Zebrafish *dazl* regulates cystogenesis and germline stem cell specification during the primordial germ cell to germline stem cell transition.

Sylvain Bertho, Mara Clapp, Torsten U. Banisch, Jan Bandemer, Erez Raz and Florence L. Marlow
DOI: 10.1242/dev.187773

Editor: Steve Wilson

Review timeline
Original submission: 31 December 2019
Editorial decision: 11 February 2020
First revision received: 15 April 2020
Editorial decision: 27 May 2020
Second revision received: 24 December 2020
Editorial decision: 23 February 2021
Third revision received: 2 March 2021
Accepted: 4 March 2021

Original submission

First decision letter

MS ID#: DEVELOP/2019/187773

MS TITLE: Zebrafish dazl regulates cystogenesis upstream of the meiotic transition and germline stem cell specification and independent of meiotic checkpoints.

AUTHORS: Sylvain Bertho, Mara Clapp, Torsten U Banisch, Jan Bandemer, Erez Raz, and Florence L. Marlow

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 (two of whom sign their reviews) express considerable interest in your work, but have some criticisms and quite a lot of suggestions for improving the manuscript. If you are able to revise the manuscript along the lines suggested, I will be happy receive a revised version of the manuscript. Please also note that Development will normally permit only one round of major revision.

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

Comments:

Zebrafish provides an exciting molecular genetic system to examine the maternal control of germline development by germ plasm and sex chromosome-independent mechanisms of sex determination in a vertebrate animal. This manuscript contributes to this field through its examination of the early formation of germline cysts and the finding that the conserved RNA-binding protein Dazl is required for this process. Strengths of the manuscript include the generation of two strong loss-of-function dazl mutant alleles using genome editing technology and the finding that these mutants are sterile, likely through a failure of germ cells to properly undergo cystogenesis. The weaknesses in the manuscript are largely presentational in nature and could be rather easily fixed by providing clarification and textual modifications. Neither does the manuscript clarify the underlying biochemical mechanisms by which Dazl proteins function nor does it address the cell biological modifications of cell division needed to ensure germline cyst formation. These are clearly complex issues of high interest beyond the scope of the present study, which does provide useful data for the field. The authors should consider the following points.

Major Points
1. Throughout the manuscript, the authors claim that Dazl functions upstream of meiotic entry and that Dazl mutant germ cell fail to differentiate. This claim is not well established by the data because meiotic markers (e.g., synaptonemal complex markers, Sycp3 etc.) were not examined.
Even if the dazl mutant germ cells fail to enter the meiotic pathway of development, this could be a secondary consequence of defects in germline cyst formation.
2. A major contribution of the manuscript is its examination of early cystogenesis. From the presentation, it wasn't always clear how many embryos or larvae or images of a specific stage were examined. I wonder whether there are transmission electron microscopic images (or grids) available of these stages?
3. The claim that interconnected germ cells contain fusome-like structures is not well established by the data.
4. Materials and methods should include the details of the microscopic imaging.
5. Figure 2 is very nice and represents a key result of the manuscript. It would be helpful to know how many 10-day larval gonads were examined and how many cysts of each type were documented. In Figure 2E', the "plus sign" obscures the image. Please present an unlabeled image in the supplemental data. Since cell boundaries were indirectly inferred, ideally in the future transmission electron microscopy of serial sections should be analyzed.
6. Figure 4. Ideally, the Vasa-positive cells should be counted and then the genotype determined for each embryo. Since zebrafish apparently does not yet have good balancer chromosomes, this would have to be done by PCR of the fixed and stained embryos--not sure whether this is feasible though it probably is. The statistics in Figure 4 are not compelling because the number of each specific genotype in each sample and its corresponding number of germ cells is not known.
7. Page 23. "Dazl…acts upstream of meiosis…." Whether this is an indirect consequence of the earlier defects is unclear. This applies also to the statement on page 24 that "dazl is required for gametogenesis and meiotic progression in zebrafish."
8. The claim that ring canals might collapse in dazl mutants might be tempered a bit because the analysis relies solely on static images.
9. The title of the manuscript might be crisper and more accurately reflect its key findings.

Minor Points
1. Use RNA-binding protein.
2. Introduction. Consider citing Lawson et al. (1999) for the finding that Bmp4 is required for formation of primordial germ cells in mice. Also, distinguish between primary literature and reviews
(e.g., by adding "reviewed by…," etc.).
3. Page 4. The sentence, "Arrest of the cytokinetic furrow and maintenance…" could be more clearly written.

4. Page 4. Next sentence, “In mice…”
5. Page 9. Perhaps use “single-guide RNAs” instead of “gRNAs.”
6. Page 9. “Cleaned up” is jargon.
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9. Page 13. In the text accompanying Figure 1, the authors refer to “presumptive gonadal somatic cells.” If possible, it might be helpful also to utilize an antibody specific to the somatic cells. Alternatively, point out that these cells do not express Vasa 10. In Figure 3, please use Courier font for the DNA sequences so that the sequences from the wild type and mutants are in register.
11. Figure 5K. Please use a different shading scheme to make the key result clearer.
12. Page 18. “We measured cell size…”

David Greenstein

Comments for the author

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David Greenstein

Reviewer 2

Advance summary and potential significance to field

Reviewer: Bruce Draper
During germ cell development, germ cells undergo a conserved developmental stage that is characterized by cell division with incomplete cytokinesis that form cyst of clonal interconnected cells. The resulting cells within a cyst have synchronized division and entry into meiosis. What regulates the transition of germ cells from a division pattern that created two separate cells to one that form cyst by incomplete cytokinesis is largely unknow. In this manuscript, Bertho et al. investigate when germ cell first enter the cyst-forming stage and ask if the conserved germline protein Dazl is involved in cyst formation as data from other organisms suggest. They investigate the timing of cyst formation by assaying germ cell morphology at different times during larval development following antibody labeling to identify germ cells and labeling of actin to help define cell boundaries. The assay the involvement of Dazl using newly produced mutations (via genome editing). Their major conclusions are that cyst formation can first be detected in wild-type larvae as early as 10 dpf, and that dazl mutants fail to form cyst. They also show that germ cells in dazl mutants eventually disappear and all dazl mutants are sterile males as adults.

Comments for the author
Major comments:

1. In general, the data that attempts to establish the time when germ cell cyst first appear during larval development are low resolution and hard to interpret. In Drosophila specific markers that lable the ring canals allow one to unambiguously identify which cells are members of an interconnected cyst. Unfortunately, and no fault of the authors, no such markers have been identified in any vertebrate, including zebrafish. Though ring canals have been clearly identified between zebrafish ovarian germ cells by TEM (Marlow and Mullens), no methods have been established for unambiguously identifying them in the light microscope. Unfortunately, I am not convinced that the methods presented here have solved this problem.

2. A significant weakness in the paper is that there is no data that establishes when dazl is expressed during larval development. The only data on dazl expression that I am aware has investigated its expression during oogenesis in the adult ovary and then the localization of maternal dazl mRNA in early embryos. Given that the authors find that dazl is required for germ cell survival during larval development, they need to establish when and where dazl is expressed during larval development. Presumable, it will be expressed in pre-meiotic germ cells and perhaps in germline stem cells. This will require in situ hybridization. This data also is important for establishing the motivation behind asking if zygotic dazl is required for germ cell migration during embryogenic (i.e. Fig. 4; specific comments on this below).

Other comments/suggestions, ordered as they appear in the text:

Title: Zebrafish dazl regulates cystogenesis upstream of the meiotic transition and germline stem cell specification and independent of meiotic checkpoints.

1. No markers of meiosis were assayed so “upstream of meiotic transition” has not been established.
2. “…and germline stem cell specification…” The expression of nanos2 was not assayed so the requirement of daz for GAS specification has not been established.
3. “…and independent of meiotic checkpoints.” Miotic checkpoints were not assayed so this has not been established.

Fig 1 is distracting as it does not have the resolution or necessary quantitative date to support the claims made in the text. Supp Figure 4 has quantification, but if it is based on the methods of Fig. 1, these are not convincing. By contrast, the methods used in Fig. 2 would likely have the required resolution to adequately identify cystocytes, but it does not appear that this method was used for any of the quantitative analysis.

