Genetic and structural analysis of the *in vivo* functional redundancy between murine NANOS2 and NANOS3

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MS TITLE: Genetic and structural analysis of the *in vivo* functional redundancy between murine NANOS2 and NANOS3

AUTHORS: Danelle Wright, Makoto Kiso, and Yumiko Saga

I have now received all the referees' reports on the above manuscript, and have reached a decision. The referees' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

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

We are aware that you may 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.

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

NANOS2 and NANOS3 are structurally related germ cell-specific proteins. NANOS2 is crucial in male germ cell differentiation in embryonic testis, while NANOS3 plays a role in survival of primordial germ cells. In the current study, the authors investigated structural requirements of NANOS2 for its distinct roles from those of NANOS3 in germ cells in mouse embryos.

They first showed that NANOS3 was dispensable in male germ cell differentiation by generating the Nanos3 cKO mouse, but NANOS3 was necessary for germ cell maintenance in the Nanos2 deficient condition by using Nanos2/3 dKO mice, suggesting functional redundancy of NANOS2 and NANOS3 in terms of germ cell survival in embryonic testis .

DND1, a binding partner of NANOS2 is important for the functions of NANOS2, and the expression of DND1 can be maintained either by NANOS2 or NANOS3. The zinc finger domain (ZF) of NANOS2 is needed for interaction with DND1, and the authors consistently found that a chimeric protein of NANOS3 N-terminal and NANOS2 ZF more efficiently interacted with DND1 compared with that of NANOS2 N-terminal and NANOS3 ZF in cultured cells. However, the expression of both the chimeric proteins failed to rescue Nanos2 deficiency in germ cells in embryonic testis, indicating that both the N-terminal and ZF of NANOS2 are crucial for its functions.

Finally, the authors attempted to identify critical amino acids in the ZF of NANOS2 for interaction with DND1 and found that a mutation in Y111 in NANOS2 and the triple mutations in NANOS3 partially reduced and increased, respectively, the interaction with DND1. They also tested whether NANOS3 could interacted with CNOT1, another partner of NANOS2, but neither a CNOT interaction motif of NANOS3 nor its mutated form failed to bind to CNOT1.

This study provided important information concerning structural basis for the distinct functions of NANOS2 and NANOS3. The results revealed that both the N-terminal region and the ZF of NANOS2 were crucial for its function in male germ cell differentiation.

Comments for the author

Even though the authors demonstrated the importance of both the N-terminal and the ZF are crucial for the NANOS2 functions, structural requirements of NANOS2 for its interaction with its partner molecules still remain unclear.

Major concerns;
In the experiments of the amino acid replacements of NANOS2 and NANOS3, more comprehensive studies likely provide definite conclusions. Concerning the ZF of Nanos2 (Fig.6B), the authors discussed that ‘even all three mutations together failed to increase the strength to NANOS2 levels’ (lines 336-337). If so, multiple replacements including the additional three candidate amino acids may be worth doing. In addition, in a CNOT interaction motif in NANSO3 (Fig.6F), replacement of the additional two amino acids which are different between human and mouse, and their combinations for interaction with CNOT1 as well as for rescuing Nanos2 deficiency in gem cells by the chimeric protein expression, may be worth trying.

Additional comments;
1. Lines 318-319 (‘However, as 3xF-NANOS2-N58-NANOS3-ZF(ΔC46) was expressed at levels similar to endogenous NANOS2 in vivo)’: Comparison with the endogenous NANOS2 expression is not found in the figures.
2. Lines 395-398 (‘inability to bind to the CCR4-NOT deadenylation complex correctly (3xF-Nanos3N54-
Nanos2ZF)’, ‘In the case of 3xF-Nanos3N54-396 Nanos2ZF germ cells, the phenotype was milder than 3xF-Nanos2N58-Nanos3ZF(ΔC46) germ cells’): Corresponding data cannot be found in the figures.
3. Fig. 1B, Fig.2B, D: Please briefly describe the methods for quantitative evaluation of the positive cells.

4. Fig.4: Please explain the colored amino acids other than red in the panel A. The color codes in the panels C, D are confusing. Please explain a reason why some FLAG negative cells are DAZL positive in the panel E.

Reviewer 2

Advance summary and potential significance to field

This manuscript by Wright et al. revealed function of Nanos3 in mouse germ cells during sex determination.
The authors previously found that Nanos3 is upregulated in Nanos2-null germ cells in the germ cells at the midgestation stage. However, the function of Nanos3 is not fully understood, since Nanos2-null germ cells feminized and eventually died. To address this issue, the authors provided Nanos2- and Nanos3-conditional knockout (cKO) and found that the double cKO mice rapidly lost their germ cells after the sex determination.
The cKO mice suggested a distinct role of Nanos3, as germ cells in the double cKO did not show feminization represented by Stra8 expression. To see the molecular mechanism of the distinct function, the authors tested interaction of DND1 and CNOT1 with NANOS proteins. Biochemical analyses demonstrated that the zinc finger domain and N-terminal region in NANOS2 are essentially required for binding to DND1 and CNOT1 respectively. Based on these results, the authors concluded that Nanos3 has an independent role on preventing apoptosis from germ cells during sex determination, which could be caused by the difference in the interactors.

Comments for the author

This paper provides novel findings of Nanos3 on germ cell differentiation during sex determination. Although the function is recognized under a Nanos2-null condition, these findings are potentially interesting. However there are several experimental flaws that greatly attenuate the value of this work. In principal, quantitative analysis with the correct control experiment is overall required. Specifically, it is important to exclude a possibility that the rapid loss of germ cells in the double cKO is caused by early loss of germ cells due to precocious loss of Nanos3 before E13.5. As described below, the authors should consider additional experiment to support their interpretation in this study.

Specific comments:
1. The authors concluded that there was no difference in the number of germ cells in Nanos3-cKO, when Nanos3 was deleted at E11.5. It is necessary to show the efficiency of Cre-mediated gene deletion in the embryos for this analysis. The comparable germ cell number could be due to few cells having the deleted allele.

2. Based on Figure 1D, it looks that DAZL expression is enhanced in Nanos3 KO. Is it possible to quantify the results?

3. The authors concluded that there is no difference in the germ cell number between the double cKO and the control. However, this is too preliminary. How can author make such conclusion with only one E13.5 dKO embryo? Also, it is concerned that the germ/soma ratio is an optimal way to interpret data. Why not the authors count the absolute number of the germ cells? This is crucial, because the image in the Figure 2A gives an impression that the number is decreased in E13.5 dKO.

4. Again, the authors observation of apoptosis, Stra8 and Ki67 expression in cKO (Figure 2C-F) should be confirmed by quantitative analysis using multiple, at least three, embryos. Is the number of E13.5 dKO in these examinations only one?

5. The authors should compare the frequency of apoptosis and the number of germ cells between Nanos2 and dKO. Otherwise it is impossible to interpret the authors conclusion.

