parametric statistical methods analyzed the data shown in **Figure.3C-G**. We use Mann-Whitney U-test in which allows two groups to compared without assuming that values normally distributed. The data calculated by using an online tool (https://www.socscistatistics.com/tests/mannwhitney/). If the result is significant at $P < 0.05$, we labeled *. If $P < 0.01$, we labeled **. We added the non- parametric statistical method into the method section in the revision.

- fig3d : it appears that the authors defined arbitrary bins for this histogram and then found a statistically significant difference in a certain bin (1.2-1.5). If this is not the case, the rationale for defining these uneven/specific bins needs to be explained.

Reply: The length distribution of mitochondria and the aspect ratio is a standard method used in many published papers (Schneeberger et al., 2013; Naon et al., 2016; Boutant et al., 2017) to analyze the morphological change of mitochondria. Our data shown mitochondria with aspect ratio 1.2-1.5 is relatively close to the circle, indicating shorter mitochondria increased in *Mfn2*-cKO compared to wild type.

- fig3j : how many replicates were examined for these PCR experiments?

Reply: Three replicates were examined. We added the replicate numbers in the Figure legend.

- fig3k : it is difficult to see quantitative differences in staining intensities between the samples from these images.

Reply: Based on our staining results, COX staining intensities are increased in both *Mfn1*-cKO and *Mfn2*-cKO testis sections, whereas the SDH seems unaltered.

- fig4fg : control IgGs must be used for IP.

Reply: We added IgG control in **Figure.4F&G**, as suggested.

- fig5ab: it is impossible to see "the purity and morphology of isolated PS and RS" from these images (left top).

Reply: We added the new images of purified PS and RS in the revised **Figure.S6A**.

- fig5gh : again, there are no indication about how many replicates were examined for these PCRs and stats for asterisks.

Reply: Three replicates were examined also. The previous Figures.5G-H were shown as **Figure.S6C-D**.

- fig6d: the MFN2 band in MSY2 IP is very weak. Also, the ratio between input and IP samples used should be indicated.

Reply: We redid the IP experiments and added the ratio between input and IP samples in the new **Figure.6B-C**.

- fig6k : the MFN2 band in the eEF1a2 lane is clearly shifted. If this is MFN2, the reason for the shift needs to be explained.

Reply: To better focus the role of MFN2 in the regulation of the MSY2-bound mRNA translational mechanism, we now removed the eEF1A2 part in the revised version.

- fig6f : this heatmap looks very uncommon. How were the data values scaled before making the heatmap (Z-score or other)?

Reply: To make heat-maps for MSY2-bound and MSY2-nonbound mRNAs, we picked the genes out from the RNA-seq data; Z-score used for the normalization for gene expression. In detail, we made the Figure.6F heat-map (now is **Figure.6E-F** in new manuscript) using an online tool (http://www.heatmapper.ca/expression/), the clustering method is “average Linkage,” the distance measurement method is “Pearson.”

- There are no fig1, fig2 ... labels on figures.

Reply: Corrected. Thanks.

AUTHORS’ STATEMENT:

We really appreciate the constructive comments from the three expert reviewers, which have been very helpful for us to improve our manuscript. We hope that the reviewers will find that the new data added to our revised manuscript have significantly enhanced the impact of this work. Thank you so much!

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First decision letter

MS ID#: DEVELOP/2020/196295

MS TITLE: MFN2 cooperates with Nuage-associated proteins and is essential for male germ cell development by controlling mRNA fate during spermatogenesis

AUTHORS: Xiaoli Wang, Yujiao Wen, Jin Zhang, Shuangshuang Guo, Congcong Cao, Stephen A Krawetz, Zhibing Zhang, and Shuiqiao Yuan

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.

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 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.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. 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.

Reviewer 1

Advance summary and potential significance to field

(this is the same "significance" section as I wrote for the April 2020 submission of this paper... I am already late getting this review back, and any updates I include here would duplicate what I've already written in the new "suggestions to authors" PDF)

In this manuscript, Wang and colleagues begin by showing the expression pattern of MFN2 in the mouse germline, and then go on to characterize a conditional knockout of Mfn2 (and at times, Mfn1 and the double cKO). They show that Mfn2-cKO mice have reduced testis weight (this effect is earlier in Mfn1 and in the double mutant) and disrupted subcellular structures, including mitochondria, mitochondrial-associated ER membranes (MAMs), and contacts between mitochondria and ER. The authors also show that MFN2 co-immunoprecipitates with many of the proteins known to localize to the nuage in germ cells, including MIWI and DDX4. Several of these proteins show marked decrease in expression or mislocalization in the Mfn2 cKO. The authors show that Â– consistent with disruptions in the nuage Â– the overall levels of pachytene piRNAs are reduced by 50% in the Mfn2 cKO, although transposon activity appears unchanged. RNA-seq data from purified pachytene spermatocytes and round spermatids is presented, and MFN2 is shown to associate with actively-translated (polysomal) fractions. Finally, there are some nice data showing that SPATA19 is expressed 3 days early in the Mfn2 cKO.

Comments for the author

MFN2 review round 2

I think the authors did a good job dealing with my minor comments on figures and text. I might not have communicated well on Figure 3H, though – here's the relevant text from the results section: "Calreticulin displayed a diffused granular pattern in the cytoplasm of Mfn2-cKO spermatocytes at P18 instead of continuous perinuclear tubular localization exhibited in the controls (Figure.3H)" (lines 319-320). My point was that it's hard to see the calreticulin localization as 'tubular' in a 2D image. That's all.

The authors also did a bunch of experiments / extra analysis I asked for, although (as detailed below) the manuscript would benefit from some similar analysis done on control data sets, and/or would benefit from having the data displayed in a more unbiased way. I think the comparison of the set of RNAs misregulated in Mfn2 cKO vs. MIWI should be in the paper – it will add something new to the field no matter what! – but it needs to be done right and not artificially squeezed into a tidy narrative. Biology is often messy/illogical and sometimes that just needs to be acknowledged.

Figure 5 (corresponding text and actual figure)

There are a couple of places when the RNA-seq data are being discussed, where the authors say something like "of this set of RNAs*, 10% were upregulated / downregulated in the Mfn2 cKO." This might seem meaningful, but then the reader thinks: what percent of *any* set of RNAs would be found to be upregulated (or downregulated) in this data set? Possible control sets: (a) non- targets

of MIWI or (b) all transcripts (I looked for the total number of genes included in your RNA-seq data, but couldn't find it… it would be really useful to know what percentage of the total RNA-seq data set was up vs. down vs. unchanged).

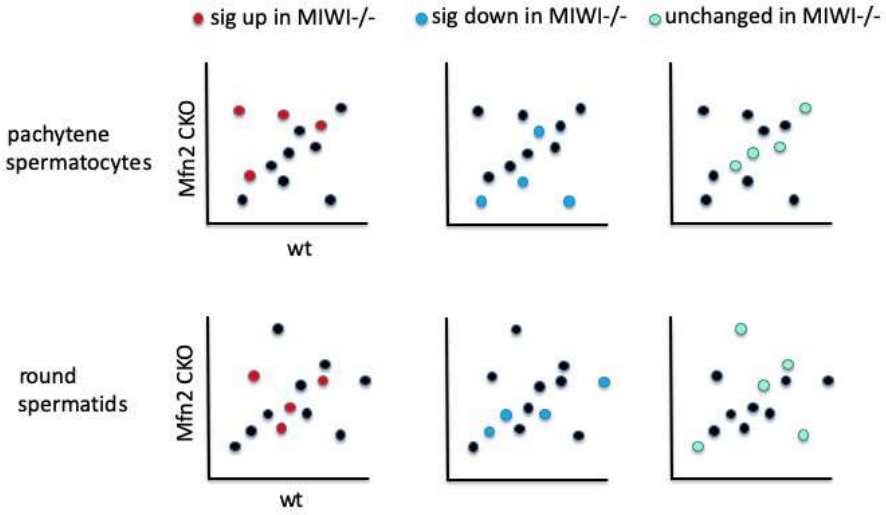
* MIWI targets, upregulated / MIWI targets, downregulated / piRNA-targeted transcripts

For C and D: Can you split these up so that it is one bright color overlaid onto the black at a time? Otherwise the reader misses some information because different colored dots are on top of each other. (it's obviously fine to have two colors at a time in A and B, since they are by definition non-overlapping).

Also, the X and Y axes should be the same for A&C and B&D. You want the overall shape of the mass of dots to look the same in A and C, and the same in B and D. Right now they don't. : / I think that the "upregulated" transcripts in C and D are held to the same standard as those in A and B, respectively – at least, that's what the text says – but visually they seem even closer to the x=y line. Maybe fixing the axes would solve that issue.