Page 12:

1. The phrase “oocyte-like cell” is confusing. What is the distinction between an oocyte-like cells and a perinucleolar oocytes (as defined by Uchida et al.,)? These cells are oocytes- fully capable of maturing if the animal becomes a female- so there is no reason for calling them “oocyte-like.”

2. “Conversely, low numbers of germ cells and OLCs drive male development.” This is not totally accurate, as stated. It is well established that it is the oocytes, not premeiotic germ cells, that produce the female promoting factor. The sentence above could be interpreted to mean that all germ cell stages are involved in promoting female development.

Page 13:

1. “…nuclear cytoplasm interface was highly convoluted…” What does this mean? Can an example of this be pointed out in one of the Fig 1C? Looks like this data is presented in Fig. 2B.

2. “Similarly, we observed a few presumptive gonadal somatic…” Because there are a lot of body wall nuclei in all of the Fig 1 panels, it would be helpful to the non-expert if examples of somatic gonad nuclei were labeled (most important in Figs 1A-C).

3. “Following clustering…” This is a vague statement. What is the definition of “clustering?”

As written, it sounds like the clusters are formed by active gonocyte migration that occurs around 7dpf. More likely, these clusters are simply the result of gonocyte proliferation. As such, cluster formation would coincide with the transition of quiescent PGC-like cells to proliferative gonocytes.

4. “This step was characterized by complex cytoplasmic and nuclear rearrangements.” What does this refer to?

5. “…individual GC boundaries were not apparent within the irregular cytoplasmic compartments devoid of Vasa and DAPI (Fig. 1c).” I can see individual germ cell boundaries but I do not know what “irregular cytoplasmic compartments devoid of Vasa and DAPI” is referring to.

6. “During this period, DAPI signal was reduced…” I do not see any difference in DAPI signal. Premeiotic germ cells, in general, stain poorly with DAPI.

7. “The simultaneous obscuring of individual cell boundaries…” Please use arrows to point out areas devoid of Vasa.

Page 14:
1. typo “…germline cyst;… should be “…germline cyst,…”

2. “The amplification step…” To define this as an amplification step one would have to show that there is an increase in germ cell numbers either by counting or by labeling with an appropriate cell cycle marker (e.g. EdU labeling, pH3). If not shown here, then reference others who have measured germ cell division during these time periods.

3. “…morphogenetic process as cystogenesis.” Cystogenesis literally means “to create a cyst.” However, as defined here, cystogenesis is the process that precedes the emergence of germline cyst. Thus, this terminology is not appropriate. Importantly, if this is to be defined as a particular developmental stage, it will be necessary to provide quantitative data that shows what percent of gonads have single vs. clustered germ cells at each of the timepoints referenced.

3. What is meant by “…patent perinuclear granules…”? A higher resolution image would allow better visualization of the perinuclear granules. Showing both a wide view (1D) vs. the cropped views (1D’-D”’) does not help much since they are all at the same magnification.

4. … early germline cysts were apparent.” Awkward sentence. The cyst are not apparent.

5. “…with a prominent nucleolus…” This is not apparent in Fig. 1E and not shown in Fig. 1G.

6. “Cysts appeared to have a shared cytoplasm…” This is not apparent at the resolution of this figure.

7. “Among the wild-type gonads examined…” How many wild-type gonad were examined?

8. “Between 12-14d, wild-type fish had pre-meiotic germline cysts...” This is not apparent at the resolution of this figure.

9. “At 10d wild-type gonads…” This is an incomplete sentence.

10. It was argued in Fig.1 that one of the hallmarks of cells competent to form cyst is the redistribution of Vasa to form perinuclear granules. However, in Fig. 2C and D, which claim to show a 2- and 4-cell cyst, Vasa does not appear to be perinuclear.

11. Fig. 2E’ has a cross that obscures the “ring canal.”

Figure 3: Figure 3B is hard to read. Please use a font whose characters are the same width (e.g. Courier).

For dazl(ae57) it is stated that there is a 9 bp insertion, yet only 8 bp are highlighted. At the end of the line is indicates (+9, 2s). This should be (-2, +9, 2s).

For dazl(ae34) allele the figure only shows the -12 bp yet it is stated that there is also a +15 bp insertion. The text states that "The dazl(ae34) allele, a 15bp insertion with a 12bp deletion resulted in a four amino acid in-frame deletion…" It also results in a 5 aa insertion correct? Typo: "…generated…" should be "…generating…"

Fig 3B: Nit-pick: Wild-type diagram should be included.
Fig. 3D: could be put in Supplemental figure. Also, I am a bit uncomfortable relying on the absence of cutting to call a fish mutant (ie for ae57 and ae34). These mutations are large enough to be able to assess them directly on a 2% gel.

Page 16 and Figure 4: "Zygotic dazl is dispensable for PGC specification and migration" It is not clear why this was even a question. dazl mRNA is maternally supplied and localizes to germ plasm. To my knowledge no one has shown that dazl is expressed zygotically during embryogenesis. The reference to Huston and King is not accurate motivation for this analysis as they were specifically depleting maternal dazl function.

Page 17:
1. "Between 7-10 d, Vasa persisted in dazl mutants, indicating zygotic Dazl is not required for…" This is only relevant if it can be shown that dazl is expressed at these stages.

2. Supp Fig. 4 is labeled Supp Fig 3

3. "Somatic gonadal cells were similarly apparent…" Please point out an example for the non-expert.

4. "…underwent a synchronous nuclear/cytoplasmic rearrangement…" What is being rearranged? Vasa localization?

5. "Some dazl mutant GCs showed evidence of amplification…(Sup fig 3,4,5)." This is not obvious in the Supp figure. Why is this not in the primary figure?

6. "…cyst cells in wild-type and dazl heterozygotes (Figure 5E)…" This makes it sound like data for both wt and hets are shown, but only hets are shown.

7. "…dazl mutant cells reverted to the earlier intermingled individual cell morphology."
Without any quantification of the percent of gonads that have cells that look like Fig. 5d vs. 5F it is hard to determine if "reversion" is an accurate conclusion.

Page 18:
1. "The cytoplasm of the mutant cells appeared convoluted in appearance," What is the definition of "convoluted cytoplasm?"

2. Supp Figure 6: What is the justification for measuring area or volume instead of simply measuring cell diameter. It makes more sense to establish a metric that is easy to measure.
The scale bars in Sup Fig A vs. A', B vs. B' and D vs. D' are difference, but the magnifications looks the same. Why also does the magnification change in C vs. C'. It would be more convincing if all panels were at the same magnification.

3. "…particularly given that Dazl interacts with cytokinesis and ring canal factors(refs)."
This sentence is very problematic as it implies that this is known for zebrafish Dazl, since zebrafish nomenclature was used (i.e. Dazl). However, the work cited was either done in mouse and humans (correct nomenclature: DAZL). This should be clearly spelled out to indicate that this is a hypothesis based solely on work in mammals.

4. It is stated that "Actin is a prominent marker of … spectrosome of GSCs, and the branched fusome that connects germline cyst cells (Refs)." I know the fusome is composed of α- and β-spectrin as well as the adducin-like HTS protein, but I am not aware that they also contain actin. The few references I checked did not mention actin when defining the component of the fusome. Please double check this and provide a specific reference to this to alleviate me from my ignorance.

Page 19:
1. "…actin was present in branched structures reminiscent of the fusome of other species (fig 6e). How are you distinguishing what is membrane-associate actin and what is intercellular "branched structures?" The lower left arrow in 6E looks like it is pointing to membrane. The structure in 6C looks to localize on opposite side of cells from a ring canal which would argue it is not a "fusome-like" structure. Please comment.

2. "…two actin aggregates were present in dazl mutant GCs…" How often was this observed?

3. Fig 7A: The arrow points to one possible ring canal, but there are two similarly looking "rings" in the image. Why is only one labeled as a ring canal? This is also true for 7B. What criteria was used? Please include in the text a general description of the method used to generate this data. This information should not be buried in the figure legend or the M&M.
This figure is hard to interpret because the magnification and resolution is low. This is likely an inherent limitation of the method. A "ring" could simply be a fluid-filled space between two adjacent cells. Rings are clear in the XY plane, but the resolution of the YZ and ZX projections is not good enough to say if they are also "bubbles or clear connections. Fig 7 shows single confocal planes. What are the chances of catching a ring in one plane? Rare.
A ring caused by a space between two cells would be easy to image as all focal planes would produce a ring-like structure. Are these rings easy or hard to find?