6. Based on the immunostaining analysis of DND1 in cKO, the authors concluded thst DND1 is destabilized in the cKO. But this is not supported by the biochemical analysis, since DND1 expression alone in HEK293T or ES cells show a similar stability of DND1, compared to co-expression

of NANOS2 or 3 protein. It is a rough interpretation that the loss of DND1 in germ cells is via “a germ cell-specific mechanism”.

7. The signal of DND1 in Figure 5A should be quantified. This is important for both interpretation of the reduced expression in the cKO with NANOS3-ZF and reproducibility of the result.

Minor comments
1. It looks redundant between Figure 3A and B.
2. The high magnification image in Figure E (top, right images) do not show precisely same view.

Reviewer 3

Advance summary and potential significance to field

In this manuscript Wright et al. elucidate the cause for the previously observed inability of NANOS3 to rescue male germ cell development in a NANOS2 null mutant, despite the close similarity of these two proteins. They show that NANOS2 and NANOS3 differ in efficiency of N-terminal binding to CNOT1 and in the efficiency of the C-terminal ZF domain to binding to DND1. They show that (1) only NANOS2 can efficiently bind to CNOT1 through its N-terminal domain, and (2) the specific structure of the C-terminal domain of NANOS2 is required to maintain DND1 expression based on IF. Although NANOS3 can rescue apoptosis of germ cells in the absence of NANOS2, a deletion of Nanos3 alone has no effect on male germ cell differentiation. The key findings enhance our understanding of the interplay between 4 key germ cell RNA binding proteins (NANOS2, NANOS3, DAZL, DND1), and will be a valuable contribution to the field.

Comments for the author

Experiments are carefully performed – the transgenics carrying chimeric NANOS proteins are particularly nice -- and the data overall is convincing. However, the authors fail to report any information about RNA levels of these genes, which is a strange omission considering that all of the genes involved are RNA binding proteins that regulate the levels of other RNAs. In Highlights and elsewhere, the authors claim that, “Expression of DND1 is dependent upon strong binding to the NANOS2 zinc finger domain”. It is very important to support this finding by investigating the RNA level of Dnd1. I recognize that the authors believe that loss of DND1 results from an instability of the protein when not bound to the NANOS2 zinc finger. However, since DND1 seems to have functions independent of NANOS2, it is unlikely that it must be bound to NANOS2 to be stable. This is an important detail that, if not addressed, could lead to misinterpretation of these results.

Overall, the manuscript is well written and clear. Below, we provide suggestions for writing corrections, additions of more references/details in the text, and improvements of figures. The method section is lacking in multiple sections, including the omission of a description of the western blotting protocol used, and antibody listing that provides catalog numbers, concentrations used, published references for gifted antibodies, etc. There are also acronyms that are not defined.

- Could the authors please explain how Nanos2mcherry/mcherry results in a null allele
- Line 54-55 – it would be nice to reference a recent review on critical roles of RBPs in germ cells
- Line 67 –this might be a good place to reference Erez Raz’s review as a comprehensive current review on the matter
- Line 164-167 - For antibodies that were not gifts, please add catalog numbers
- Please provide a description of the western blot protocol in the methods section along with vendor and catalog information for the antibodies used. This information is missing from the method section with only some details scattered into other parts of the methods section in an incomplete manner
- Line 193 – “[Glutathione-S-transferase] GST pull-down…”
- Line 208 – “confirmed by [Coomassie Brilliant Blue] CBB staining.”

- Line 216 – “As the amount of NANOS3 protein is very low in embryonic male germ cells” [ref]
- Line 243-244 – Please provide a reference to support this rationale relating SOX9-positive somatic cell number and timing of apoptotic entry by germ cells. Could the authors make a supplemental figure with the quants to support this claim?
- Line 245-246 – This is very confusing as written. Please include a reference for the assertion that Nanos2-KO germ cell also die by apoptosis after upregulation of STRA8.
- Line 264-273 - Do the authors have further support for the validity of these in vitro experiments in support of DND1 stability in germ cells? Levels of Dnd1 RNA should be reported.
- Line 284-286 – Is there any structural/functional information about NANOS3 that validates removing this 46AA C-terminal sequence without unintended consequences?
- Line 305 – this should be reference 22, it might also be prudent to reference [Chu, C. & Rana, T. M. Translation Repression in Human Cells by MicroRNA-Induced Gene Silencing Requires RCK/p54. PLoS Biol. 4, e210 (2006).] as source literature on DDX6/RCK
- Line 311 – “To [assess] this possibility…”
- Line 326-327 – specifically deletion of C61A and/or C96A? Since there are multiple cysteines in this motif could the authors please be specific?
- Line 402 – “may not have [be] functioning”
- Line 422-423 – you might also reference the recent work by Diana Laird on the apoptotic wave (Daniel H. Nguyen, Diana J. Laird. Apoptosis in the fetal testis eliminates developmentally defective germ cell clones. bioRxiv 601013; doi: https://doi.org/10.1101/601013)
- Line 565 – Insets are enlarged to what extent specifically?
- Line 575 – Sertoli cells (not somatic cells)
- Line 578 – n=1 for E13.5 quantification is inappropriate for statistical analysis
- Line 675 – “proteins in the Phyre2 engine [ref 24?]”
- Fig1A – it would be best to note on the layout that the stain in these images is anti-E-cad so that the reader does not mistake it for Nanos2-mCherry signal
- Fig1B – it would be very helpful to have a supplementary image showing the somatic cell stain that was used for counts to generate the denominator of these ratios
- Fig1B – it is unusual to produce germ cell ratios that take into account all somatic cells as the denominator, instead of only Sertoli cells. Do the authors have an explanation for this decision?
- Fig1B – the information for how this data was collected for statistical analysis is missing from the methods section
- Fig1C and D – noting the stage in the layout in addition to in the legend would clarify which stage is being shown where.
- Fig1C and D – Could the authors produce a supplemental figure for Fig1C and Fig1D that is a timecourse of E13.5, E14.5 and E15.5 as in Fig1A?
- Fig2A - are the E13.5 images exemplary of images that were actually used in counts? In these sections cords are missing from the center of most mutants. Is this an additional phenotype specific to the mutant? In this case the phenotype needs to be reported.
- Fig2B – same comments/ concerns as Fig1B, however Fig1A would seem to indicate that these are actually germ cell/Sertoli cell ratios and NOT germ cell/somatic cell ratios. Which is it?
- Fig2C – the representative image for E14.5 does not agree with the quants in Fig2D (ie this image does not show ~40% germ cell apoptosis). Do the authors have an E14.5 image that agrees with the quants in Fig2D?
- Fig2D - the information for how this data was collected for statistical analysis is missing from the methods section
- Fig2F – insets that enlarge germ cell nuclei for clearer views of KI-67 signal would be helpful
- Fig3D – Is the pair of bands for DND1 due to DND1 isoforms? Has this been reported previously with this anti-DND1 antibody? If so, please reference.
- Fig2C and D, and FigS2 – there is no reference to band size in kDa in the figure or the legend. Please add this information somewhere in the figure.
- Fig4A – the T-coffee algorithm Expresso is mentioned and referenced in the legend but a description of specifically what was done and accession numbers for the sequences used are missing from the methods section and must be supplied.
- Fig4A – the boxes used to represent distinct areas of the NANOS2/3 AA sequence are too thick and partially obstruct some of the AAs. Could the authors make these box outlines thinner or more ideally under/over-line or background shade the regions?