Of the 330 piRNA-targeted transcripts, 32 are upregulated in Mfn2 cKO round spermatids and 72 are upregulated in MIWI-/- round spermatids. Are the 32 entirely included in the set of 72? I can't tell from the text or the figure, and this seems like something that would be really critical to communicate accurately (right now the text says "~50% overlap" but I don't know if that's just a mathematical overlap, as in 10% is ~ half of 20%.

I would move E through L to a supplemental data section to join the similar analyses of the downregulated genes. In the place of E through L, I would like to see all the up-, down-, and non-regulated genes from the MIWI KO mapped onto your data sets (pachytene spermatocytes and round spermatids), kind of like this:



The benefit of doing it this way is that all of the data is displayed in a really accessible and transparent fashion for the reader. The reader can then observe whether there are any subtle trends (in my example above, there is more evidence of MFN2/MIWI overlap in spermatocytes than in spermatids. But that's just my artistic rendering as an example). Or maybe there's little correlation between the transcripts misregulated in MIWI-/- and those misregulated in the Mfn2 cKO. If that's the case, better to be open about it and admit that the Mfn2 phenotype is complex and rather distinct from that of MIWI.

Figure 6

I would move E and F to a supplemental figure and use that space in Fig 6 for pie charts (one for MSY2 targets and one for non-targets) to show proportion of MSY2 targets or non-targets that went up, down, or were unchanged in the Mfn2 cKO. Otherwise a reader just blasting their way through

the figures (and not having the patience to read the text of the results section… it happens!) would think that ALL of the MSY2 targets / non-targets changed their expression in your Mfn2 cKO data sets.

For your RT-PCR in Figure 6J: using Actin as a control has the unfortunate effect of making your SPATA19 data less convincing. Actin seems to shift a tiny bit towards the heavier fractions in the mutant, and its relative absence from the lighter polysomal fractions (2-3 ribosomes?) in both genotypes makes it harder to compare to the expression of SPATA19 in wt. I would leave Actin in the figure but try to add in a second control (maybe something that's germ cell specific – i.e. not a house-keeping gene – and not a MFN2 target!) whose levels are fairly evenly distributed across the polysomal fractions in wt and the Mfn2 cKO. (I'm assuming you still have the cDNAs from the polysome expt).

Discussion (and last paragraph of intro)

When you're talking about the significance of your findings, particularly as they pertain to MFN2 function in regulating RNAs, I think you need to be more cautious. In particular, I would recommend not making strong statements that MFN2 "works with" or "cooperates with" specific proteins (MIWI, MSY2, etc.) to "mediate/control RNA fates." I think that is getting ahead of your results a bit. One complicating factor, for example, is that MSY2 is itself upregulated in the Mfn2 cKO. So it is impossible to say that the effect on MSY2 target RNA levels in the Mfn2 cKO is due directly to the absence of MFN2. What you *can* say is that you have one great example of an MSY2 target (SPATA19) that is ectopically expressed at the protein level. And more work will be needed to determine whether that is representative of the other known MSY2 targets.

Likewise with broad statements about MFN2 working with MIWI: given the data as presented so far, I don't see support for the "working with" / "cooperating with" language.

Lines 699-704: If this is in line with what I suggested in my first review, maybe there's a better way to write it. Like,

"MSY2 is known to repress translation of and prevent degradation of target RNAs. If MFN2, as a partner protein, is required for both of those functions, we would expect that MSY2 target RNA levels would decrease in the Mfn2 cKO, even in the presence of increased MSY2. In contrast, though, we observed that 57% of the MSY2-bound RNAs showed *higher* expression in the Mfn2 cKO (vs. 12% of MSY2-nonbound RNAs). At the same time, SPATA19 protein (encoded by a MSY2 target RNA) was expressed prematurely at P25 in the Mfn2 cKO. One possible explanation is that absence of MFN2 compromises the translational repression function of MSY2 but does not disturb its ability to protect target RNAs from degradation. Thus more bulk MSY2 in the Mfn2 cKO may be more efficient at keeping target RNAs safe, but without MFN2 no longer able to prevent their ectopic translation." + need for further experiments (especially determining whether other MSY2 targets besides SPATA19 are expressed early in the Mfn2 cKO)

Line 694: "RNA transcription levels" maybe you mean "RNA transcript levels"? also line 518 I think you mean "transcript levels" …I'm pretty sure this is not a transcription paper :)

generally -

It would be helpful to have a native English speaker – either a collaborator or co-author -- to proofread so that the grammatical errors get fixed without the meaning getting changed (the *Development* copy editors are great! but sometimes things can go somewhat sideways during the editing process, even with primarily native-speaker authors).

Reviewer 2

Advance summary and potential significance to field

The authors have made considerable changes to their manuscript in response to reviews. A few concerns remain, appended below.

Comments for the author

"We also checked the papers that presented polysome gradients results by using whole testes(Grivna et al., 2006; Unhavaithaya et al., 2009; Castaneda et al., 2014; Gao et al., 2016), suggesting that using total testis lysate to do polysome gradients is widely accepted in the field." - This is not true - just because there are examples of this sloppy practice (done largely because separation procedures weren't done) doesn't make it widely accepted. This approach to doing any assay on a whole tissue and then making conclusions about a particular cell type in that tissue is dangerous. There are a variety of ways to isolate germ cells from the testis (Sta-Put, FACS based or size for spermatocytes/spermatids, some fluorescent models becoming available, etc). The authors should find a way to validate their data in germ cells of interest.

Also, would not agree that pachytene spermatocytes and round spermatids are necessarily enriched at P25 - compared to what? Please clarify in the text.

104-5: this reviewer thought that Nuage and 'intermitochondrial cement' were terms used for these non-membranous electron dense structures where mRNA processing occured in prospermatogonia, while 'chromatoid body' refers to that type of structure in spermatocytes. It is not this reviewer's understanding that the terms are interchangeable, but could be wrong. Please clarify in the text.

"In addition, since Dr. Yumiko Saga's work used E15.5 male gonads to conduct GeneChip analyses, there is no reason to make a comparison of our RNA-seq dataset with her dataset because of the different subjects." - This was a simple request, and should not be so casually ignored as "different subjects". If the authors have evidence that nuage-type structures fundamentally change their role between those times, then that should be provided. Should be done, and added to the text in the discussion.

Reviewer 3

Advance summary and potential significance to field

The resubmitted manuscript by Wang et al. shows several improvements according to the reviewers' comments on the original version of the manuscript.

Comments for the author

I now appreciate the authors' effort to cite and discuss the other researchers' work about spermatogenesis phenotypes of Mfn KO mice. The authors also provide novel interpretation of RNAseq data from the viewpoints of (1) potential overlapping changes in gene expression patterns between Mfn2-cKO and Miwi KO spermatogenic cells and (2) possible functional links among piRNA-target, MSY2-bound and Mfn2-regulated mRNAs. To address these hypotheses, the authors provide several numbers and/or percentages of overlapping genes, e.g., "~12% (74/599) and ~8% (152/1819) up-regulated genes in Miwi-KO pachytene spermatocytes and round spermatids respectively, are likely to be up-regulated in that of Mfn2-cKO" (page 16-17). However these numbers by themselves do not mean anything, i.e. for example, "12%" does not distinguish between whether such "12%" overlap is truly statistically significant or just observed by chance. To draw solid conclusions, such numbers should be very carefully evaluated by proper statistical analyses.

There also remain several points, on which I made comments in my previous review, but have not yet been addressed in this resubmitted manuscript, as follows,

- page 11 : if the authors want to describe this finding ("the thickness of IMC appeared to increase"), actual data and statistical analyses are necessary.

- page 11: lengths and ratios are different.

- page12 : is it necessary to carry out RT for mtDNA PCR?

- page 27 : it is unclear how to define the distance between mitochondria and ER.

- fig3d : it appears that the authors defined arbitrary bins for this histogram to find a statistically significant difference in a certain bin (1.2-1.5). If this is not the case, the rationale for defining these specific bins needs to be explained.

- fig3k : it is difficult to see quantitative differences in staining intensities between the samples from these images.

- There are no fig1, fig2 etc. labels on figures.

First revision

Author response to reviewers' comments

Reviewer 1 Comments for the Author:

I think the authors did a good job dealing with my minor comments on figures and text. I might not have communicated well on Figure 3H, though – here's the relevant text from the results section: "Calreticulin displayed a diffused granular pattern in the cytoplasm of Mfn2-cKO spermatocytes at P18 instead of continuous perinuclear tubular localization exhibited in the controls (Figure.3H)" (lines 319-320). My point was that it's hard to see the calreticulin localization as 'tubular' in a 2D image. That's all.