Page 20:
1. "…or sperm (Figure 2H) in wild-type siblings at 2 months of age,…" Vasa only labels premeiotic and early meiotic germ cells during spermatogenesis, it does not label sperm (e.g. see Fig. 2 in Leu and Draper, 2010). It would be more accurate to say that "Vasa labeling revealed normal testis morphology."

Page 21:
1. Typo: "…differentiation as somatic cell types." Should be "…by somatic…"

2. "Because zebrafish dazl mutant GCs are not detected after 18d…" Fig 9D contradicts this statement. Has it been established when germ cell disappear in dazl mutants?

3. "…OLCs or oocytes were present in tp53 mutants that were heterozygous for dazl(ae57) Supplemental Figure 6; n=6)." This should be Supp Fig. 7.

4. Fig. 9I, J, K: Missing data->how many gonads were examined for each of these experiments?

5. Fig. 9K: Why was Vasa staining area used instead of counting germ cell numbers as in I and J?

Reviewer 3

Advance summary and potential significance to field

Bertho et al use newly induced Crispr/cas9 mutations in the conserved germ line gene deleted in azoospermia-like (dazl) to describe a requirement for zygotic Dazl in the initiation of germline cysts in the zebrafish larvae. The work shows that zygotic Dazl is required for the transition of germ cell precursors through a mitotic amplification step into the determination of likely stem cell precursors. They also identify a specific defect in the mutants in which F-actin based ring canals, a conserved feature of early gametogenesis in a number of species, is affected in these mutants. This results in cytokinesis defects and a reversion of cellular features to an apparently more primitive germ cell stage, and more broadly in sterility. The authors also show that two pathways involved in other systems in cell death of germ cells that have failed proper cellular specification do not appear to be involved in the germ cell defects in dazl embryos, showing that dazl has an essential function independent of these known pathways.
The work also describes for the first time interesting transitions of vasa protein localization in the germline of zebrafish larvae: first as granules perinuclearly (corresponding to previously described nuage) and in the context of a highly convoluted nuclear-cytoplasmic interphase, then diffusely in

the cytoplasm during mitotic cell amplification step, and later with a re-establishment of the perinuclear pattern in the likely germ cell stem cell precursors, a transition that requires dazl function.
Also of significant interest, the work shows that zygotic dazl function is not required for primordial germ cell specification and migration in the early embryo, as well as the initial vasa perinuclear pattern and somatic cell recruitment in early larvae, implying that it is maternal, and not zygotic, dazl that may have a role in these earlier processes.
The work also reports that the above-mentioned cell cycle factor chek2, tested for a potential involvement in the death of dazl mutant germ cells, when depleted on its own results in no overt phenotypes.

Comments for the author

It is unclear whether the role of Dazl in ring canal maintenance is direct or a secondary consequence of more general cellular defects. It is understandable if additional research beyond this manuscript is required to determine this in detail, but at the very least the manuscript should address potential mechanisms and what is known in other systems regarding the interaction of Dazl and cytokinesis and ring canal factors.

Is the transition from perinuclear at day 8 to “diffusely cytoplasmic” really a redistribution of vasa or could it involve degradation of vasa protein and reinitiation of expression”? Does it coincide with change in convoluted nucleus?

Additional markers for spectrosomes would be useful, and/or how the branched structure allows to identify the observed structures as spectrosomes

Only one marker of ring canals is used, and additional markers for ring canals and related midbodies, might help confirm the findings and possibly add additional insights into the underlying defects How are male and females assessed from a morphological and gonad perspective in fig 8?

Minor issues
It would be helpful if the introduction or discussion addressed the origin of the unusual convoluted pattern of nuclear-cytoplasm interface, for added context, since these features do not appear to occur in the very early embryos.

The description of the cellular changes at the amplification step seemed somewhat unclear at least to this reviewer, for example the statement
“The amplification step is characterized by cystogenic cells with a compact round nucleus and patent perinuclear Vasa granules circumscribed by scant cytoplasm (Figure 1 D,G). At 10-12d (n=7 gonads), early germline cysts were apparent”
seems somewhat contradictory to the description that perinuclear cytoplasm becomes cytoplasmic and diffuse. The answer is likely in the precise staging and perhaps the text could be revised for clarity.

First revision

Author response to reviewers' comments

We thank you and the reviewers for your time and appreciate your positive views and constructive evaluation of our manuscript reporting Dazl functions in the zebrafish germline. We appreciate your constructive comments and have carefully considered and have addressed each point. Specifically, we have performed additional experiments, made revisions to the main figures, added new main figures, tables, and supplemental figures, and made revisions to the text to address each matter. Our detailed point-by-point responses and a description of the revisions are provided below.

We have addressed all of the issues that the reviewers raised, but, due to the lab shut down, we were unable to complete some of the experiments that were suggested (indicated in purple text). However, in those cases we either addressed the issue with alternative experiments or revised the writing to temper claims for which we could not provide additional supporting data at this time. Even without these experiments, we hope that the reviewers will agree that the new results and revisions have strengthened the manuscript and we hope that you will find our revised manuscript suitable for publication in Development.

Reviewer 1 Advance summary and potential significance to field
Comments:

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We have samples, primers and markers on hand but were unable to process prior to the shutdown; therefore, we have revised the text as follows.

The stage (12-14 dpf), when germ cells are lost in dazl mutants, occurs before expression of the germline stem cell marker has been reported. Thus, Dazl acts upstream of or before stem cell specification. Nonetheless, we agree that upstream could be construed as direct regulation, and thus we have revised the text, replacing “upstream” with “prior to” or “before” to be more precise.

2.A major contribution of the manuscript is its examination of early cystogenesis. From the presentation, it wasn’t always clear how many embryos or larvae or images of a specific stage were examined. I wonder whether there are transmission electron microscopic images (or grids) available of these stages?

The numbers of embryos or larvae are not presented on the figure panels for clarity. However, the numbers examined are stated in each figure legend. Unfortunately, TEM images were not available at this time and won’t be possible for the foreseeable future but will be pursued in follow-up studies.

3.The claim that interconnected germ cells contain fusome-like structures is not well established by the data.
We have the samples on hand, and we have acquired and tested some antibodies that work in other species. So far this has not been fruitful. We have others that remain to be tested because we ran out of time before the labs were closed. Given that we do not know how long the shut-down will last and that we do not know if these antibodies will cross-react and that we cannot perform EM at this time, we are unable to address this experimentally. Therefore, we have revised the text to call these actin-rich structures throughout the text.

4.Materials and methods should include the details of the microscopic imaging.
We have added details of the microscopic imaging as requested by the reviewer.

“Confocal images were acquired using a Leica SP5 DMI with an 40x or 63x objective. The acquisition setting was set between sample and experiments to XY resolution 1024x1024 (or 512x512 as indicated) , zoom: 2.5x, pinhole was adjusted to 1.1 µm of the Z thickness, increments between images in stacks were 0.2 µm. Laser power and gain were set for each antibody or fluorescent compound to 2-10% below saturation condition.”

5.Figure 2 is very nice and represents a key result of the manuscript. It would be helpful to know how many 10-day larval gonads were examined and how many cysts of each type were documented. In Figure 2E’, the “plus sign” obscures the image. Please present an unlabeled image in the supplemental data. Since cell boundaries were indirectly inferred, ideally in the future transmission electron microscopy of serial sections should be analyzed.

The plus sign in Figure 2 has been moved and an unlabeled version has been added to the revised manuscript as supplemental Figure 9 as requested.

Figure 2 shows a single representative gonad at 10dpf with the different cyst stages indicated. We have added a supplemental Figure 10 with 3 additional representative gonads with stages indicated. Additionally, the quantification of cells/cyst and number of gonads examined can be found in Figure 5L. We have also clarified the label on panel 5L to state “cells/cyst”. Unfortunately, obtaining TEM images is not possible at this time, but will be pursued in the future.