- Fig4A - What is the alignment and coloring scheme used for this alignment? It does not seem to be one AA over the other in the alignment and the coloring does not seem to apply to individual AAs, both of which seem unconventional. Perhaps the authors could better define this in the text or add a method section
- Fig4B – the box outlines denoting the different regions of NANOS proteins need to be "Nudged" in Photoshop so that they are consistently spaced (specifically, either overlapping or adjacent box outlines in all parts of figure and the removal of all gaps between boxes)
- Fig4C and D – It is difficult to draw conclusions based on these single composites, can the authors provide breakouts of these channels ideally as enlarged/zoomed insets?
- Fig4C, D and E - Please add time point labels to the actual images to avoid stage confusion for this figure
- Fig4D and Line 294 - Did the authors extend their timecourse to E17.5 for this image because this is the earliest that STRA8 is observed? The timing of STRA8 up-regulation in both versions of the chimeric NANOS proteins may be worth reporting.
- FigS3 and Line 295-296 - Is the same anti-NANOS3 antibody used for both IF and western blot? Is the C-terminus detection of this antibody specific to the 46AA C-terminus region of NANOS3 that is deleted in the chimera?
- FigsS4A - Please box the cells that are featured in the enlarged breakout to make clear which cells are being shown.
- Fig5A and FigS4 and Line 307-308 – Some of the chimeric germ cells seem to have high levels of DND1 that is restricted to the nucleus. Do the authors feel that DND1 expression is always reduced in chimeric germ cells or that efficiency of nuclear export of DND1 may be hindered in some cases?
- Fig5A and FigS4 – The authors have previously reported that E15.5 is the period when male germ cells have the highest levels of P-bodies. Did the authors image other timepoints to see if peak P-body levels are shifted earlier or later in the context of chimeric NANOS?
- Fig6A – similar comments to Fig4A
- Fig6D – What are the green and yellow highlights?
- Fig6E and F - do the authors have an explanation for why GST-CNOT1-3 was detected only with Coomassie and not an antibody?
- Fig6E and F – the cartoon "plus" signs are confusing and distracting. Please consider a way to reformat this layout.

First revision

Author response to reviewers' comments

Responses to Reviewer 1:
We would like to thank the reviewer for taking the time to review our paper. Our responses to each of your suggestions are presented below.

Reviewer 1 Comments for the Author:
Even though the authors demonstrated the importance of both the N-terminal and the ZF are crucial for the NANOS2 functions, structural requirements of NANOS2 for its interaction with its partner molecules still remain unclear.

Major concerns;
In the experiments of the amino acid replacements of NANOS2 and NANOS3, more comprehensive studies likely provide definite conclusions. Concerning the ZF of Nanos2 (Fig.6B), the authors discussed that 'even all three mutations together failed to increase the strength to NANOS2 levels' (lines 336-337). If so, multiple replacements including the additional three candidate amino acids may be worth doing. In addition, in a CNOT interaction motif in NANSO3 (Fig.6F), replacement of the additional two amino acids which are different between human and mouse, and their combinations for interaction with CNOT1 as well as for rescuing Nanos2 deficiency in gem cells by the chimeric protein expression, may be worth trying.

Response:

Thank you for this important comment. We additionally created the NANOS3 mutant containing all 6 candidate amino acids and performed IP experiments. However, even the 6-mutant NANOS3 was unable to further increase DND1 precipitation compared with the F107Y mutant and 3-mutant NANOS3. We replaced the IP data in Figure 6C with representative experimental Western blotting data containing the 6-mutant NANOS3. Regarding the CNOT1 interaction motif, the chimeric protein consisting of the NANOS2 N-terminal, which can bind CNOT1, and NANOS3 zinc finger was unable to rescue NANOS2 function *in vivo*. Therefore, even if CNOT1 is bound, without the specific NANOS2 zinc finger, NANOS2 function cannot be rescued. Compared with our chimera analysis, GST pull-down using the mutated recombinant CNOT1 is unlikely to provide additional information.

Additional comments;
1. Lines 318-319 ('However, as 3xF-NANOS2-N58-NANOS3-ZF(ΔC46) was expressed at levels similar to endogenous NANOS2 in vivo)': Comparison with the endogenous NANOS2 expression is not found in the figures.

Response:

Thank you for this comment. We performed immunostaining for FLAG using testes from 3xF-NANOS2 heterozygous mice as a control, and 3xF-NANOS2-N58-NANOS3-ZF(ΔC46) and 3xF-NANOS3-N54-NANOS2-ZF chimeric mice. The levels of FLAG staining were similar between chimeric protein-expressing cells and 3xF-NANOS2-expressing cells, confirming that both chimeric proteins were expressed to a similar degree as control 3xF-NANOS2. Immunostaining images were added as Supplementary Figure 3A.

2. Lines 395-398 ('inability to bind to the CCR4-NOT deadenylation complex correctly (3xF-Nanos3N54- Nanos2ZF)', 'In the case of 3xF-Nanos3N54-396 Nanos2ZF germ cells, the phenotype was milder than 3xF-Nanos2N58-Nanos3ZF(ΔC46) germ cells'): Corresponding data cannot be found in the figures.

Response:

Thank you for this comment. The milder phenotype described refers to the higher expression of DNMT3L, smaller degree of DND1 protein loss and delayed STRA8 upregulation observed in the 3xF-NANOS3-N54- NANOS2-ZF-expressing germ cells compared with 3xF-NANOS2-N58-NANOS3-ZF(ΔC46)-expressing germ cells. This so-called "milder" phenotype was most likely caused by the retention of DND1 expression and the NANOS2 ZF. We quantified DNMT3L expression, as shown in new Figure 4D. Although a direct comparison of DNMT3L expression between chimeras is difficult because the DNMT3L expression level varies even in wild- type cells, more 3xF-NANOS3-N54- NANOS2-ZF-expressing germ cells were DNMT3L-positive, whereas almost all 3xF-NANOS2-N58-NANOS3-ZF(ΔC46)-expressing germ cells were negative for DNMT3L. DND1 expression was also quantified and data were added as Figure 5B. When the two types of chimeric testes were stained together, DND1 expression was significantly higher in 3xF-NANOS3-N54- NANOS2-ZF-expressing germ cells than in 3xF-NANOS2-N58-NANOS3-ZF(ΔC46)-expressing germ cells. We described this in the results and discussion sections. (p. 12-13, lines 317-325; p. 18 lines 440-443)

3. Fig. 1B, Fig.2B, D: Please briefly describe the methods for quantitative evaluation of the positive cells.

Response:

We added the methods for quantification to the Statistical analysis section on p. 10 lines 222-226.