Reply: We appreciate this reviewer for his/her valuable suggestions in the first round, which enabled us to improve the manuscript. We apologize for our misunderstanding of your suggestion about Figure 3H. Now, we deleted "tubular" in the description because it's hard to see the "tubular" structure in 2D images and replaced this image with a clearer one as attached (also see the new **Fig.3H**).

The authors also did a bunch of experiments / extra analysis I asked for, although (as detailed below) the manuscript would benefit from some similar analysis done on control data sets, and/or would benefit from having the data displayed in a more unbiased way. I think the comparison of the set of RNAs misregulated in Mfn2 cKO vs. MIWI should be in the paper – it will add something new to the field no matter what! – but it needs to be done right and not artificially squeezed into a tidy narrative. Biology is often messy/illogical and sometimes that just needs to be acknowledged.
Reply: This is a good point. We have re-analyzed the data and re-organized the Figures as your suggestions detailed in the following. Thank you so much!

Figure 5 (corresponding text and actual figure)
There are a couple of places when the RNA-seq data are being discussed, where the authors say something like "of this set of RNAs*, 10% were upregulated / downregulated in the Mfn2 cKO." This might seem meaningful, but then the reader thinks: what percent of *any* set of RNAs would be found to be upregulated (or downregulated) in this data set? Possible control sets: (a) non- targets of MIWI or (b) all transcripts (I looked for the total number of genes included in your RNA-seq data, but couldn't find it… it would be really useful to know what percentage of the total RNA-seq data set was up vs. down vs. unchanged).
Reply: Thank you for this great suggestion. We re-analyzed the RNA-seq data and made some new Figures in this revision, as suggested.

MIWI targets, upregulated / MIWI targets, downregulated / piRNA-targeted transcripts
Reply: We re-analyzed the RNA-seq data and made new **Fig.5C-J** show all the upregulated, downregulated, and unchanged genes in *Miwi*-KO spermatocytes and round spermatids mapped onto *Mfn2*-KO data sets as suggested, and also added the description in manuscript accordingly (lines 412-416, 419-421).

For C and D: Can you split these up so that it is one bright color overlaid onto the black at a time?

Otherwise the reader misses some information because different colored dots are on top of each other. (it's obviously fine to have two colors at a time in A and B, since they are by definition non-overlapping).
Reply: This is a good suggestion. We re-analyzed the data and made new **Fig.5C-D** to clearly show the situation of all piRNA-targeting mRNAs based on Figure 5A-B, which can distinguish the upregulated targets and non-upregulated targets in *Mfn2*-cKO datasets and avoid the different colored dots are on top of each other.

Also, the X and Y axes should be the same for A&C and B&D. You want the overall shape of the mass of dots to look the same in A and C, and the same in B and D. Right now they don't. : / I think that the "upregulated" transcripts in C and D are held to the same standard as those in A and B, respectively – at least, that's what the text says – but visually they seem even closer to the x=y line. Maybe fixing the axes would solve that issue.
Reply: Thank you for your careful review, we have modified the X and Y axes of C&D, now the X and Y-axes are the same for A&C and B&D.

Of the 330 piRNA-targeted transcripts, 32 are upregulated in Mfn2 cKO round spermatids and 72 are upregulated in MIWI-/- round spermatids. Are the 32 entirely included in the set of 72? I can't tell from the text or the figure, and this seems like something that would be really critical to communicate accurately (right now the text says "~50% overlap" but I don't know if that's just a mathematical overlap, as in 10% is ~ half of 20%.)
Reply: Based on our analyses, only a few transcripts overlapped between 32 upregulated piRNA targeted mRNAs in *Mfn2*-cKO round spermatids and the 72 upregulated piRNA targeted mRNAs in *Miwi*-KO round spermatids. To avoid the misunderstanding, we mentioned this comparison in the text of this revision (lines 412-416).

I would move E through L to a supplemental data section to join the similar analyses of the downregulated genes. In the place of E through L, I would like to see all the up-, down-, and non-regulated genes from the MIWI KO mapped onto your data sets (pachytene spermatocytes and round spermatids), kind of like this:

We have removed unpublished data provided for the referees in confidence.

The benefit of doing it this way is that all of the data is displayed in a really accessible and transparent fashion for the reader. The reader can then observe whether there are any subtle trends (in my example above, there is more evidence of MFN2/MIWI overlap in spermatocytes than in spermatids. But that's just my artistic rendering as an example). Or maybe there's little correlation between the transcripts misregulated in MIWI-/- and those misregulated in the Mfn2 cKO. If that's the case, better to be open about it and admit that the Mfn2 phenotype is complex and rather distinct from that of MIWI.

Reply: Thank you for your thoughtful suggestion. Now Figure 5E-L is moved to Supplementary Figure 7, as suggested. We also showed all the up-, down-, and non- regulated genes from the *Miwi*-KO mapped onto our RNA-seq datasets in both pachytene spermatocytes and round spermatids following your suggestion (see the new **Fig.5E-J**). From the analysis results, only a small number of misregulated genes are overlapped between *Mfn2*-cKO and *Miwi*-KO spermatogenic cells, indicating the complexity of MFN2 in regulating spermatogenesis, which is distinct from that of MIWI. We also mentioned this explanation in the text (see lines 412-416).

Figure 6
I would move E and F to a supplemental figure and use that space in Fig 6 for pie charts (one for MSY2 targets and one for non-targets) to show proportion of MSY2 targets or non-targets that went up, down, or were unchanged in the Mfn2 cKO. Otherwise a reader just blasting their way through the figures (and not having the patience to read the text of the results section… it happens!) would think that ALL of the MSY2 targets / non-targets changed their expression in your Mfn2 cKO data sets.
Reply: We moved E and F to Supplementary Figure 8 in the revision following your suggestion.

For your RT-PCR in Figure 6J: using Actin as a control has the unfortunate effect of making your SPATA19 data less convincing. Actin seems to shift a tiny bit towards the heavier fractions in the mutant, and its relative absence from the lighter polysomal fractions (2-3 ribosomes?) in both genotypes makes it harder to compare to the expression of SPATA19 in wt. I would leave Actin in the

figure but try to add in a second control (maybe something that's germ cell specific – i.e. not a house-keeping gene – and not a MFN2 target!) whose levels are fairly evenly distributed across the polysomal fraction in wt and the Mfn2 cKO. (I'm assuming you still have the cDNAs from the polysome expt).
Reply: This is a good suggestion. We indeed detected several other control genes such as *Gapdh* and *Arbp*, and their distribution is similar to *Actin*. The reason for their not fairly even distribution across polysomal fractions might be that we entirely manually performed the polysome profiling assays due to the lack of an automated fractionation system. However, the distribution shift of *Spata19* is obvious enough to show the differences.

Discussion (and last paragraph of intro)
When you're talking about the significance of your findings, particularly as they pertain to MFN2 function in regulating RNAs, I think you need to be more cautious. In particular, I would recommend not making strong statements that MFN2 "works with" or "cooperates with" specific proteins (MIWI, MSY2, etc.) to "mediate/control RNA fates." I think that is getting ahead of your results a bit. One complicating factor, for example, is that MSY2 is itself upregulated in the Mfn2 cKO. So it is impossible to say that the effect on MSY2 target RNA levels in the Mfn2 cKO is due directly to the absence of MFN2. What you *can* say is that you have one great example of an MSY2 target (SPATA19) that is ectopically expressed at the protein level. And more work will be needed to determine whether that is representative of the other known MSY2 targets.
Likewise with broad statements about MFN2 working with MIWI: given the data as presented so far, I don't see support for the "working with" / "cooperating with" language.
Reply: We now changed the "working with"/ "cooperating with" to "interacting with" in the revision following suggestion. Thank you very much for your careful review again.

Lines 699-704: If this is in line with what I suggested in my first review, maybe there's a better way to write it. Like,
"MSY2 is known to repress translation of and prevent degradation of target RNAs. If MFN2, as a partner protein, is required for both of those functions, we would expect that MSY2 target RNA levels would decrease in the Mfn2 cKO, even in the presence of increased MSY2. In contrast, though, we observed that 57% of the MSY2-bound RNAs showed *higher* expression in the Mfn2 cKO (vs. 12% of MSY2-nonbound RNAs). At the same time, SPATA19 protein (encoded by a MSY2 target RNA) was expressed prematurely at P25 in the Mfn2 cKO. One possible explanation is that absence of MFN2 compromises the translational repression function of MSY2 but does not disturb its ability to protect target RNAs from degradation. Thus more bulk MSY2 in the Mfn2 cKO may be more efficient at keeping target RNAs safe, but without MFN2 no longer able to prevent their ectopic translation." + need for further experiments (especially determining whether other MSY2 targets besides SPATA19 are expressed early in the Mfn2 cKO)
Reply: Thank you for giving us this elaborate discussion and we have modified the discussion part following your suggestion (see lines 737-753 in revision).