6.Figure 4. Ideally, the Vasa-positive cells should be counted and then the genotype determined for each embryo. Since zebrafish apparently does not yet have good balancer chromosomes, this would have to be done by PCR of the fixed and stained embryos--not sure whether this is feasible, though it probably is. The statistics in Figure 4 are not compelling because the number of each specific genotype in each sample and its corresponding number of germ cells is not known.
The experiment was done as described by the reviewer. The embryos were immunostained, imaged, and analyzed blinded to genotype, and then were subsequently genotyped. For the experiments in panel I the data are grouped; however, for the experiments in panel J the data are displayed according to each specific genotype. The corresponding statistics are in the figure legend. In addition, a supplemental table (Supplemental Table 2 in the revised manuscript) has been prepared and added to show the number of PGCs per genotype and statistics.

7.Page 23. “Dazl…acts upstream of meiosis….” Whether this is an indirect consequence of the earlier defects is unclear. This applies also to the statement on page 24 that “dazl is required for gametogenesis and meiotic progression in zebrafish.”

The stage (12-14 dpf), when germ cells are lost in dazl mutants, occurs before expression of the germline stem cell marker nanos2 has been reported. Thus, Dazl acts upstream of or before stem cell specification. Nonetheless, we agree that upstream could be construed as direct regulation, and thus we have revised the text, replacing “upstream” with “prior to” or “before” to prevent confusion.

8.The claim that ring canals might collapse in dazl mutants might be tempered a bit because the analysis relies solely on static images.

We agree and have revised the text accordingly. For example:

“ring canal integrity was compromised, possibly due to…”

“In contrast, ring canals appeared compromised, possibly collapsed….”

“but ring canals are not maintained,…”

9.The title of the manuscript might be crisper and more accurately reflect its key findings.

We have revised the title as suggested. “Zebrafish dazl regulates cystogenesis prior to the meiotic transition and independent of meiotic checkpoints.”

Minor Points
1.Use RNA-binding protein.

The text has been revised accordingly.

2.Introduction. Consider citing Lawson et al. (1999) for the finding that Bmp4 is required for formation of primordial germ cells in mice. Also, distinguish between primary literature and reviews (e.g., by adding “reviewed by…,” etc.).

The text has been revised accordingly.

3.Page 4. The sentence, “Arrest of the cytokinetic furrow and maintenance…” could be more clearly written.

We have revised the sentence as follows: “During normal cell division, sister cells are separated by the cytokinetic furrow and a severing of the transient connection between sister cells called the midbody. For incomplete cytokinesis, a complex regulatory network stabilizes the midbody actomyosin meshwork to block abscission and maintain the contractile ring such that ring canals are formed between sister cells and they remain connected as reviewed in (reviewed in Greenbaum et al., 2011; Haglund et al., 2011; Hime et al., 1996; Robinson and Cooley, 1996).”

4.Page 4. Next sentence, “In mice…”

The text has been revised accordingly.

5.Page 9. Perhaps use “single-guide RNAs” instead of “gRNAs.”

The text has been revised accordingly.

6.Page 9. “Cleaned up” is jargon.

“cleaned up” has been replaced with “purified”.

7.In Figure 1, panel B’’, the DAPI image of the clustered germ cells appears to be out of focus on my computer screen. Alternatively, brightness and contrast might need to be enhanced in the photographic reproduction of the data.

The brightness and contrast have been enhanced as recommended by the reviewer.

8.Figure 1, panel G is not labeled as such. The figure legend could more clearly describe the drawings. Further, the drawings might be improved upon. As one example, where the cell boundaries and the ring canals are not clearly indicated. The grey regions in the clustering cells at the left should be designated.

The figure has been revised as recommended by the reviewer and a corresponding description has been added to the figure legend. In addition, to revising the schematics (now panel H), we have added images of beta-Catenin immunostaining as panel G, which was used to delineate cell boundaries and have revised the text accordingly.

“(G) ß-Catenin immunostaining (Magenta) revealed some partitioning between cells labeled with the germ cell marker Vasa (Green) and nuclear marker DAPI (greyscale) within a cyst (Figure 1G). (H) Schematics representing the cystogenesis process from individual cells that cluster, then divide, to form a premeiotic cyst. Germ cells at all stages are represented with green cytoplasm, and a grey nucleus with a dark green nuclear envelope. At d7, single germ cells characterized by their folded, raisin-like nuclear morphology cluster together. Next, the germ cells divide mitotically (transit-amplification) to form germline cysts between 7 to 12 days. During this period, the nucleus and cytoplasm adopt several different configurations, culminating in cells with a round nucleus and

a defined cytoplasm and perinuclear Vasa granules. Incomplete cytokinesis leads to formation of ring canals (pink). The early cyst (12-14d) progress through to advanced cyst stages (12-14d) through rounds of incomplete cell divisions. Finally, nucleoli emergence indicates the transition from mitotic cyst to premeiotic cyst."

9.Page 13. In the text accompanying Figure 1, the authors refer to "presumptive gonadal somatic cells." If possible, it might be helpful also to utilize an antibody specific to the somatic cells. Alternatively, point out that these cells do not express Vasa

We have revised the text accordingly.

"Similarly, we observed a few presumptive gonadal somatic cells, which do not express the germ cell marker Vasa, but surround the Vasa antibody labeled GC during this period (Figure 1)."

10.In Figure 3, please use Courier font for the DNA sequences so that the sequences from the wild type and mutants are in register.

The figure has been revised accordingly.

11.Figure 5K. Please use a different shading scheme to make the key result clearer.
12.Page 18. "We measured cell size…"

The figure has been revised accordingly.

David Greenstein

Reviewer 2 Advance summary and potential significance to field
Reviewer: Bruce Draper
During germ cell development, germ cells undergo a conserved developmental stage that is characterized by cell division with incomplete cytokinesis that form cyst of clonal, interconnected cells. The resulting cells within a cyst have synchronized division and entry into meiosis. What regulates the transition of germ cells from a division pattern that created two separate cells to one that form cyst by incomplete cytokinesis is largely unknow. In this manuscript, Bertho et al. investigate when germ cell first enter the cyst-forming stage and ask if the conserved germline protein Dazl is involved in cyst formation, as data from other organisms suggest. They investigate the timing of cyst formation by assaying germ cell morphology at different times during larval development following antibody labeling to identify germ cells and labeling of actin to help define cell boundaries. The assay the involvement of Dazl using newly produced mutations (via genome editing). Their major conclusions are that cyst formation can first be detected in wild-type larvae as early as 10 dpf, and that dazl mutants fail to form cyst. They also show that germ cells in dazl mutants eventually disappear and all dazl mutants are sterile males as adults.

Reviewer 2 Comments for the author
Major comments:
1. In general, the data that attempts to establish the time when germ cell cyst first appear during larval development are low resolution and hard to interpret. In Drosophila, specific markers that label the ring canals allow one to unambiguously identify which cells are members of an interconnected cyst. Unfortunately, and no fault of the authors, no such markers have been identified in any vertebrate, including zebrafish. Though ring canals have been clearly identified between zebrafish ovarian germ cells by TEM (Marlow and Mullins), no methods have been established for unambiguously identifying them in the light microscope. Unfortunately, I am not convinced that the methods presented here have solved this problem.

We wish we had additional markers. We acquired and purchased antibodies that mark these structures in other species and fixed the necessary samples; however, those we were able to test thus far do not seem to cross react with the zebrafish proteins. We have others that remain to be tested; however, we were unable to do so due to the lab shut down associated with the pandemic. It is currently unclear how long the labs will be shut down so we have no idea when we will be able to complete this analysis. Moreover, even when we are able to do so, we have no guarantee that these antibodies will cross react with the fish proteins.

We hope that the reviewer finds the data presented in the revised manuscript, including the new Figure 9 and supplemental movie files, to be more convincing.

2. A significant weakness in the paper is that there is no data that establishes when dazl is expressed during larval development. The only data on dazl expression that I am aware has investigated its expression during oogenesis in the adult ovary and then the localization of maternal dazl mRNA in early embryos. Given that the authors find that dazl is required for germ cell survival during larval development, they need to establish when and where dazl is expressed during larval development. Presumable, it will be expressed in pre-meiotic germ cells and perhaps in germline stem cells. This will require in situ hybridization. This data also is important for establishing the motivation behind asking if zygotic dazl is required for germ cell migration during embryogenic (i.e. Fig. 4; specific comments on this below).