"To quantify immunofluorescence, signals on testis sections stained on the same slide from 3 embryos for each genotype were measured in Fiji. Cell counts were also similarly performed using Fiji. Significant differences between genotypes were assessed using the unpaired Student's t-test with GraphPad Prism 8."

4. Fig.4: Please explain the colored amino acids other than red in the panel A. The color codes in

the panels C, D are confusing. Please explain a reason why some FLAG negative cells are DAZL positive in the panel E.

Response:

We apologize for the lack of description about the coloring. The protein sequences in Fig. 4A were aligned by amino acid structural similarity by the T-Coffee ESPRESSO algorithm. Red indicates a high degree of similarity, yellow is moderate, and green is low similarity. We added this information to the figure legend.

DAZL is a germ cell-specific protein and is used as a marker of germ cells. All germ cells express DAZL.

Responses to Reviewer 2:

We would like to thank the reviewer for taking the time to review our manuscript. Our responses to your suggestions are presented below.

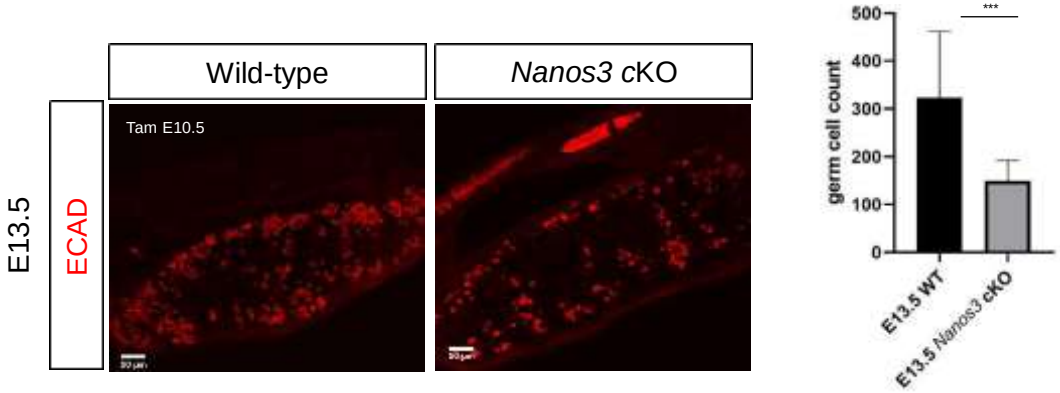
Reviewer 2 Comments for the Author:
This paper provides novel findings of Nanos3 on germ cell differentiation during sex determination. Although the function is recognized under a Nanos2-null condition, these findings are potentially interesting. However, there are several experimental flaws that greatly attenuate the value of this work. In principal, quantitative analysis with the correct control experiment is overall required. Specifically, it is important to exclude a possibility that the rapid loss of germ cells in the double cKO is caused by early loss of germ cells due to precocious loss of Nanos3 before E13.5. As described below, the authors should consider additional experiment to support their interpretation in this study.

Thank you for this important comment.

In *Nanos3* straight KO, germ cells do not immediately die but undergo apoptosis while migrating to the gonad. As such, at E13.5, if NANOS3 were lost precociously before germ cells entered the testis, there would be few if any germ cells remaining. In order to exclude the effects of precocious NANOS3 deletion, we injected tamoxifen at E11.5 and confirmed that NANOS3 is already removed at E13.5 without changing cell number (Supplemental Fig. 1C). We also tested tamoxifen injection at E10.5, a day earlier, and collected *Nanos3* cKO embryos at E13.5. As expected, there were fewer germ cells than in the WT E13.5 testes.

Therefore, the number of germ cells in the dKO was not different from WT at E13.5, indicating that NANOS3 was not lost before injecting tamoxifen at E11.5.

We are providing images for E10.5-injected cKO embryos for the reviewer below



Specific comments:
1. The authors concluded that there was no difference in the number of germ cells in Nanos3-cKO, when Nanos3 was deleted at E11.5. It is necessary to show the efficiency of Cre-mediated gene deletion in the embryos for this analysis. The comparable germ cell number could be due to few cells having the deleted allele.

Response:

As we already answered your question above, we confirmed effective Cre-mediated knockout.

2. Based on Figure 1D, it looks that DAZL expression is enhanced in Nanos3 KO. Is it possible to quantify the results?

Response:

Thank you for pointing this out. We quantified DAZL expression in control, *Nanos3* cKO and *Nanos2* KO germ cells, and found that DAZL expression was indeed upregulated in *Nanos3* cKO, although this increase was still not as great as that in *Nanos2* KO. Therefore, although the loss of NANOS3 did not affect male differentiation, it did cause a slight phenotype. In wild-type testes, NANOS3 is present until NANOS2 becomes expressed, and as we consider NANOS3 to be a "weak" version of NANOS2, it may be able to slightly repress *Dazl* even though it is not a specific target mRNA.

We added the quantification data as Figure 1E, and newly described this point in the Results and Discussion. (p. 10-11 lines 245-249; p. 17 line 414)

3. The authors concluded that there is no difference in the germ cell number between the double cKO and the control. However, this is too preliminary. How can author make such conclusion with only one E13.5 dKO embryo? Also, it is concerned that the germ/soma ratio is an optimal way to interpret data. Why not the authors count the absolute number of the germ cells? This is crucial, because the image in the Figure 2A gives an impression that the number is decreased in E13.5 dKO.

Response:

Thank you for this comment. We apologize for the miswording. We used the SOX9-positive Sertoli cell number to calculate ratios, not the overall somatic cell number, which is a standard method. We revised this throughout the manuscript and figures. At the time of submission, we only had one E13.5 embryo, i.e., two testes. We have since collected more E13.5 embryos, so all analyses are n=3. We added quantitative data and images using the newly collected embryos.

4. Again, the authors observation of apoptosis, Stra8 and Ki67 expression in cKO (Figure 2C-F) should be confirmed by quantitative analysis using multiple, at least three, embryos. Is the number of E13.5 dKO in these examinations only one?

Response:

Please see the above response.

5. The authors should compare the frequency of apoptosis and the number of germ cells between Nanos2 and dKO. Otherwise it is impossible to interpret the authors conclusion.

Response:

Thank you for this important comment. We newly compared cPARP staining among wild-type, dKO and *Nanos2* KO at E14.5. The number of cPARP signals did not significantly differ between wild-type and Nanos2 KO testes, which is consistent with previous reports on *Nanos2* KO stating that germ cells do not immediately die. However the number of cPARP signals was nearly double in the dKO, reflecting the high rate of cell death due to the additional loss of NANOS3. Immunostaining images and quantification of cPARP signals were added as new Fig. 2C,D.