Line 694: "RNA transcription levels" maybe you mean "RNA transcript levels"? also line 518 I think you mean "transcript levels" …I'm pretty sure this is not a transcription paper :)
Reply: We changed the word "transcription" as "transcript".

generally -
It would be helpful to have a native English speaker – either a collaborator or co-author -- to proofread so that the grammatical errors get fixed without the meaning getting changed (the *Development* copy editors are great! but sometimes things can go somewhat sideways during the editing process, even with primarily native-speaker authors).
Reply: The co-author, Dr. Stephen A Krawetz has checked the grammar and corrected all typos again.

Reviewer 2 Advance Summary and Potential Significance to Field:
The authors have made considerable changes to their manuscript in response to reviews. A few concerns remain, appended below.
Reply: Thanks to this Reviewer for his/her evaluation of our work again.

Reviewer 2 Comments for the Author:
"We also checked the papers that presented polysome gradients results by using whole testes(Grivna et al., 2006; Unhavaithaya et al., 2009; Castaneda et al., 2014; Gao et al., 2016), suggesting that

using total testis lysate to do polysome gradients is widely accepted in the field." - This is not true - just because there are examples of this sloppy practice (done largely because separation procedures weren't done) doesn't make it widely accepted. This approach to doing any assay on a whole tissue and then making conclusions about a particular cell type in that tissue is dangerous. There are a variety of ways to isolate germ cells from the testis (Sta-Put, FACS based or size for spermatocytes/spermatids, some fluorescent models becoming available, etc). The authors should find a way to validate their data in germ cells of interest.
Reply: We appreciate your strict review and quite agreed with your opinion. It is a better way to use isolated germ cells for polysome profiling, especially when comparing the difference between adult WT and cKO samples since there are multiple different cell types. In this work, we performed two experiments (three times per each) by using the whole testis after careful thinking. One is Figure 6A, we used WT adult testis to show the distribution of MFN2 protein through the gradients for two reasons: firstly, MFN2 is highly expressed in spermatocytes and round spermatids; secondly, it is general to use the whole testis to show the expression distribution of interested protein across the gradients, including piRNA pathway component MAEL, MIWI, and MILI, etc. (Castaneda et al., 2014; Grivna et al., 2006; Wang et al., 2009). Another experiment is Figure 6I and J, we used P25 WT and cKO testis to compare the distribution of SAPTA19 in WT and cKO gradients. The reason for using the whole testis is that: 1) the cell types at this time point are comparable between WT and cKO testes; 2) mainly composed of spermatocytes and round spermatids at this time point, where MFN2 highly expressed.
Besides, since polysome profiling is a time-consuming experiment, if we redid all these experiments using isolated germ cells, it will be significantly delayed the publication of our work. We hope this Reviewer will understand and sympathized with us.

Also, would not agree that pachytene spermatocytes and round spermatids are necessarily enriched at P25 - compared to what? Please clarify in the text.
Reply: Done as suggested.

- : this reviewer thought that Nuage and 'intermitochondrial cement' were terms used for these non-membranous electron dense structures where mRNA processing occured in prospermatogonia, while 'chromatoid body' refers to that type of structure in spermatocytes. It is not this reviewer's understanding that the terms are interchangeable, but could be wrong. Please clarify in the text.
Reply: We agreed with the reviewer's point that these terms are not interchangeable since they are specific structures in different cell types. We have clarified this description in the text to avoid misunderstanding (see lines 94-107 in revision).

"In addition, since Dr. Yumiko Saga's work used E15.5 male gonads to conduct GeneChip analyses, there is no reason to make a comparison of our RNA-seq dataset with her dataset because of the different subjects." - This was a simple request, and should not be so casually ignored as "different subjects". If the authors have evidence that nuage-type structures fundamentally change their role between those times, then that should be provided. Should be done, and added to the text in the discussion.
Reply: This reviewer's suggestion is very inspiring, thought-provoking, and very grateful. However, at present, we have not compared *Mfn2* RNA-seq data with the NANOS2 data set for the following reasons:
- As described in previous literature, Nuage are non-membrane cloudy granules in germ cells and its location and morphology are dynamic in different cell types across the process of spermatogenesis, mainly including IMC (also named as pi-body, mainly containing piRNA pathway components), piP-body (simultaneously containing both the piRNA pathway and P-body components), and chromatoid body (CB). Some proteins can localize in different Nuage structures, such as MVH/DDX4, expressed in IMC, piP-body, and CB from embryonic gonocytes to adult round spermatids. However, there are also many components in which only expressed in specific Nuage and/or in specific time- windows, such as piRNA protein MIWI, MILI, and MIWI2. For example, MILI and MIWI2 began their expression in fetal gonocytes and located in IMC. In contrast, MIWI started its expression in pachytene spermatocytes in the postnatal testes and played a different role in regulating piRNA pathway during spermatogenesis, as reviewed in our recent paper(Wang et al., 2020).
- NONOS2 protein was first detectable at E13.5 in the cytoplasm of male mouse gonocytes as granular foci co-staining with P-body markers but not IMC markers. The signal increased until about E16.5 and then slightly decreased by E17.5. In addition, the expression of NANOS2 decreased just before birth, and only a small number of spermatogonia expressed this gene after birth in Dr.Yumiko

Saga' work. However, in our present study, we only tried to explore the function of MFN2 in postnatal testis, and MFN2 is mainly located at mitochondria, not on P-body, which is totally different from the distribution of NANOS2. Moreover, we still don't know whether MFN2 is expressed in embryonic testis and which type of cell expressed. Therefore, at present, it is not convincingly to compare the dataset of MFN2 and NANOS. However, whether MFN2 is expressed in the Nuage of male mouse gonocytes is worthy to expected, which will prompt us to explore it in the future.

Reviewer 3 Advance Summary and Potential Significance to Field:
The resubmitted manuscript by Wang et al. shows several improvements according to the reviewers' comments on the original version of the manuscript.
Reply: Thank you so much for evaluation of our work again. We appreciate your suggestions to further improve our study.

Reviewer 3 Comments for the Author:
I now appreciate the authors' effort to cite and discuss the other researchers' work about spermatogenesis phenotypes of Mfn KO mice. The authors also provide novel interpretation of RNAseq data from the viewpoints of (1) potential overlapping changes in gene expression patterns between Mfn2-cKO and Miwi KO spermatogenic cells and
(2) possible functional links among piRNA-target, MSY2-bound and Mfn2-regulated mRNAs. To address these hypotheses, the authors provide several numbers and/or percentages of overlapping genes, e.g., "~12% (74/599) and ~8% (152/1819) up- regulated genes in Miwi-KO pachytene spermatocytes and round spermatids, respectively, are likely to be up-regulated in that of Mfn2-cKO" (page 16-17). However, these numbers by themselves do not mean anything, i.e. for example, "12%" does not distinguish between whether such "12%" overlap is truly statistically significant or just observed by chance. To draw solid conclusions, such numbers should be very carefully evaluated by proper statistical analyses.
Reply: Thanks to this reviewer for his/her valuable comments. We had re-analyzed the RNA-seq data and re-structured the **Fig.5** to show all up-regulated, down-regulated and unchanged genes from *Miwi*-KO spermatogenic cells mapped onto our *Mfn2*-cKO RNA- seq dataset (**Fig.5E-J** and **Fig.S7**). From the analysis results, only a small number of misregulated genes are overlapped between *Mfn2*-cKO and *Miwi*-KO spermatogenic cells, indicating the complexity of MFN2 in regulating spermatogenesis, which is distinct from that of MIWI. In addition, since the percentage of overlapped genes (e.g. ~12%, 8%) means the overall proportion of the overlapped upregulated genes between *Mfn2*-cKO and *Miwi*-KO spermatogenic cells, there is no necessary to make statistical analyses. To avoid the misunderstanding of our conclusions for the readers, we carefully revised the text according to the new analyses (see lines 412-421).