We have included new data showing Dazl protein distribution in the developing germline (New Figure 4 and supplemental Fig. 3). These data show that Dazl protein is not detectable in early PGCs and is first detectable just before cyst forming stages.

Other comments/suggestions, ordered as they appear in the text:
Title: Zebrafish dazl regulates cystogenesis upstream of the meiotic transition and germline stem cell specification and independent of meiotic checkpoints.
1. No markers of meiosis were assayed so “upstream of meiotic transition” has not been established.

We have samples, primers and markers on hand but were unable to process prior to the shutdown; therefore, we have revised the text as indicated below.

The stage (12-14 dpf), when germ cells are lost in dazl mutants, occurs before expression of the germline stem cell marker has been reported. Thus, Dazl acts upstream of or before stem cell specification. Nonetheless, we agree that upstream could be construed as direct regulation, and thus we have revised the text, replacing “upstream” with “prior to” or “before” to be more precise.

In addition, in leiu of RTPCR analysis, we have added new data showing that a reporter of zygotic germ cell transcriptional program activation, ziwi:GFP, is activated in dazl mutants (New Fig. 7). Based on activation of the ziwi reporter and persistent expression of Vasa in dazl mutant germ cells, we conclude that at least some zygotic transcriptional programs are activated in the absence of Dazl.

2. “…and germline stem cell specification…” The expression of nanos2 was not assayed so the requirement of daz for GAS specification has not been established.

We planned on nos2 in situ and RT and have samples on hand but were unable to process due to the shutdown; therefore, we have revised the text as indicated below.

The stage (12-14 dpf), when germ cells are lost in dazl mutants, occurs before expression of the germline stem cell marker, nanos2 has been reported at day 21 (Beer et al 2013). Thus, Dazl acts upstream of or more conservatively before stem cell specification; thus, we have tempered this conclusion in the revised text by replacing “upstream” with “prior to” or “before” to be more precise.

3. “…and independent of meiotic checkpoints.” Miotic checkpoints were not assayed so this has not been established.

p53 and chk2 are regulators of meiotic checkpoints (Miao et al., 2017; Rodriguez-Mari et al., 2010; Shive et al., 2010; Abdu et al., 2002; Bolcun-Filas et al., 2014; Sperka et al., 2012). The observation that loss of dazl mutant germ cells is not suppressed in dazl-/-; p53-/- or dazl-/-; chk2-/- double mutants, suggests that dazl is required independent of these checkpoint regulators and checkpoints.

Fig 1 is distracting as it does not have the resolution or necessary quantitative date to support the claims made in the text. Supp Figure 4 has quantification, but if it is based on the methods of Fig. 1, these are not convincing. By contrast, the methods used in Fig. 2 would likely have the required resolution to adequately identify cystocytes, but it does not appear that this method was used for any of the quantitative analysis.

Figure 1 was intended to provide the full overview of the distribution/organization of the germ cells along the body axis and overall gonad morphology at the indicated stages, which cannot be appreciated in the higher resolution/magnification confocal images. We felt providing a description and overview was important as these steps have not been previously described. The quantification was performed on the confocal images as in Figure 2 and is found in Figure 5L and Supplemental Figure 6.

Page 12:

1. The phrase “oocyte-like cell” is confusing. What is the distinction between an oocyte-like cells and a perinucleolar oocytes (as defined by Uchida et al.,)? These cells are oocytes- fully capable of maturing if the animal becomes a female- so there is no reason for calling them “oocyte-like.”

We now simply call the cells “early oocytes” throughout the text.

2. “Conversely, low numbers of germ cells and OLCs drive male development.” This is not totally accurate, as stated. It is well established that it is the oocytes, not premeiotic germ cells, that produce the female promoting factor. The sentence above could be interpreted to mean that all germ cell stages are involved in promoting female development.

We did not intend to be inaccurate or cause confusion. We have revised the text as follows:

“Conversely, low numbers of germ cells results in fewer early oocytes and male development”

Page 13:

1. “…nuclear cytoplasm interface was highly convoluted…” What does this mean? Can an example of this be pointed out in one of the Fig 1C? Looks like this data is presented in Fig. 2B.

An arrow has been added to point out an example, and “convoluted” has been replaced with “folded” in the revised text. In addition, this can be seen at higher resolution in Figure 2B (as noted by the reviewer) and the new Figure 6, which shows Laminin staining in gonads of wild-type and mutant genotypes.

2. “Similarly, we observed a few presumptive gonadal somatic…” Because there are a lot of body wall nuclei in all of the Fig 1 panels, it would be helpful to the non-expert if examples of somatic gonad nuclei were labeled (most important in Figs 1A-C).

As recommended by the reviewer, we have revised the figure to include blue dashed lines to indicate the somatic cells and yellow dashed lines for the germ cells. We have also added arrows and yellow dashed lines to panels D-F.

3. “Following clustering…” This is a vague statement. What is the definition of “clustering?” As written, it sounds like the clusters are formed by active gonocyte migration that occurs around 7dpf. More likely, these clusters are simply the result of gonocyte proliferation. As such, cluster formation would coincide with the transition of quiescent PGC-like cells to proliferative gonocytes.

Initially individual PGCs are spatially separated, the clustered individual cells are close together, this could indeed be either due to the cells moving closer to one another or coincide with the transition of quiescent PGC-like cells to proliferative gonocytes. The text has been revised to reflect these possibilities:

“PGCs transition from spatially separated individual cells to more closely positioned GC clusters, either due to relocation or a consequence of division, that undergo complex cytoplasmic and nuclear morphological changes to form germline cysts.”

“Individual GCs were dispersed within the gonad, while clustered GCs were closely spaced groups of individual cells (Figure 1B,H). It is unclear whether clusters form due to cell movement or division.”

4. “This step was characterized by complex cytoplasmic and nuclear rearrangements.”
What does this refer to?

This sentence has been revised replacing “rearrangements” with “nuclear morphological changes”.

5. “…individual GC boundaries were not apparent within the irregular cytoplasmic compartments devoid of Vasa and DAPI (Fig. 1c).” I can see individual germ cell boundaries, but I do not know what “irregular cytoplasmic compartments devoid of Vasa and DAPI” is referring to.

An arrow has been added to the revised figure to indicate these compartments.

6. “During this period, DAPI signal was reduced…” I do not see any difference in DAPI signal. Premeiotic germ cells, in general, stain poorly with DAPI.

As shown in Figure 1 C, Figure 5 C-D’, and supp. Fig. 7 DAPI staining is weaker in the “dividing cells” even though these are still mitotic cells. We have revised the text as follows:
“During this period, germ cells stain poorly with DAPI…”

7. “The simultaneous obscuring of individual cell boundaries…” Please use arrows to point out areas devoid of Vasa.

Arrows have been added as requested.

Page 14:
1. typo “…germline cyst;… should be “…germline cyst,…”

This has been corrected.

2. “The amplification step…” To define this as an amplification step one would have to show that there is an increase in germ cell numbers either by counting or by labeling with an appropriate cell cycle marker (e.g. EdU labeling, pH3). If not shown here, then reference others who have measured germ cell division during these time periods.

We have revised the text as recommended by the reviewer.

“Indeed, previous EdU and phospho-Histone labeling studies indicate that GC proliferation occurs during this stage (Leerberg et al., 2017).”

3. “…morphogenetic process as cystogenesis.” Cystogenesis literally means “to create a cyst.” However, as defined here, cystogenesis is the process that precedes the emergence of germline cyst. Thus, this terminology is not appropriate. Importantly, if this is to be defined as a particular developmental stage, it will be necessary to provide quantitative data that shows what percent of gonads have single vs. clustered germ cells at each of the timepoints referenced.
The distinction between create and precede the emergence seems semantic. We do think that this is the process of creating the cyst and that Dazl is required for this process. PGCs resemble siblings initially, initiate morphological changes, and activate gene expression (ziwi promoter), but fail to complete the process/generate cysts. The quantitative data showing the number of gonads with single versus clustered germ cells are provided in Supplemental Figure 6.

3. What is meant by “…patent perinuclear granules…”? A higher resolution image would allow better visualization of the perinuclear granules. Showing both a wide view (1D) vs. the cropped views (1D’-D”’) does not help much since they are all at the same magnification.

We have revised the figure and have added a small white arrow to better indicate the granules.