6. Based on the immunostaining analysis of DND1 in cKO, the authors concluded that DND1 is destabilized in the cKO. But this is not supported by the biochemical analysis, since DND1 expression alone in HEK293T or ES cells show a similar stability of DND1, compared to co-expression of NANOS2 or 3 protein. It is a rough interpretation that the loss of DND1 in germ cells is via "a germ cell-specific mechanism".

Response:

Thank you for this comment. We agree that this statement was speculative, and hence removed it from the text.

7. The signal of DND1 in Figure 5A should be quantified. This is important for both interpretation of the reduced expression in the cKO with NANOS3-ZF and reproducibility of the result.

Response: Thank you for this comment. We quantified the DND1 fluorescence signal in both chimeras and added the data as new Figure 5B.

Minor comments

1. It looks redundant between Figure 3A and B

Response:

According to the reviewer's comment, we removed Figure 3B.

2. The high magnification image in Figure E (top, right images) do not show precisely same view.

Response:

We revised the figure accordingly

Responses to Reviewer 3:

We would like to thank the reviewer for taking the time to review our manuscript. Our responses to your comments are presented below.

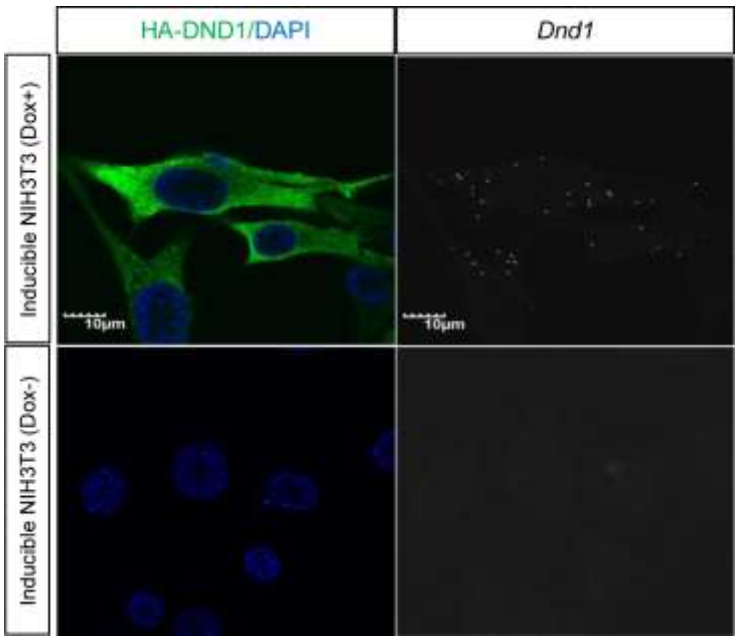
Reviewer 3 Comments for the Author:
Experiments are carefully performed – the transgenics carrying chimeric NANOS proteins are particularly nice -- and the data overall is convincing. However, the authors fail to report any information about RNA levels of these genes, which is a strange omission considering that all of the genes involved are RNA binding proteins that regulate the levels of other RNAs. In Highlights and elsewhere, the authors claim that, "Expression of DND1 is dependent upon strong binding to the NANOS2 zinc finger domain". It is very important to support this finding by investigating the RNA level of Dnd1. I recognize that the authors believe that loss of DND1 results from an instability of the protein when not bound to the NANOS2 zinc finger. However, since DND1 seems to have functions independent of NANOS2, it is unlikely that it must be bound to NANOS2 to be stable. This is an important detail that, if not addressed, could lead to misinterpretation of these results.

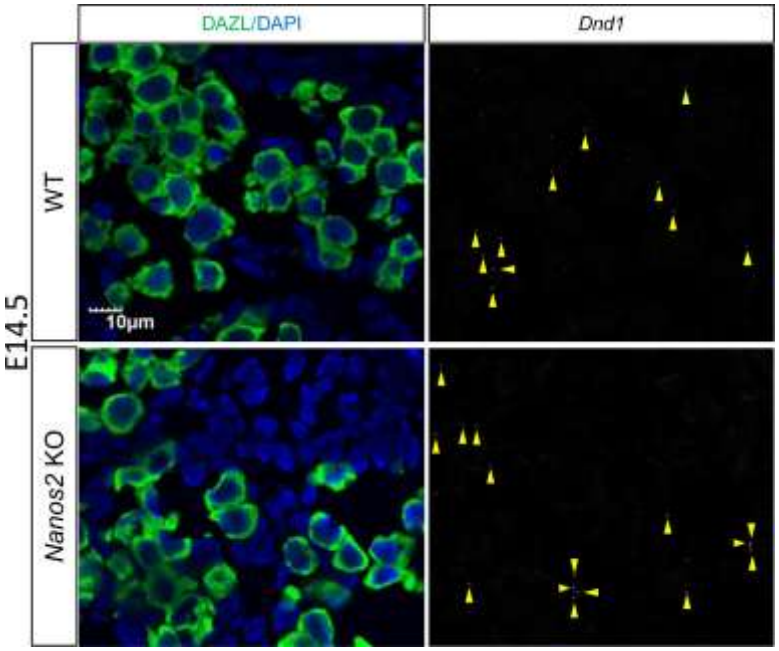
Overall, the manuscript is well written and clear. Below, we provide suggestions for writing corrections, additions of more references/details in the text, and improvements of figures. The method section is lacking in multiple sections, including the omission of a description of the western blotting protocol used, and antibody listing that provides catalog numbers, concentrations used, published references for gifted antibodies, etc. There are also acronyms that are not defined.

Response:
Thank you for your important comments and suggestions. We agree that addressing the mRNA levels of *Dnd1* is essential. According to our current single-cell RNAseq data of wild-type and *Nanos2* KO, *Dnd1* mRNA levels are unchanged at E14.5 even though the DND1 protein level is lower in *Nanos2* KO than in wild-type germ cells. As we cannot prepare mRNA from chimeric embryos, we

tried to detect *Dnd1* mRNA directly using ViewRNA technology. However, the expression of *Dnd1* mRNA was not high enough to detect the signals in tissue sections for quantification. In addition, as there are so few germ cells remaining at E14.5 in the dKO, it is not possible to collect enough cells to measure the RNA level. Thus, although the analyses of *Nanos2* KO germ cells support our idea that DND1 without partner protein NANOS2 is destabilized, we tried not to overstate the mechanism. We added single-cell RNAseq data as Supplementary figure 2B to show this.

ViewRNA results are presented for the reviewer below





•Could the authors please explain how Nanos2mcherry/mcherry results in a null allele

Response:

We apologize for our lack of description regarding this point. The schema for creating the Nanos2 ^{mcherry} mouse line was newly added as Supplementary Figure 1A.

•Line 54-55 – it would be nice to reference a recent review on critical roles of RBPs in germ cells

Response:

We added a reference.

Licatalosi, D.D., 2016. Roles of RNA-binding Proteins and Post-transcriptional Regulation in Driving Male Germ Cell Development in the Mouse, in: Yeo, G.W. (Ed.), RNA Processing: Disease and Genome-Wide Probing, Advances in Experimental Medicine and Biology. Springer International Publishing, Cham, pp. 123–151. https://doi.org/10.1007/978-3-319-29073-7_6

•Line 67 –this might be a good place to reference Erez Raz's review as a comprehensive current review on the matter

Response:

We added the reference.