There also remain several points, on which I made comments in my previous review, but have not yet been addressed in this resubmitted manuscript, as follows,
- page 11: if the authors want to describe this finding ("the thickness of IMC appeared to increase"), actual data and statistical analyses are necessary.
Reply: Thanks for your good advice. We didn't quantify the thickness because we can see the increased thickness of IMC by naked eyes based on the TEM images. We labeled the IMC as blue color in the TEM images to better visualize the defects (see **Fig.3A-B**).

- page 11: lengths and ratios are different.
Reply: Corrected in the revision.

-page12 : is it necessary to carry out RT for mtDNA PCR?
Reply: Thank you for your careful review. Probably, we misunderstood your suggestion for the first revision, and we apologize if so. Basically, for RT in mtDNA copy number analysis, we performed quantitative real-time PCR, not reverse transcription. Sorry for our wrong label and misunderstanding for your last review. We have corrected it in this revision as "quantitative real-time PCR" (see line 349) and also added the method in the "Materials and methods" (see lines 868-872).

- page 27 : it is unclear how to define the distance between mitochondria and ER.
Reply: As shown in the following cartoon (Naon et al., 2016), the shortest distance between the mitochondrial membrane and ER membrane is defined as the distance between mitochondria and ER. We measured it using Image J software.

We have removed unpublished data provided for the referees in confidence.

- fig3d: it appears that the authors defined arbitrary bins for this histogram to find a statistically significant difference in a certain bin (1.2-1.5). If this is not the case, the rationale for defining these specific bins needs to be explained. **Reply:** We referred to previous literature (Santoro et al., 2017; Schneeberger et al., 2013) using aspect ratio (AR) to measure the mitochondrial length. In fact, the AR is a standard method to analyze the morphological change of mitochondria. In our case, AR in 1.2-1.5 range, which relatively tends to be round shape, indicating shorter mitochondria increased in *Mfn2*-cKO compared to that of controls.

- fig3k : it is difficult to see quantitative differences in staining intensities between the samples from these images.
Reply: Figure.3K shows the COX (cytochrome c oxidase, or Complex IV) activity in which reflect the function of mitochondrial respiratory complexes using SDH (succinate dehydrogenase) Complex II, which is entirely encoded by nuclear DNA) staining as control. Based on our staining results, COX staining intensities are increased in both Mfn1-cKO and Mfn2-cKO testis sections, whereas the SDH seems unaltered, consistent with the mtDNA copy number result. We added the method of COX/SDH staining enzyme histochemistry in the revision.

- There are no fig1, fig2 etc. labels on figures.
Reply: Done as suggested.

AUTHORS' STATEMENT:

We thank the Editor and Reviewers for their careful consideration of our revised manuscript and for their helpful comments, which have allowed us to significantly improve the study again. We hope that the reviewers will find that our revised manuscript have significantly enhanced the impact of this work and consider that our manuscript is suitable for publication. Thank you so much!

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Second decision letter

MS ID#: DEVELOP/2020/196295

MS TITLE: MFN2 interacts with Nuage-associated proteins and is essential for male germ cell development by controlling mRNA fate during spermatogenesis

AUTHORS: Xiaoli Wang, Yujiao Wen, Jin Zhang, Shuangshuang Guo, Congcong Cao, Stephen A Krawetz, Zhibing Zhang, and Shuiqiao Yuan

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.

Please address the points raised by reviewer 3 properly. If you are able to revise the manuscript along the lines suggested, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by the reviewer 3, 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

In this manuscript, Wang and colleagues begin by showing the expression pattern of MFN2 in the mouse germline, and then go on to characterize a conditional knockout of Mfn2 (and at times, Mfn1 and the double cKO). They show that Mfn2-cKO mice have reduced testis weight (this effect is earlier in Mfn1 and in the double mutant) and disrupted subcellular structures, including mitochondria, mitochondrial-associated ER membranes (MAMs), and contacts between mitochondria and ER. The authors also show that MFN2 co-immunoprecipitates with many of the proteins known to localize to the nuage in germ cells, including MIWI and DDX4. Several of these proteins show marked decrease in expression or mislocalization in the Mfn2 cKO. The authors show that Â– consistent with disruptions in the nuage Â– the overall levels of pachytene piRNAs are reduced by 50% in the Mfn2 cKO, although transposon activity appears unchanged. RNA-seq data from purified pachytene spermatocytes and round spermatids is presented, and MFN2 is shown to associate with actively-translated (polysomal) fractions. Finally, there are some nice data showing a connection to MSY2, via the RNA-seq data and evidence of SPATA19 protein misexpression. This paper is much improved from its original incarnation and only needs a few tweaks to be publication-ready.

Comments for the author

MFN2 review round 3

This is better (Figures 5 and 6 are much improved!), and I only have a few changes – mostly in the writing.

text

I would still like the description of the RNA-seq data to include the total number of transcripts analyzed in (a) primary spermatocytes and (b) round spermatids. Good #s to have in the body of the text.

421-442: I would be a very happy reviewer if this section got cut/rewritten, especially now that I've seen the clear data in your updated Figure 5. What every reader will take away from Figure 5 is that there really doesn't seem to be any meaningful correlation between the MIWI targets and the MFN2 targets. I mean, except the ones that don't change in either mutant (but that doesn't tell you much). The section in 421-442 is focused on small subsets of RNAs at the margins of your data (which aren't representative of the data set as a whole) and analyzing them to death. This part distracts from your more significant data on MSY2 later in the manuscript. Instead, I would like to see something like this (it may need a little polishing):

We took the Mfn2 cKO vs wt scatter plots and mapped the genes upregulated in Miwi KO PS (Figure 5E) and RS (Figure 5F), downregulated in Miwi KO PS (Figure 5G) and RS (Figure 5H), and unchanged in Miwi KO (Figure 5I and 5J). We found no significant correlation between MIWI targets and MFN2 targets in either spermatocytes or spermatids, suggesting that while MFN2 and MIWI bind in testis, they regulate mostly non-overlapping sets of mRNAs.

(this echoes what you say in lines 417-419 about MFN2, MIWI, and piRNA targets)

444-445: "mRNA translational machinery" almost always means (a) the initiation complex (b) elongation factors (c) termination factors – in other words, the core & canonical proteins that regulate translation in all eukaryotes. I would rewrite the heading from

MFN2 associates with mRNA translational machinery to regulate gamete-specific mRNA fates by interacting with MSY2 in testes

to

MFN2 associates with polysomes and interacts with translational regulator MSY2

"translational machinery" also makes an appearance in lines 34-35… maybe remove this:

and is involved translational machinery that controls the fate of gamete-specific mRNAs in spermatogenesis

Figures

Figure 5C and 5D
Make all the piRNA target RNA dots the same color (i.e. don't artificially separate out the upregulated ones by color). Either all green or all red. The reader will easily see which overlaid dots are in the upregulated / no change / downregulated portions of the Mfn2 cKO RNA-seq data.

Figure 6 legend/title (lines 1174-5) change
MFN2 associates during mRNA translation regulating gamete-specific mRNA fates.

to

MFN2 is associated with polysomes and regulates the fate of gamete-specific mRNAs.

Reviewer 2

Advance summary and potential significance to field

Previously provided.

Comments for the author

The authors appropriately responded to my requests.

Reviewer 3

Advance summary and potential significance to field

In this 2nd revision of the manuscript by Wang et al, the authors chose not to properly respond to several of my previous comments.

Especially, I was very surprised and disappointed to see the authors' reply "In addition since the percentage of overlapped genes (e.g. ~12%, 8%) means the overall proportion of the overlapped upregulated genes between Mfn2-cKO and Miwi-KO spermatogenic cells, there is no necessary to make statistical analyses."

I now doubt that the authors (or al least some of them) may lack very basic understanding of statistics and the critical importance of statistical analyses.

In my opinion, I must conclude that this manuscript does not reach the high standard required for publication in Development.

Comments for the author

The followings are just very elementary explanation of stats relevant to gene overlap interpretation.

First, assume that there are 1000 genes (probes) on a microarray platform, and 100 genes are detected for sample A, and similarly, 100 genes are detected for sample B.
Next, assume that the overlap (%) of the detected genes both in sample A and sample B is "10%", i.e., 10 genes are commonly detected in sample A and sample B, while 90 genes are uniquely detected only in sample A, and other 90 genes are only detected in sample B. Then remaining 810 genes are not detected in both samples. The summary of this assumption is as follows

The total number of genes on a microarray = 1000 genes
sample A detected, sample B detected = 10 genes
sample A not detected, sample B detected = 90 genes
sample A detected, sample B not detected = 90 genes
sample A not detected, sample B not detected = 810 genes

In the above example, the overlap of the detected genes is again "10 %." The VERY important point is that the p-value of this 10% overlap is "1" (Fisher exact test, for an example), and thus is statistically MEANINGLESS. This level of overlap can happen solely by chance, so there is no biological meaning to discuss AT ALL.