4. … early germline cysts were apparent." Awkward sentence. The cyst are not apparent.
The sentence has been revised and we have added a small white arrow to point out the cyst in the revised figure.

"At 10-12d (n=7 gonads), early germline cysts were observed."

5. "…with a prominent nucleolus…" This is not apparent in Fig. 1E and not shown in Fig. 1G.

A turquoise arrow was added to the figure to indicate the nucleolus in 1E" and 1 F'' and the schematics which are now panel 1G.

6. "Cysts appeared to have a shared cytoplasm…" This is not apparent at the resolution of this figure.

We have added images of beta-Catenin immunostaining, which was used to delineate cell boundaries as panel 1G and have revised the text accordingly.

"ß-Catenin immunostaining revealed some partitioning between cells within a cyst (Figure 1G); but it was unclear if cyst cells had a shared cytoplasm or were interconnected by cytoplasmic bridges such as those observed in juvenile ovaries (Marlow and Mullins, 2008)."

7. "Among the wild-type gonads examined…" How many wild-type gonad were examined?

The numbers of gonads examined, n=8 are indicated in Supp. Fig. 6, which has been indicated in the revised text.

8. "Between 12-14d, wild-type fish had pre-meiotic germline cysts..." This is not apparent at the resolution of this figure.

As reported in (Beer and draper, 2013). premeiotic germ cells have distinct DNA morphology characterized by a lack of condensed chromosomes and the presence of nucleoli , which have been indicated by blue arrows in the revised figure and corresponding legend.

9. "At 10d wild-type gonads…" This is an incomplete sentence.

The sentence has been revised as follows:
"At 10d cysts of various stages were observed in wild-type gonads (Figure 2A, B-B"), including GCs in 2-cell (Figure 2A, C-C") and 4-cell (Figure 2A, D-D") configurations."

10. It was argued in Fig.1 that one of the hallmarks of cells competent to form cyst is the redistribution of Vasa to form perinuclear granules. However, in Fig. 2C and D, which claim to show a 2- and 4-cell cyst, Vasa does not appear to be perinuclear.

We apologize for the confusion, we did not intend to imply that Vasa redistribution was a "hallmark of cells competent to form cysts", rather that perinuclear Vasa is lost before cyst formation and reestablished later once the cysts form. It is likely that it redistributes from granules or is lost during the divisions and is reestablished as granules after divisions. Accordingly, a cyst that is about to divide would be expected to lose perinuclear Vasa granules. We make no claims as to the cause or effect of this change.
In addition, the new data showing labeling with LaminB1 (new Figure 6), a nuclear envelope marker and Vasa show its perinuclear localization.

11. Fig. 2E' has a cross that obscures the "ring canal."

The cross has been removed and replaced with an arrow in the revised figure.

Figure 3: Figure 3B is hard to read. Please use a font whose characters are the same width (e.g. Courier).

The figure has been revised using courier font as recommended.

For dazl(ae57) it is stated that there is a 9 bp insertion, yet only 8 bp are highlighted.

This has been corrected.

At the end of the line is indicates (+9, 2s). This should be (-2, +9, 2s).

This has been corrected.

For dazl(ae34) allele the figure only shows the -12 bp yet it is stated that there is also a +15 bp insertion. The text states that “The dazl(ae34) allele, a 15bp insertion with a 12bp deletion resulted in a four amino acid in-frame deletion…” It also results in a 5 aa insertion, correct?

This has been corrected.

Typo: “…generated…” should be “…generating…”

This has been corrected.

Fig 3B: Nit-pick: Wild-type diagram should be included.

We did not include this in the panel originally because the wild-type diagram was above in panel 3A; however, as requested by the reviewer we have now added the wild-type diagram to panel 3B as well.

Fig. 3D: could be put in Supplemental figure. Also, I am a bit uncomfortable relying on the absence of cutting to call a fish mutant (ie for ae57 and ae34). These mutations are large enough to be able to assess them directly on a 2% gel.
I share your discomfort, but we tried many assays and it was not possible to develop one that cuts in the mutant. Moreover, we do not rely on one assay. In addition to typically using a 3% mix of metaphor/agarose gel, which provides better resolution than 2% agarose gels, we also developed the independent HRM assay that is included in Supp. Fig. 2

Page 16 and Figure 4: “Zygotic dazl is dispensable for PGC specification and migration” It is not clear why this was even a question. dazl mRNA is maternally supplied and localizes to germ plasm. To my knowledge no one has shown that dazl is expressed zygotically during embryogenesis. The reference to Huston and King is not accurate motivation for this analysis as they were specifically depleting maternal dazl function.

We have included new data showing Dazl protein distribution in the developing germline (New Fig.4). These data show that Dazl protein is not detectable in early PGCs and is first detectable just before cyst forming stages. We have moved analyses of PGC specification migration to the supplemental data. Taken together our data and conclusions are in agreement with Houston and King that if Dazl has a role in PGC specification and migration, then it must be fulfilled by maternal Dazl, which cannot currently be examined because the germline is lost and no ovary forms in dazl mutants.

Page 17:
1. “Between 7-10 d, Vasa persisted in dazl mutants, indicating zygotic Dazl is not required for…” This is only relevant if it can be shown that dazl is expressed at these stages.

As mentioned above, we have included new data showing Dazl protein distribution in the developing germline (New Fig.4 and supplemental Fig. 3).

2. Supp Fig. 4 is labeled Supp Fig 3

This figure is now Supp. Fig. 5 in the revised manuscript.

3. “Somatic gonadal cells were similarly apparent…” Please point out an example for the non-expert.

We have revised the figure to point out the somatic gonadal cells as requested.

4. “…underwent a synchronous nuclear/cytoplasmic rearrangement…” What is being rearranged? Vasa localization?

We hope that this is clearer in the revised manuscript. We have added new data, including analysis of Laminin in the New Figure 6, and have revised the text to more precisely describe the changes morphology observed.

5. “Some dazl mutant GCs showed evidence of amplification…(Sup fig 3,4,5).” This is not obvious in the Supp figure. Why is this not in the primary figure?

The amplification step is shown in Figures 1C, 5 C-D’ of the revised manuscript.

6. “…cyst cells in wild-type and dazl heterozygotes (Figure 5E)…” This makes it sound like data for both wt and hets are shown, but only hets are shown.

We observed no differences between wild-type and heterozygotes. Additional cysts of Wild-type genotypes are now shown in new supplemental Figure 13.

7. “…dazl mutant cells reverted to the earlier intermingled individual cell morphology.”
Without any quantification of the percent of gonads that have cells that look like Fig. 5d vs. 5F it is hard to determine if “reversion” is an accurate conclusion.
These data are found in panel K.

We have revised the text as follows:
“instead the dazl mutant cells adopted to the earlier intermingled individual cell morphology”

Page 18:
1. “The cytoplasm of the mutant cells appeared convoluted in appearance,” What is the definition of “convoluted cytoplasm?”

We have clarified this description in the revised text.

“germ cell nuclei had a raisin-like folded appearance”

2. Supp Figure 6: What is the justification for measuring area or volume instead of simply measuring cell diameter. It makes more sense to establish a metric that is easy to measure.
The scale bars in Sup Fig A vs. A’, B vs. B’ and D vs. D’ are difference, but the magnifications looks the same. Why also does the magnification change in C vs. C’. It would be more convincing if all panels were at the same magnification.

It is more accurate to measure the cell area or volume because this gives us the information about the whole cell. In contrast, the cell diameter can differ according to the plane, thus measuring diameter is less reliable. The 3D images (surface) correspond to the original. The magnification does not change, only the angle of view changes. However, image A-A’ was at resolution 512x512 instead of 1024x1024 like the others. This is now indicated in the legend and methods.

3. “…particularly given that Dazl interacts with cytokinesis and ring canal factors(refs).”
This sentence is very problematic as it implies that this is known for zebrafish Dazl, since zebrafish nomenclature was used (i.e. Dazl). However, the work cited was either done in mouse and humans (correct nomenclature: DAZL). This should be clearly spelled out to indicate that this is a hypothesis based solely on work in mammals.

This has been revised accordingly.