•Line 164-167 - For antibodies that were not gifts, please add catalog numbers

Response:

Thank you for this comment. We added the catalog numbers for antibodies. (p.6-7 lines 166-171)

•Please provide a description of the western blot protocol in the methods section along with vendor and catalog information for the antibodies used. This information is missing from the method section with only some details scattered into other parts of the methods section in an incomplete manner

Response:

Thank you for this comment. We added a description of the Western blotting protocol and information for the used antibodies in the methods section. (p.6-7 lines 166-188)

•Line 193 – "[Glutathione-S-transferase] GST pull-down…"

Response:

We revised the manuscript accordingly.

•Line 208 – "confirmed by [Coomassie Brilliant Blue] CBB staining."

Response:

We revised the manuscript accordingly

•Line 216 – "As the amount of NANOS3 protein is very low in embryonic male germ cells" [ref]

Response:

We added the relevant reference for this point.

•Line 243-244 – Please provide a reference to support this rationale relating SOX9-positive somatic cell number and timing of apoptotic entry by germ cells. Could the authors make a supplemental figure with the quants to support this claim?

Response:

Thank you for this comment. We apologize for the confusing wording. The Sertoli cell number was

not related to the timing of germ cell death. We wished to describe that more germ cells in the dKO had died via apoptosis by E14.5 when compared with wild-type. In response to the comment from Reviewer 2, we also added data for *Nanos2* KO for comparison. The numbers of cPARP signals were compared among wild-type, Nanos2 KO, and dKO testes at E14.5 in the new Fig. 2C, D.

•Line 245-246 – This is very confusing as written. Please include a reference for the assertion that Nanos2-KO germ cell also die by apoptosis after upregulation of STRA8.

Response:

We apologize for the confusing wording and added the reference for *Nanos2* KO germ cells dying by apoptosis.

Suzuki, A., Saga, Y., 2008. Nanos2 suppresses meiosis and promotes male germ cell differentiation. Genes Dev. 22, 430–435. https://doi.org/10.1101/gad.1612708

•Line 264-273 - Do the authors have further support for the validity of these in vitro experiments in support of DND1 stability in germ cells? Levels of Dnd1 RNA should be reported.

Response:

Please see the response to comment 1.

•Line 284-286 – Is there any structural/functional information about NANOS3 that validates removing this 46AA C-terminal sequence without unintended consequences?

Response:

Thank you for this comment. To our knowledge, no study to date has examined the role of the 46AA NANOS3 C terminal sequence.

•Line 305 – this should be reference 22, it might also be prudent to reference [Chu, C. & Rana, T. M. Translation Repression in Human Cells by MicroRNA-Induced Gene Silencing Requires RCK/p54. PLoS Biol. 4, e210 (2006).] as source literature on DDX6/RCK

Response:

Thank you for pointing this out. We added the reference.

•Line 311 – “To [assess] this possibility…”
Response:

We revised the manuscript accordingly.

•Line 326-327 – specifically deletion of C61A and/or C96A? Since there are multiple cysteines in this motif could the authors please be specific?

Response:

We specified the cysteines as C61 and C96 in the text (p. 15 line 365)

•Line 402 – “may not have [be] functioning”
Response:

We revised the manuscript accordingly.

•Line 422-423 – you might also reference the recent work by Diana Laird on the apoptotic wave (Daniel H. Nguyen, Diana J. Laird. Apoptosis in the fetal testis eliminates developmentally defective germ cell clones. bioRxiv 601013; doi: https://doi.org/10.1101/601013)

Response:

We added the reference.

•Line 565 – Insets are enlarged to what extent specifically?

Response:

Insets are enlarged by 2x. We added this to the figure legend.

•Line 575 – Sertoli cells (not somatic cells)

Response:

We revised the manuscript accordingly.

•Line 578 – n=1 for E13.5 quantification is inappropriate for statistical analysis

Response:

Thank you for this comment. At the time of submission, we only had one E13.5 embryo, i.e., two testes. We have since collected more E13.5 embryos so all time points are n=3.

•Line 675 – "proteins in the Phyre2 engine [ref 24?]"

Response:

We apologize for this mistake. We revised the reference.

•Fig1A – it would be best to note on the layout that the stain in these images is anti-E-cad so that the reader does not mistake it for Nanos2-mCherry signal

Response:

Thank you for this comment. We indicated E-cadherin staining in the figure.

•Fig1B – it would be very helpful to have a supplementary image showing the somatic cell stain that was used for counts to generate the denominator of these ratios

Response:

We apologize for the miswording. We used the SOX9-positive Sertoli cell number to generate the ratios.

•Fig1B – it is unusual to produce germ cell ratios that take into account all somatic cells as the denominator, instead of only Sertoli cells. Do the authors have an explanation for this decision?

Thank you for this comment. We apologize for our confusing wording. Only Sertoli cells were used to measure the ratios. We revised the manuscript and figures accordingly.

•Fig1B – the information for how this data was collected for statistical analysis is missing from the methods section

Response:

We added the methods for quantification to the Statistical analysis section on p. 10 lines 223-226.

"To quantify immunofluorescence, signals on testis sections stained on the same slide from 3 embryos for each genotype were measured in Fiji. Cell counts were also similarly performed using Fiji. Significant differences between genotypes were assessed using the unpaired Student's t-test

with GraphPad Prism 8."

•Fig1C and D – noting the stage in the layout in addition to in the legend would clarify which stage is being shown where.

Response:

We added the stage labels accordingly.

•Fig1C and D – Could the authors produce a supplemental figure for Fig1C and Fig1D that is a time course of E13.5, E14.5 and E15.5 as in Fig1A?

Response:

We added a time course of E13.5, E14.5, and E15.5 *Nanos3* cKO and control for DNMT3L staining as Supplemental figure 1D.

•Fig2A - are the E13.5 images exemplary of images that were actually used in counts? In these sections cords are missing from the center of most mutants. Is this an additional phenotype specific to the mutant? In this case the phenotype needs to be reported.

Response: Thank you for this comment. We replaced the image in Fig. 2A. The absence of cords in the center was only observed in one testis of one embryo, but not a specific phenotype. We apologize for the poor image choice. To count cells, multiple sections from the same testis were used to account for anatomical variability such as the lack of cords in a given area.

•Fig2B – same comments/ concerns as Fig1B, however Fig1A would seem to indicate that these are actually germ cell/Sertoli cell ratios and NOT germ cell/somatic cell ratios. Which is it?

Response:

Please see the response to the comment for Fig. 1B

•Fig2C – the representative image for E14.5 does not agree with the quants in Fig2D (ie this image does not show ~40% germ cell apoptosis). Do the authors have an E14.5 image that agrees with the quants in Fig2D?