Second revision

Author response to reviewers' comments

POINT-BY-POINT RESPONSE TO REVIEWERS

Reviewer #1
MFN2 review round 3

This is better (Figures 5 and 6 are much improved!), and I only have a few changes – mostly in the

writing.
RESPONSE: We appreciate the reviewer's evaluation and valuable comments during the review process, which had significantly improved our manuscript's quality. We also revised the text following your suggestions. Thank you very much.

text
I would still like the description of the RNA-seq data to include the total number of transcripts analyzed in (a) primary spermatocytes and (b) round spermatids. Good #s to have in the body of the text.

421-442: I would be a very happy reviewer if this section got cut/rewritten, especially now that I've seen the clear data in your updated Figure 5. What every reader will take away from Figure 5 is that there really doesn't seem to be any meaningful correlation between the MIWI targets and the MFN2 targets. I mean, except the ones that don't change in either mutant (but that doesn't tell you much). The section in 421-442 is focused on small subsets of RNAs at the margins of your data (which aren't representative of the data set as a whole) and analyzing them to death. This part distracts from your more significant data on MSY2 later in the manuscript. Instead, I would like to see something like this (it may need a little polishing):

We took the Mfn2 cKO vs wt scatter plots and mapped the genes upregulated in Miwi KO PS (Figure 5E) and RS (Figure 5F), downregulated in Miwi KO PS (Figure 5G) and RS (Figure 5H), and unchanged in Miwi KO (Figure 5I and 5J). We found no significant correlation between MIWI targets and MFN2 targets in either spermatocytes or spermatids, suggesting that while MFN2 and MIWI bind in testis, they regulate mostly non-overlapping sets of mRNAs.

(this echoes what you say in lines 417-419 about MFN2, MIWI, and piRNA targets)
RESPONSE: Thank you again for your detailed suggestions. We appreciate your polishing the description and added this description in the revision (lines 422-424). In addition, we performed additional statistical analyses to test the probability of finding these overlaps between the *Mnf2*-cKO and *Miwi*-KO genes in PS and RS following Reviewer#3 's suggestion. The results showed that while fewer genes overlapped than expected (representation factor = 0.8 and 0.7 respectively), the overlap remained significant. We also revised the text according the statistical analyses (lines 430-431, 438-446).

444-445: "mRNA translational machinery" almost always means (a) the initiation complex (b)elongation factors (c) termination factors – in other words, the core & canonical proteins that regulate translation in all eukaryotes. I would rewrite the heading from

MFN2 associates with mRNA translational machinery to regulate gamete- specific mRNA fates by interacting with MSY2 in testes
To
MFN2 associates with polysomes and interacts with translational regulator MSY2
RESPONSE: We agreed with this modification. Thank you.

"translational machinery" also makes an appearance in lines 34-35… maybe remove this *and is involved translational machinery that controls the fate of gamete-specific mRNAs in spermatogenesis*
RESPONSE: Done as suggested. Thanks again.

Figures
Figure 5C and 5D
Make all the piRNA target RNA dots the same color (i.e. don't artificially separate out the upregulated ones by color). Either all green or all red. The reader will easily see which overlaid dots are in the upregulated / no change / downregulated portions of the Mfn2 cKO RNA-seq data.
RESPONSE: All piRNA target RNA dots are now changed to red color in the revised Figure 5C&D. Thank you for your careful review.

Figure 6 legend/title (lines 1174-5) Change
MFN2 associates during mRNA translation regulating gamete-specific mRNA fates.
to

MFN2 is associated with polysomes and regulates the fate of gamete-specific mRNAs.
RESPONSE: Done as suggested. Thanks.

Reviewer #2 Comments for the Author:
The authors appropriately responded to my requests.
RESPONSE: Thank you very much for your satisfaction with our revision of the manuscript.

Reviewer #3 Advance Summary and Potential Significance to Field:
In this 2nd revision of the manuscript by Wang et al, the authors chose not to properly respond to several of my previous comments.
RESPONSE: We are deeply grateful to this reviewer for his/her multiple valuable comments in the revision process, especially for the professional statistical analysis, from which we learned a lot. We sincerely apologize that we did not fully grasp your points thoroughly and subsequently did not properly respond to the comments. Therefore, we made a supreme effort to address the comments from the 2nd round review in more detail this time. In this revision, we consulted Dr. Grace Swanson, who is experienced in statistical analyses of RNA-seq data and asked for help to analyze the overlapping data. We hope you will be satisfied with our revision and statistical analysis in this round.

Especially, I was very surprised and disappointed to see the authors' reply "In addition, since the percentage of overlapped genes (e.g. ~12%, 8%) means the overall proportion of the overlapped upregulated genes between Mfn2-cKO and Miwi-KO spermatogenic cells, there is no necessary to make statistical analyses."
I now doubt that the authors (or at least some of them) may lack very basic understanding of statistics and the critical importance of statistical analyses.
In my opinion, I must conclude that this manuscript does not reach the high standard required for publication in Development.
RESPONSE: We sincerely apologize for our misleading description due to not understanding your aim fully. To be honest, none of the authors are professional statisticians and didn't realize the critical importance of statistical analyses for the overlapping number. Hence, we sought help from Dr. Grace Swanson as described above to analyze the data in this revision.

Dr. Grace Swanson performed additional calculations to test the probability of finding these overlaps between the *Mnf2*-cKO and *Miwi*-KO genes in PS and RS. First, a representation factor was calculated to determine if the number of overlaps was expected. As indicated in the revised manuscript (lines 409-413, 422-431, 438-445), the number of overlaps found were < 1.0 in all cases expect for the 304 overlapping down-regulated genes in PS cells (lines 441-443) which was > 1.0. This indicates that the number of overlaps were less than expected, with the one exception. The 304 down-regulated overlapping genes was greater than expected. The probability of these overlaps was then calculated using the exact hypergeometric probability (distribution) and normal approximation (where appropriate) (lines 409-413, 422-431 and 854-872). The hypergeometric distribution is comparable to the one-tailed Fisher's exact test. It is a common method to evaluate whether the probability of the gene overlap is significant. This calculation is used to describe the expected number of successful draws from a finite population without replacement. When possible, the exact hypergeometric distribution is calculated. Normal approximation of the hypergeometric distribution is a method used under circumstances that the number of genes in experiment 1 (here taken to be the up-/down-regulated Mnf2-cKO genes) * 10 < the total genes (total sequenced genes in PS/RS in the Mnf2-cKO).
From these calculations, the 34 (PS)/32 (RS) up-regulated genes overlapping the 330 putative genes from Goh et al., 2015, did not meet significance due to a low number of overlaps. The remaining overlaps of concern however, met significance thresholds indicating they are not by chance. Together, although MFN2 and MIWI only regulate a smaller overlapping set of mRNAs than expected, this ontological analysis suggests that MFN2 and MIWI (a Nuage protein) are involved in some similar cellular pathways that control mRNA fates during spermatogenesis and at least partly contribute to regulating meiotic transcripts in spermatogenic cells (lines 445-449).
Besides, the statistical analysis method was also added to the "*Materials and Methods*" section in the revision (lines 854-872).

Reviewer 3 Comments for the Author:
The followings are just very elementary explanation of stats relevant to gene overlap interpretation. First, assume that there are 1000 genes (probes) on a microarray platform, and 100 genes are detected for sample A, and similarly, 100 genes are detected for sample B. Next, assume that the

overlap (%) of the detected genes both in sample A and sample B is "10%", i.e., 10 genes are commonly detected in sample A and sample B, while 90 genes are uniquely detected only in sample A, and other 90 genes are only detected in sample B. Then remaining 810 genes are not detected in both samples. The summary of this assumption is as follows

The total number of genes on a microarray = 1000 genes sample A detected, sample B detected = 10 genes sample A not detected, sample B detected = 90 genes sample A detected, sample B not detected = 90 genes
sample A not detected, sample B not detected = 810 genes
In the above example, the overlap of the detected genes is again "10 %." The VERY important point is that the p-value of this 10% overlap is "1" (Fisher exact test, for an example), and thus is statistically MEANINGLESS. This level of overlap can happen solely by chance, so there is no biological meaning to discuss AT ALL.
RESPONSE: Thank you very much for giving us an example to illustrate how to conduct statistical analysis. We consulted with Dr. Grace Swanson, and asked for help to analyze our data as following processes:

http://nemates.org/MA/progs/representation.stats.html
probability by Exact hypergeometric probability and normal approximation where appropriate.