4. It is stated that “Actin is a prominent marker of … spectrosome of GSCs, and the branched fusome that connects germline cyst cells (Refs).” I know the fusome is composed of α- and β-spectrin as well as the adducin-like HTS protein, but I am not aware that they also contain actin. The few references I checked did not mention actin when defining the component of the fusome. Please double check this and provide a specific reference to this to alleviate me from my ignorance.

Additional references have been added supporting actin as prominent marker of the fusome.

Page 19:
1. “…actin was present in branched structures reminiscent of the fusome of other species (fig 6e). How are you distinguishing what is membrane-associate actin and what is intercellular “branched structures?” The lower left arrow in 6E looks like it is pointing to membrane. The structure in 6C looks to localize on opposite side of cells from a ring canal, which would argue it is not a “fusome-like” structure. Please comment.

We have revised the text to call these actin-rich structures throughout the text.

“By 12d, when cysts are abundant in wild-type, actin was also present in branched structures but it is unclear if these are analogous to fusomes of other species (Hime et al., 1996; Kloc et al., 2004; Warn et al., 1985).”

2. “…two actin aggregates were present in dazl mutant GCs…” How often was this observed?

Revised Panel 8J shows quantification of aggregates and double aggregates, which has also been indicated in the corresponding text.

3. Fig 7A: The arrow points to one possible ring canal, but there are two similarly looking “rings” in the image. Why is only one labeled as a ring canal? This is also true for 7B. What criteria was used? Please include in the text a general description of the method used to generate this data. This information should not be buried in the figure legend or the M&M.

The others would also be considered ring canals if they connect two cells. We only pointed out one example per panel to avoid crowding the field with arrows.

“Although the whole ring canal structures were not entirely visible within a single plane of a Z-stack, examination of max projections of 10 slices spanning the top and the bottom of the circular structures and supplemental 3D projections revealed the circular actin-rich structures present between cells (Fig. 9 and Supplemental videos 1-6).”

This figure is hard to interpret because the magnification and resolution is low. This is likely an inherent limitation of the method. A “ring” could simply be a fluid-filled space between two adjacent cells. Rings are clear in the XY plane, but the resolution of the YZ and ZX projections is not good enough to say if they are also “bubbles or clear connections. Fig 7 shows single confocal planes. What are the chances of catching a ring in one plane? Rare. A ring caused by a space between two cells would be easy to image as all focal planes would produce a ring-like structure. Are these rings easy or hard to find?

We agree with the reviewer that better spatial resolution is needed to clearly observe the ring canal. We have addressed this in the new Fig. 8 which shows only a single plane in XY and maximum projection of 10 planes to provide a more-clear view of this unique and discernable membrane structure.

Page 20:
1. “…or sperm (Figure 2H) in wild-type siblings at 2 months of age,…” Vasa only labels premeiotic and early meiotic germ cells during spermatogenesis, it does not label sperm (e.g. see Fig. 2 in Leu and Draper, 2010). It would be more accurate to say that “Vasa labeling revealed normal testis morphology.”

We have revised the text accordingly.

Page 21:
1. Typo: “…differentiation as somatic cell types.” Should be “…by somatic…”

This has been corrected.

2. “Because zebrafish dazl mutant GCs are not detected after 18d…” Fig 9D contradicts this statement. Has it been established when germ cell disappear in dazl mutants?

Healthy germ cells are not apparent after 14 dpf, although individual Vasa positive cells with unusual morphologies that appear vacuolated (as in Fig 10D) are infrequently detected after 18d, all Vasa positive or Ziwi:GFP positive cells are lost prior to sexual differentiation. We have revised the text as follows:

“Because zebrafish dazl mutant GCs are lost before sexual differentiation,”

3. “…OLCs or oocytes were present in tp53 mutants that were heterozygous for dazl(ae57) Supplemental Figure 6; n=6).” This should be Supp Fig. 7.

This has been corrected.

4. Fig. 9I, J, K: Missing data->how many gonads were examined for each of these experiments?

The numbers are indicated in the text.

5. Fig. 9K: Why was Vasa staining area used instead of counting germ cell numbers as in I and J?

Because the germ cells of wild-type become smaller after the PGC to GC transition and the cells lacking dazl appeared larger, we wanted to measure the size of the cells to determine if they resembled the size of PGCs prior to cyst formation or were larger. Vasa was used as a marker because the germ cells could only be definitively identified by a germ cell marker due to their morphological (e.g. vacuolization) phenotypes in dazl-/-;chk2-/- compared to dazl-/-;chk2+/-.

Reviewer 3 Advance summary and potential significance to field
Bertho et al use newly induced Crispr/cas9 mutations in the conserved germ line gene deleted in azoospermia-like (dazl) to describe a requirement for zygotic Dazl in the initiation of germline cysts in the zebrafish larvae. The work shows that zygotic Dazl is required for the transition of germ cell precursors through a mitotic amplification step into the determination of likely stem cell precursors. They also identify a specific defect in the mutants in which F-actin based ring canals, a conserved feature of early gametogenesis in a number of species, is affected in these mutants. This results in cytokinesis defects and a reversion of cellular features to an apparently more primitive germ cell stage, and more broadly in sterility. The authors also show that two pathways involved in other systems in cell death of germ cells that have failed proper cellular specification do not appear to be involved in the germ cell defects in dazl embryos, showing that dazl has an essential function independent of these known pathways.
The work also describes for the first time interesting transitions of vasa protein localization in the germline of zebrafish larvae: first as granules perinuclearly (corresponding to previously described nuage) and in the context of a highly convoluted nuclear-cytoplasmic interphase, then diffusely in the cytoplasm during mitotic cell amplification step, and later with a re-establishment of the perinuclear pattern in the likely germ cell stem cell precursors, a transition that requires dazl function.
Also of significant interest, the work shows that zygotic dazl function is not required for primordial germ cell specification and migration in the early embryo, as well as the initial vasa perinuclear pattern and somatic cell recruitment in early larvae, implying that it is maternal, and not zygotic, dazl that may have a role in these earlier processes.
The work also reports that the above-mentioned cell cycle factor chek2, tested for a potential involvement in the death of dazl mutant germ cells, when depleted on its own results in no overt phenotypes.

Reviewer 3 Comments for the author
It is unclear whether the role of Dazl in ring canal maintenance is direct or a secondary consequence of more general cellular defects. It is understandable if additional research beyond this manuscript is required to determine this in detail, but at the very least the manuscript should address potential mechanisms and what is known in other systems regarding the interaction of Dazl and cytokinesis and ring canal factors.

This is a good question. Since the initial steps appear to be intact and expression of some late germ cell transcripts occurs (e.g. activation of the ziwi promoter) we think that up to that point general cellular processes are intact. Moreover, Dazl is known to bind to ring canal regulators in other contexts; therefore, it is possible that ring canals do not fully form or are not maintained because Dazl (DAZL in mammals) regulates a ring canal protein such as TEX14. However, dazl mutant phenotypes are more severe than those reported for known ring canal components in vertebrates; thus, is it likely that Dazl regulates additional processes, possibly allocation of germ cell fates, and that when these fail a cell death program is activated. We have revised the discussion to include these possibilities.
Is the transition from perinuclear at day 8 to “diffusely cytoplasmic” really a redistribution of vasa or could it involve degradation of vasa protein and reinitiation of expression”? Does it coincide with change in convoluted nucleus?
While it could involve degradation and re-initiation of Vasa expression, we have previously shown that Vasa protein produced from maternal sources persists through at least 10 dpf and zygotic expression begins around 21 dpf (Hartung et al. Mol Reprod Dev 81, 946-961. 2014). It does seem to coincide with the change to the folded nuclear morphology, which may reflect activation of a new transcription program or could be due to changes to the nuclear membrane observed with LaminB1 (New Fig. 6). Indeed, a recent bioRxiv paper from Rene Ketting’s group indicates that transcription in the germline initiates around this time. We have revised the text to include this possibility.