Response:

Thank you for this comment. We newly compared cPARP staining among wild-type, dKO and *Nanos2* KO at E14.5. The number of cPARP signals did not significantly differ between wild-type and Nanos2 KO testes, which is consistent with previous reports on *Nanos2* KO stating that germ cells do not immediately die.
However the number of cPARP signals was nearly double in the dKO, reflecting the high rate of cell death due to the additional loss of NANOS3. Immunostaining images and quantification of cPARP signals were added as new Fig. 2C,D.

•Fig2D - the information for how this data was collected for statistical analysis is missing from the methods section

Response:

Please see response to the comment for Fig. 1B

•Fig2F – insets that enlarge germ cell nuclei for clearer views of KI-67 signal would be helpful

Response:

We revised the figure to show enlarged images to more clearly see the Ki-67 signal.

•Fig3D – Is the pair of bands for DND1 due to DND1 isoforms? Has this been reported previously with this anti- DND1 antibody? If so, please reference.

Response:

Thank you for this comment. For Western blotting, the anti-HA antibody was used, not anti-DND1. We specified this in the figure legend. However, the reason for the double bands with the ES cell lysate is unknown.

•Fig2C and D, and FigS2 – there is no reference to band size in kDa in the figure or the legend. Please add this information somewhere in the figure.

Response:

We added the band size to the figures.

•Fig4A – the T-coffee algorithm Expresso is mentioned and referenced in the legend but a description of specifically what was done and accession numbers for the sequences used are missing from the methods section and must be supplied.

Response:

We revised the manuscript accordingly.

•Fig4A – the boxes used to represent distinct areas of the NANOS2/3 AA sequence are too thick and partially obstruct some of the AAs. Could the authors make these box outlines thinner or more ideally under/over-line or background shade the regions?

Response:

We revised the figure accordingly.

•Fig4A - What is the alignment and coloring scheme used for this alignment? It does not seem to be one AA over the other in the alignment and the coloring does not seem to apply to individual AAs, both of which seem unconventional. Perhaps the authors could better define this in the text or add a method section

Response:

We apologize for the lack of description about the coloring. The protein sequences in Fig. 4A were aligned by amino acid structural similarity by the T-Coffee ESPRESSO algorithm. Red indicates a high degree of similarity, yellow is moderate, and green is low similarity. We added this information to the figure legend.

•Fig4B – the box outlines denoting the different regions of NANOS proteins need to be “Nudged” in Photoshop so that they are consistently spaced (specifically, either overlapping or adjacent box outlines in all parts of figure and the removal of all gaps between boxes)

Response:

We revised the figure accordingly.

•Fig4C and D – It is difficult to draw conclusions based on these single composites, can the authors provide breakouts of these channels ideally as enlarged/zoomed insets?

Response:

Thank you for this comment. We revised this figure and newly added the individual channels for STRA8 and FLAG staining to more clearly show the time course.

•Fig4C, D and E - Please add time point labels to the actual images to avoid stage confusion for this figure

Response:

We added time point labels.

•Fig4D and Line 294 - Did the authors extend their time course to E17.5 for this image because this is the earliest that STRA8 is observed? The timing of STRA8 up-regulation in both versions of the chimeric NANOS proteins may be worth reporting.

Response:

Thank you for this important comment. In *Nanos2* KO, upregulated STRA8 expression can be seen from E14.5. In the Nanos2N-Nanos3-ZF(del46) chimeras, STRA8 expression was observed at E15.5, which is the time point we started analysis. However, STRA8 upregulation was not observed in the Nanos3N-Nanos2ZF chimeras at E15.5. By E17.5, both chimeric protein-expressing germ cells had upregulated STRA8 expression. We added images of E15.5 STRA8 immunostaining to Figure 4E to show this delay in STRA8 expression and revised the text regarding this point. (p. 14 lines 321-325)

•FigS3 and Line 295-296 - Is the same anti-NANOS3 antibody used for both IF and western blot? Is the C- terminus detection of this antibody specific to the 46AA C-terminus region of NANOS3 that is deleted in the chimera?

Response:

Thank you for this comment. The same NANOS3 antibody was used for IHC and Western blotting. This antibody detects the 46AA C-terminal of NANOS3. Please see Supplementary Figure 3B for Western blotting confirming this.

•FigsS4A - Please box the cells that are featured in the enlarged breakout to make clear which cells are being shown.

Response:

We revised the figure accordingly.

•Fig5A and FigS4 and Line 307-308 – Some of the chimeric germ cells seem to have high levels of DND1 that is restricted to the nucleus. Do the authors feel that DND1 expression is always reduced in chimeric germ cells or that efficiency of nuclear export of DND1 may be hindered in some cases?

Response:

Thank you for this comment. Regarding DND1 expression, even in wild-type testes, the amount of DND1 in the nucleus versus in the cytoplasm on immunohistochemistry varies among cells. As DND1 is known to shuttle between the nucleus and cytoplasm, the time of fixation likely affects how much DND1 is localized at a certain area at a given time. In the Nanos2N-Nanos3-ZF(del46) chimera, the overall amount of DND1, both cytoplasmic and nuclear, was reduced in all chimeric germ cells. As most of the remaining DND1 was localized to the cytoplasm, our current hypothesis, albeit only speculative at this point, is that DND1 becomes trapped in the cytoplasm. We wish to address this point in future studies.

•Fig5A and FigS4 – The authors have previously reported that E15.5 is the period when male germ cells have the highest levels of P-bodies. Did the authors image other time points to see if peak P-body levels are shifted earlier or later in the context of chimeric NANOS?

Response:

We also performed immunostaining for P-bodies using E16.5 and E17.5 chimeric testes. However,

there was no notable change in P-body number.

•Fig6A – similar comments to Fig4A

Response:

Please see the response for Fig. 4A.

•Fig6D – What are the green and yellow highlights?

Response:
Fig. 6D shows the NIM domains aligned by amino acid structural similarity by the T-Coffee ESPRESSO algorithm. Red indicates a high degree of similarity, yellow is moderate, and green is low similarity. We added this information to the figure legend.

•Fig6E and F - do the authors have an explanation for why GST-CNOT1-3 was detected only with Coomassie and not an antibody?

Response:

Thank you for this comment. As GST-CNOT1-3 was only shown with Coomassie staining in the previous report our analysis was based off of, we presented the results in the same manner. Suzuki, A., Niimi, Y., Saga, Y., 2014. Interaction of NANOS2 and NANOS3 with different components of the CNOT complex may contribute to the functional differences in mouse male germ cells. Biol. Open 3, 1207–1216. https://doi.org/10.1242/bio.20149308

•Fig6E and F – the cartoon “plus” signs are confusing and distracting. Please consider a way to reformat this layout.

Response:
We revised this figure accordingly.

Second decision letter

MS ID#: DEVELOP/2020/191916

MS TITLE: Genetic and structural analysis of the *in vivo* functional redundancy between murine NANOS2 and NANOS3

AUTHORS: Danelle Wright, Makoto Kiso, and Yumiko Saga

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

In the revised manuscript, the authors have addressed all of my concerns.