- In **Fig5C**: 34/330 in PS cells:

Set1: 4046 (Significant up in *Mfn2*-cKO) Set1: 330 (piRNA targets)
Overlap: 34
Total number of genes: 31021
Representation factor: 0.8
***p*-value < 0.077**

- In **Fig5D**: 32/330 in RS cells:

Set1: 3756 (Significant up in *Mfn2*-cKO) Set2: 330 (piRNA targets)
Overlap: 32
Total number of genes: 33726
Representation factor: 0.9
***p*-value < 0.230**

- In **Fig.5E** and **Fig.S7A**: 74/680 in PS cells: Set1: 4046 (Significant up in *Mfn2*-cKO) Set2: 680 (Significant up in *Miwi*-KO) Overlap: 74

Total number of genes: 31021
Representation factor: 0.8
***p*-value < 0.049**

- In **Fig.5F** and **Fig.S7C**: 152/1971 in RS cells: Set1: 3756 (Significant up in *Mfn2*-cKO) Set2: 1971 (Significant up in *Miwi*-KO) Overlap: 152

Total number of genes in MFN2 dataset: 33726
Representation factor: 0.7
***p*-value < 1.137e-07**

- In **Fig.5G** and **Fig.S7G** 304/1541 in PS cells: Set1: 5324 (Significant down in *Mfn2*-cKO) Set2: 1541 (Significant down in *Miwi*-KO) Overlap: 304

Total number of genes: 31021
Representation factor: 1.1
***p*-value < 0.004**

- In **Fig.5H** and **Fig.S7I**: 108/1757 in RS cells: Set1: 3186 (Significant down in *Mfn2*-cKO) Set2: 1757 (Significant down in *Miwi*-KO)

Overlap: 108
Total number of genes: 33726
Representation factor: 0.7

***p*-value < 1.846e-07**

Thereinto, the total number of genes used above is the total detected genes in *Mfn2*-cKO RNAseq dataset, including significantly up-regulated, down- regulated, and nonsignificant genes, the number is 31021 in PS cells and 33726 in RS cells.

RESPONSE AGAIN TO REVIEWER #3's COMMENTS IN 2nd ROUND

Reviewer 3 Advance Summary and Potential Significance to Field:
The resubmitted manuscript by Wang et al. shows several improvements according to the reviewers' comments on the original version of the manuscript.

RESPONSE: Thank you very much for your evaluation of our work. We appreciate your suggestions to further improve our study.

Reviewer 3 Comments for the Author:
I now appreciate the authors' effort to cite and discuss the other researchers' work about spermatogenesis phenotypes of Mfn KO mice. The authors also provide novel interpretation of RNAseq data from the viewpoints of (1) potential overlapping changes in gene expression patterns between Mfn2-cKO and Miwi KO spermatogenic cells and (2) possible functional links among piRNA-target, MSY2-bound and Mfn2-regulated mRNAs. To address these hypotheses, the authors provide several numbers and/or percentages of overlapping genes, e.g., "~12% (74/599) and ~8% (152/1819) up-regulated genes in Miwi-KO pachytene spermatocytes and round spermatids, respectively, are likely to be up-regulated in that of Mfn2-cKO" (page 16-17). However, these numbers by themselves do not mean anything, i.e. for example, "12%" does not distinguish between whether such "12%" overlap is truly statistically significant or just observed by chance. To draw solid conclusions, such numbers should be very carefully evaluated by proper statistical analyses.

RESPONSE: Thanks to this reviewer for his/her valuable comments and concerns. We have asked an experienced statistician for help to do statistical analyses of those overlapping genes. Please see our response in detail as above (see 3rd round response).

There also remain several points, on which I made comments in my previous review, but have not yet been addressed in this resubmitted manuscript, as follows,

- page 11: if the authors want to describe this finding ("the thickness of IMC appeared to increase"), actual data and statistical analyses are necessary.

RESPONSE: This is a good suggestion. We quantified the IMC thickness as suggested using Image J software, and the method is similar to that of mito-ER distance calculation. As shown in Figure A, white lines are used to mark IMC thickness, then we used Image J to calculate the values and make statistical analysis. We found that the increased thickness in *Mfn2*-cKO is significant, as shown in B after statistical analyses by *t*-test (*P*-value = 0.024).

We have removed unpublished data provided for the referees in confidence.

- page 11: lengths and ratios are different.

RESPONSE: Corrected in the revision. Thank you.

-page12: is it necessary to carry out RT for mtDNA PCR?
Reply: Thank you for your careful review. Probably, we misunderstood your suggestion for the first revision, and we apologize if so. Basically, for RT in mtDNA copy number analysis, we performed quantitative real-time PCR, not reverse transcription. Sorry for our wrong label and misunderstanding for your last review. We have corrected it in this revision as "quantitative real-time PCR" (line 306) and also added the method in the "Materials and methods" (see lines 746-750).

- page 27: it is unclear how to define the distance between mitochondria and ER.

RESPONSE: To define and calculate the distance between mitochondria and ER, we referred to the published literature (Naon et al., 2016) and measured them using Image J software. As shown in the following schematic Figure (A), the average shortest distance between the mitochondrial membrane and ER membrane is defined as mito-ER distance. If the ER-mitochondria contact site is long, multiple sites were chosen to get the average distance (site a in A); if the ER-mitochondria contact site is short, the shortest distance will be calculated (sites b-d in A). Figure B is an example to illustrate how to calculate the mito-ER distance in TEM images.

We have removed unpublished data provided for the referees in confidence.

- fig3d: it appears that the authors defined arbitrary bins for this histogram to find a statistically significant difference in a certain bin (1.2-1.5). If this is not the case, the rationale for defining these specific bins needs to be explained.

RESPONSE: We referred to previous literature (Santoro et al., 2017; Schneeberger et al., 2013) using aspect ratio (AR) to measure the mitochondrial length. Although the AR is a common method to analyze the morphological change of mitochondria, there is no absolute standard to define what range should be used. In the published literature, authors usually showed a difference according to their own data. In our case, AR in the 1.2-1.5 range tends to be round in shape, indicating shorter mitochondria increased in *Mfn2*- cKO than that of controls.

- fig3k: it is difficult to see quantitative differences in staining intensities between the samples from these images.

RESPONSE: Figure 3K shows the COX (cytochrome c oxidase, or Complex IV) activity in which reflect the function of mitochondrial respiratory complexes using SDH (succinate dehydrogenase) Complex II, which is entirely encoded by nuclear DNA) staining and usually should have no change, thereafter SDH signals are used as negative control. Based on our staining results, the brown signals are darker in both *Mfn1*-cKO and *Mfn2*-cKO testis sections, indicating the COX staining intensities are increased, whereas the SDH seems unaltered, consistent with the mtDNA copy number result. Indeed, the increased signals of COX are not hugely obvious but only mildly to moderately, but we still can see the difference. We added the method of COX/SDH staining enzyme histochemistry in the revision.

- There are no fig1, fig2 etc. labels on figures.

RESPONSE: We added the labels on the Figures. Thank you.

Third decision letter

MS ID#: DEVELOP/2020/196295

MS TITLE: MFN2 interacts with Nuage-associated proteins and is essential for male germ cell development by controlling mRNA fate during spermatogenesis

AUTHORS: Xiaoli Wang, Yujiao Wen, Jin Zhang, Grace Swanson, Shuangshuang Guo, Congcong Cao, Stephen A Krawetz, Zhibing Zhang, and Shuiqiao Yuan

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 referee appreciated to your consultation with the statistics expert in this third version. The reviewer, however, found the interpretation of the results still quite ambiguous and suggests modifications in this part. You thus need to amend this part before we can consider publication. If you are able to revise the manuscript along the lines suggested, I will be happy receive a revised version of the manuscript. Your revised paper will be re-reviewed by this referee, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers'

major concerns. Please note that this "is" a last opportunity I can offer you to further revise the manuscript.

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 3

Advance summary and potential significance to field

In this revised manuscript (4th round?), the authors consulted Dr. Grace Swanson who is (according to the authors) experienced in statistical analyses.

Comments for the author

Actually, the numbers and stats shown in the "authors responses to the reviewers letters" now look good and appropriate, such like (for an example) 4. In Fig.5F and Fig.S7C: 152/1971 in RS cells:
Set1: 3756 (Significant up in Mfn2-cKO)
Set2: 1971 (Significant up in Miwi-KO)
Overlap: 152 Total number of genes in MFN2 dataset: 33726 Representation factor: 0.7 p-value < 1.137e-07 However, its interpretation described in the main text "While fewer genes overlapped than expected (representation433 factor = 0.8 and 0.7 respectively), the overlap remained significant" (page 15, lines 432-433) is unfortunately and totally wrong.
I think Dr. Grace Swanson surely did good stats, but probably did not check/interfere in the original authors' manuscript sentences/biological interpretation.
Please ask Dr. Grace whether the two gene sets are really significantly overlapping, OR, mutually exclusive.

Third revision

Author response to reviewers' comments

RESPONSE TO REVIEWER #3

Reviewer 3 Advance Summary and Potential Significance to Field:
In this revised manuscript (4th round?), the authors consulted Dr. Grace Swanson, who is (according to the authors) experienced in statistical analyses.

RESPONSE: We appreciate this reviewer's evaluation and valuable comments during the whole review process, which had significantly improved our manuscript's quality. At this round, we asked Dr. Grace Swanson to carefully check the statistical analyses of two gene sets and revise the text to interpret the results accurately. Thank you again for your careful review.

Reviewer 3 Comments for the Author:
Actually, the numbers and stats shown in the "authors responses to the reviewers letters" now look

good and appropriate, such like (for an example) 4. In Fig.5F and
Fig.S7C: 152/1971 in RS cells:
Set1: 3756 (Significant up in Mfn2-cKO) Set2: 1971 (Significant up in Miwi-KO) Overlap: 152
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However, its interpretation described in the main text "While fewer genes overlapped than expected (representation433 factor = 0.8 and 0.7 respectively), the overlap remained significant" (page 15, lines 432-433) is unfortunately and totally wrong.
I think Dr. Grace Swanson surely did good stats, but probably did not check/interfere in the original authors' manuscript sentences/biological interpretation.
Please ask Dr. Grace whether the two gene sets are really significantly overlapping, OR, mutually exclusive.

RESPONSE: We thank this Reviewer for his/her careful scrutiny of our work again. We consulted Dr. Swanson and confirmed the statistical result changes. To make the biological interpretation more rigorous, only overlapping gene numbers with representation factor >1.0 and p-value <0.05 will be considered significant.
Based on this criterion, only the 304 overlapping downregulated genes identified in PS were meaningful with significantly changed representation factor and P-value (p-value = 0.004, representation factor = 1.1). In contrast, the other overlapped genes in *Mfn2*-cKO and *Miwi*-KO (upregulated genes in both PS and RS, and downregulated genes in RS) are significantly less than expected, considered to be overlapped by random chance.
In detail, for the upregulated genes, the overlap of ~10% (74/680, representation factor = 0.8, p-value = 0.049) and ~7% (152/1971, representation factor = 0.7, p-value = 1.137e-7) upregulated genes, are significantly less than was expected. As such, these overlapping upregulated genes in PS and RS of *Mfn2*-cKO and *Miwi*-KO were obtained by random chance, which indicates that they are unlikely to be co-regulated by MIWI and MFN2 in testes.
For the downregulated genes, the overlaps account for ~25% (304/1541, p-value = 0.004, representation factor = 1.1) and ~6% (108/1757, p-value = 1.846e-7, representation factor = 0.7) of downregulated genes in *Miwi*-KO PS and RS, separately. Therefore, the 304 overlapping genes identified in PS was significantly more than expected, while the 108 overlapping genes in RS were significantly less than expected, indicating that the 304 overlapping downregulated genes in PS are related while those overlapping downregulated genes in RS are found by chance.
We revised the text and interpreted these statistical analyses in this revision accordingly (**See lines 405-406, 423-428, 435-438, 441-443, and highlighted it with yellow color**). In addition, making the data interpretation accurate for the readers, we removed the heatmaps and GO analysis of the overlapping upregulated genes in PS/RS and overlapping downregulated genes in RS as these genes are by chance rather than related. Only the heatmap and GO term data for overlapping downregulated genes in PS were kept (**See new Fig.S7**).

Once again, we thank this Reviewer for his/her time and valuable, helpful comments. We hope that this reviewer will be satisfied with our revision and considered that our manuscript is suitable for publication.

Fourth decision letter

MS ID#: DEVELOP/2020/196295

MS TITLE: MFN2 interacts with Nuage-associated proteins and is essential for male germ cell development by controlling mRNA fate during spermatogenesis

AUTHORS: Xiaoli Wang, Yujiao Wen, Jin Zhang, Grace Swanson, Shuangshuang Guo, Congcong Cao, Stephen A Krawetz, Zhibing Zhang, and Shuiqiao Yuan

I have now received the referee report 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.

The reviewer appreciated to your efforts and recognized the manuscript improved. Nonetheless, she/he was still unsatisfied as you see in her/his comments. Despite of her/his unsatisfaction, I found the manuscript sufficiently improved through the multiple rounds of revision. We, thus, would like to publish a revised manuscript in Development, provided that the referees' comments can be editorially incorporated. Please attend to the reviewers' comments in your revised 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.

We are aware that you may currently be unable to access the lab to undertake experimental revisions. 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.

Reviewer 3

Advance summary and potential significance to field

In this revised version of the manuscript. the authors made an improvement on their presentation of gene overlap analyses (fig 5).

Comments for the author

However, unfortunately, I have to point out that the authors still severely misunderstand the statistical interpretation of their own data. In the responses to reviewers letter, for an example, the authors state that "In detail, for the upregulated genes, the overlap of ~10% (74/680, representation factor = 0.8, p-value = 0.049) and ~7% (152/1971, representation factor = 0.7, p-value = 1.137e-
7) upregulated genes, are significantly less than was expected. As such, these overlapping upregulated genes in PS and RS of Mfn2-cKO and Miwi-KO were obtained by random chance ..."

This interpretation is incorrect. The point is that "p-value = 1.137e-7"
indicates "the observation is very UNLIKELY obtained by random chance." In other words, "representation factor = 0.7" should probably have some meaning (the two gene sets are regulated by INDEPENDENT/MUTUALLY EXCLUSIVE pathways etc.)

This is not a trivial issue, but is relevant to basic understanding of statistics, which is a prerequisite when publishing biological omics data and analyses.

Fourth revision

Author response to reviewers' comments

POINT-BY-POINT RESPONSE TO REVIEWER #3

Reviewer 3 Advance Summary and Potential Significance to Field:
In this revised version of the manuscript. the authors made an improvement on their presentation of gene overlap analyses (fig 5).

RESPONSE: We appreciate this reviewer for his/her rigorous evaluation and comments during the whole review process, which had significantly improved our manuscript's quality.

Reviewer 3 Comments for the Author:
However, unfortunately, I have to point out that the authors still severely misunderstand the

statistical interpretation of their own data. In the responses to reviewers letter, for an example, the authors state that "In detail, for the upregulated genes, the overlap of ~10% (74/680, representation factor = 0.8, p-value = 0.049) and ~7% (152/1971, representation factor = 0.7, p-value = 1.137e-7) upregulated genes, are significantly less than was expected. As such, these overlapping upregulated genes in PS and RS of Mfn2-cKO and Miwi-KO were obtained by random chance ..."

This interpretation is incorrect. The point is that "p-value = 1.137e-7" indicates "the observation is very UNLIKELY obtained by random chance." In other words, "representation factor = 0.7" should probably have some meaning (the two gene sets are regulated by INDEPENDENT/MUTUALLY EXCLUSIVE pathways etc.)

This is not a trivial issue, but is relevant to basic understanding of statistics, which is a prerequisite when publishing biological omics data and analyses.

RESPONSE: Thank you for your careful scrutiny. Indeed, as you said, "p-value = 1.137e-7" indicates "the observation is very UNLIKELY obtained by random chance.", so we modified the statistical interpretation following the suggestion to avoid misunderstanding (See lines 427 and 438). Meanwhile, since the upregulated overlapping gene number in PS (representation factor = 0.8) and RS (representation factor = 0.7) is far less than expected (¤1.0), they are unlikely to be co-regulated by MIWI and MFN2 in testes no matter whether the two gene sets might be regulated by independent or mutually exclusive pathways.

Thanks again for this reviewers' valuable comments in the revision process, especially for the statistical analysis, which enriched our statistical experience and significantly benefited our future research work.

Fifth decision letter

MS ID#: DEVELOP/2020/196295

MS TITLE: MFN2 interacts with Nuage-associated proteins and is essential for male germ cell development by controlling mRNA fate during spermatogenesis

AUTHORS: Xiaoli Wang, Yujiao Wen, Jin Zhang, Grace Swanson, Shuangshuang Guo, Congcong Cao, Stephen A Krawetz, Zhibing Zhang, and Shuiqiao Yuan
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.