Comments for the author

In the revised manuscript, the authors have addressed all of my concerns.

Reviewer 2

Advance summary and potential significance to field

In the revised manuscript, the authors properly addressed to concerns raised in the first round of reviews. The quantification outcomes support the authorÂ's conclusions. This reviewer therefore thinks that the revised version of the manuscript satisfies scientific novelty and experimental quality for publication in Development.

Comments for the author

It could attract a general interest if the authors speculate an evolutional trait of Nanos2 and 3, since this study indicates that Nanos3 is a weak version of Nanos2, as the authors recognize. Anyway, this is a minor suggestion.

Reviewer 3

Advance summary and potential significance to field

This manuscript is greatly improved by the authorsÂ' attention to reviewersÂ' comments and corrections and clarifications throughout. Overall, I feel the manuscript makes an important contribution to our understanding of the roles of Nanos2 and Nanos3 and their interactions with DND1.

Comments for the author

I have a few remaining queries and comments:

Lines 268 and following – This statement is contradictory with the statement in lines 276-278. Do Nanos2 KO germ cells resume "mitotic activity" or is the cell cycle arrested "in a large proportion of germ cells"? I don't understand how to evaluate the conclusion (lines 278-279) that "in the absence of NANOS2, NANOS3 may suppress apoptosis by regulating genes related to the cell cycle". Although Fig.2F supports this view, the paragraph should be clarified.

Line 281 – It would be better to be specific: NANOS2 is required for the maintenance of DND1 protein expression in vivo.

Lines 294-298 and Fig. 3B – I agree with the main point here, but Is 3xF-NANOS2 destabilized, or is it affected by cycloheximide treatment because it has a shorter half-life than DND1? This might be an important consideration.

Lines 310-311 – I don't understand the rationale for deleting the longer C-terminal of NANOS3. Could the authors try explaining this again? Do they have evidence that N3N58-N3-ZF(DC46) can rescue Nanos3 in vivo?

Line 432 – crystallization of the huDND1- RRM domain has been done: https://www.biorxiv.org/content/10.1101/2020.03.05.978023v1.full

Line 475 – Ki-67 indicates reentry into active cell cycle, but not necessarily progress through M-phase. This might be important for the interpretation of Ki67 staining. Perhaps this is the S-phase preceding meiosis.

Line 485 – should be mRNAs

Second revision

Author response to reviewers' comments

We would like to thank the reviewers for their additional comments and queries, which further improved our revised manuscript. Our responses are provided in a point-by-point format below and all changes to the manuscript are indicated in red. We hope that our responses sufficiently answer their concerns.

Reviewer 2 Comments for the Author:
It could attract a general interest if the authors speculate an evolutional trait of Nanos2 and 3, since this study indicates that Nanos3 is a weak version of Nanos2, as the authors recognize. Anyway, this is a minor suggestion.

Thank you for this suggestion. We also agree that the evolution of NANOS2 and 3 is of great interest, especially across species. We know that NANOS3 is required in mammals, but its actual function remains unknown and the expression pattern of NANOS2 in the human embryonic testis is unclear. There are limited studies on this topic, making speculation difficult. Although we described mouse NANOS3 as a weak version of NANOS2, this may not be the case for human NANOS3. The mouse is a good model to investigate the conservation of functions of Nanos family proteins, for example by knocking human NANOS3 into the mouse *Nanos2* locus. We hope that future studies can address this and help form an evolutionary basis for the separation of their functions.

Reviewer 3 Comments for the Author:
I have a few remaining queries and comments:

Lines 268 and following – This statement is contradictory with the statement in lines 276-278. Do Nanos2 KO germ cells resume "mitotic activity" or is the cell cycle arrested "in a large proportion of germ cells"? I don't understand how to evaluate the conclusion (lines 278-279) that "in the absence of NANOS2, NANOS3 may suppress apoptosis by regulating genes related to the cell cycle". Although Fig.2F supports this view, the paragraph should be clarified.

We apologize for the confusion. In the *Nanos2* KO case, the cell cycle is arrested at E14.5 in many germ cells, but this arrest cannot be maintained and they resume the cell cycle from E15.5. In the dKO case, this temporary arrest at E14.5 is not observed. We considered one of reasons for this to be the presence of NANOS3. We added a statement that the cell cycle arrest observed in *Nanos2* KO germ cells is only temporary at E14.5 on line 277.

Line 281 – It would be better to be specific: NANOS2 is required for the maintenance of DND1 protein expression in vivo.

We revised the heading accordingly.

Lines 294-298 and Fig. 3B – I agree with the main point here, but Is 3xF-NANOS2 destabilized, or is it affected by cycloheximide treatment because it has a shorter half-life than DND1? This might be an important consideration.

Thank you for this comment. Indeed, we cannot say whether 3xF-NANOS2 was destabilized or it had a faster turnover rate. We therefore revised the text from “destabilized” to “decreased”.

Lines 310-311 – I don’t understand the rationale for deleting the longer C-terminal of NANOS3. Could the authors try explaining this again? Do they have evidence that N3N58-N3-ZF(DC46) can rescue Nanos3 in vivo?

We apologize for the confusing description. The C-terminal of NANOS3 was deleted to assess the possibility of the extra length interfering with DND1 binding. The chimeric protein lacking the C-terminal was expressed *in vivo* without interfering with interaction with DND1, but was unexpectedly poorly expressed *in vitro*. As we wanted to assess DND1 interaction by immunoprecipitation, we added back the C terminal and found that the protein was well expressed *in vitro*. The C-terminal affected protein stability under *in vitro* conditions only, and we unfortunately do not have any evidence about the removal of the C-terminal of NANOS3 *in vivo*. This was a minor point that was only described in the text to explain the immunoprecipitation results. We revised the text on line 311 to state that we only deleted the C-terminal to check if it interfered with DND1 binding.

Line 432 – crystallization of the huDND1- RRM domain has been done: https://www.biorxiv.org/content/10.1101/2020.03.05.978023v1.full

We added the reference.

Line 475 – Ki-67 indicates reentry into active cell cycle, but not necessarily progress through M-phase. This might be important for the interpretation of Ki67 staining. Perhaps this is the S-phase preceding meiosis.

Thank you for this comment. As STRA8 was not expressed, we did not consider the dKO germ cells to be entering meiosis. We changed our wording regarding Ki-67 staining to “cell cycle” rather than “proliferation” as it is unclear exactly which phase the germ cells are in.

Line 485 – should be mRNAs

We revised the text accordingly.

Third decision letter

MS ID#: DEVELOP/2020/191916

MS TITLE: Genetic and structural analysis of the <em style=3D"font-style: italic; margin: 0px; padding: 0px; border: 0px; outline: 0px; font-size: 100%; vertical-align: baseline; background-color: transparent;">in vivo functional redundancy between murine NANOS2 and NANOS3

AUTHORS: Danelle Wright, Makoto Kiso, and Yumiko Saga
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.