“During this period, DAPI signal was reduced and Vasa distribution, either due to re-localization or degradation and de novo Vasa expression, shifted from the perinuclear aggregates typical of PGCs……”

“The unusual nuclear morphologies we observed during the transition from individual PGC to cyst stages prompted us to examine the nuclear envelope. To do so we performed immunostaining on dazlΔ7/+ or dazlΔ7/Δ7 gonads at 8d and dazlae57/+, dazlΔ7/+ or dazlΔ7/ae57 at 10d with LaminB1 to mark the nuclear envelope (Knaut et al., 2000; Strasser et al., 2008), DAPI to mark DNA and Vasa to label germ cells. (Figure 6). In dazlΔ7/+ at 8dpf germ cell nuclei had a raisin-like folded appearance (Figure 6A-A”). No differences were detected in dazlΔ7/Δ7 mutants at this stage (Figure 6B-B”). At 10dpf, the germ cell nuclei of dazlae57/+ became smooth and unfolded in appearance (Figure 6C-C”) as they entered the amplification state. This change in nuclear appearance corresponds to the amplification step during cyst formation and post- mitotic germ cell nuclei emerged with a smooth nuclear membrane and a large nucleolus (Figure 6E-E’’). Although dazlae57/Δ7 mutants entered the amplification step (Figure 6D-D’) (also see Figure 5D and Supplemental Figures 3 and 4) mutant GCs did not maintain a smooth nuclear morphology at 10dpf or thereafter and instead of forming cysts were detected as individual cells with a folded raisin-like nuclear membrane like the PGCs at 10dpf (Figure 6F-F”).”

Additional markers for spectrosomes would be useful, and/or how the branched structure allows to identify the observed structures as spectrosomes. Only one marker of ring canals is used, and additional markers for ring canals and related midbodies, might help confirm the findings and possibly add additional insights into the underlying defects

We wish we had additional markers. We acquired and purchased antibodies that mark these structures in other species and fixed the necessary samples; however, those we were able to test thus far do not seem to cross react with the zebrafish proteins. We have others that remain to be tested; however, we were unable to do so due to the lab shut down associated with the pandemic. It is currently unclear how long the labs will be shut down so we have no idea when we will be able to complete this analysis. Moreover, even when we are able to do so, we have no guarantee that these antibodies will cross react with the fish proteins.

Because we were unable to complete these analyses, we replaced “spectrosome-like” aggregates with “actin-rich aggregates” or “actin-rich structures” and made similar modifications throughout the text. See examples below.

“Close inspection of actin (labeled with Phalloidin) in gonads with wild-type genotypes at 8 and 10d revealed an actin rich density within GCs, reminiscent of spectrosomes observed in other species (Figure 8A-A’; C-C’).”

“By 12d, when cysts are abundant in wild-type, actin was also present in branched structures but it is unclear if these are analogous to fusomes of other species (Hime et al., 1996; Kloc et al., 2004; Warn et al., 1985).”

“In contrast, at this stage in dazl mutants the actin-rich aggregates persist in Vasa+ cells (Figure 8F-F’).”

“Figure 8. dazl mutant cells retain actin-rich structures. Single confocal plane of dazlae57/+ or dazlae57/ae57 gonad between 8-14d labeled with Vasa and fluorescent conjugated phalloidin which marks actin-rich structures and nuclei labeled with DAPI. (A-A’, C-C’, E-E’, G-G’) The actin-rich structures indicated by the white arrow is present transiently between 8-12d in dazlae57/+ gonads. (B-B’, D-D’, F-F’, H-H’) Actin-rich structures (white arrow) are present in dazl mutant cells between 8-14d. (H-H’) At 14d, actin-rich doublets are observed. Scale bar, 10μm. (I) Quantification of the number of cells containing an actin-rich structure within each gonad. (J) Quantification of actin aggregates per cell.”

How are male and females assessed from a morphological and gonad perspective in fig 8

Males and females are distinguished based on secondary sex traits, color, and presence of tubercles on fins of males. In addition, gonad morphology is unique between males and females, oocytes are significantly larger than male germ cells and are morphologically distinct. Germ cells of both sexes are Vasa positive when present. The criteria used to distinguish between males and females has been added to the revised figure legend (Now Fig. 10).

Minor issues
It would be helpful if the introduction or discussion addressed the origin of the unusual convoluted pattern of nuclear-cytoplasm interface, for added context, since these features do not appear to occur in the very early embryos.
Nuclear indentations have been reported in other cell types concomitant with cell state or behavioral changes. This has now been added to the discussion as requested by the reviewer.

“Nuclear indentations have been reported in other cell types concomitant with cell state or behavioral changes. For example, lymphocytes upon activation (Hulspas et al., 1994), mouse rod cells (Seirin-Lee et al., 2019), cell cycle progression in cultured cells (Aureille et al., 2019), and most recently transcriptional modulation in mouse oocytes (Almonacid et al., 2019). Although it is unclear if the transformation of nuclear morphology from the smooth appearance of early PGCs (Knaut et al., 2000; Strasser et al., 2008) to the raisin-like morphology that precedes the smooth unfolded nuclear compartment during the amplification step represents activation of transcriptional programs necessary for the PGC to premeiotic or germline stem cell remains to be determined. That this step is grossly intact without Dazl and de novo expression from the ziwi promoter and persistent expression of Vasa indicate that zygotic transcriptional programs are initiated but this transition subsequently fails without Dazl.”

The description of the cellular changes at the amplification step seemed somewhat unclear at least to this reviewer, for example the statement “The amplification step is characterized by cystogenic cells with a compact round nucleus and patent perinuclear Vasa granules circumscribed by scant cytoplasm (Figure 1 D,G). At 10-12d (n=7 gonads), early germline cysts were apparent” seems somewhat contradictory to the description that perinuclear cytoplasm becomes cytoplasmic and diffuse. The answer is likely in the precise staging and perhaps the text could be revised for clarity.

We apologize for the confusion and have tried to more clearly describe these stages in the revised manuscript.

“During the subsequent amplification steps germ cells within forming cysts adopted a compact round nucleus and perinuclear Vasa granules are reestablished between cells divisions within germline cysts (Figure 1 ¬D,H). At 10-12d (n=7 gonads), cyst cell nuclei were compact with a prominent nucleolus and perinuclear Vasa aggregates were detected (Figure 1 E,H).”

Second decision letter

MS ID#: DEVELOP/2019/187773

MS TITLE: Zebrafish *dazl* regulates cystogenesis prior to the meiotic transition and independent of meiotic checkpoints

AUTHORS: Sylvain Bertho, Mara Clapp, Torsten U Banisch, Jan Bandemer, Erez Raz, and Florence L. Marlow

I hope all is well.

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 appreciate the revisions that you have made but still have some significant criticisms and suggestions for revisions. Of most concern is that in their review and comments to the editor, reviewer 2 expresses the opinion that some aspects of the data do not allow you to unambiguously draw the conclusions you state and that in absence of more robust data, the interpretations of the data may later prove to wrong. This is of course hard for me to assess as both you and the referee are far more experienced on this topic than I am. Consequently, you will need to consider the reviewers comments, which are all meant to be constructive, in deciding how to further revise the manuscript.

We are aware that you may currently be unable to access the lab to undertake experimental revisions and, if necessary, we would be happy to extend revision timeframes. Also happy to discuss your revisions further if you think this would be helpful.

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

The authors have done a commendable job in revising their prior submission given the constraints of the current public health crisis. The manuscript has several strengths. The authors generate mutant alleles of the zebrafish Dazl gene and show that they result in recessive sterility. The expected effect on sex determination—masculinization—is observed. Although the authors did not conduct transgenic rescue experiments, they can exclude off-target effects because independently generated alleles behave similarly and mutant alleles fail to complement as expected. The Dazl alleles are a good contribution to the field and contribute to our understanding of the roles for this gene in germline development of vertebrates and invertebrates. A key contribution of this manuscript is its documentation of the process of cystogenesis in zebrafish germline development and the finding that Dazl plays a key role. No doubt it will be important to examine this process at an ultrastructural level and in real time, but the videos provided by the authors clearly document

that ring canals can form in the mutant but are likely not maintained for some reason. The manuscript also reports specific antibodies to Dazl and its specificity for the germline. The authors mention that Dazl protein was not detectable in germ cells at the 30 hpf stage and thus any maternally contributed protein is likely to be removed before germline defects are first observed. It would have been nice for the authors to stain early embryos to determine what extent if any maternally contributed Dazl might play. I was confused by the citation of the Giraldez et al. (2006) paper saying that Dazl mRNA is removed from early embryos in a Dicer-dependent fashion. I waded through the main text and the supplemental data of that paper and couldn't find the cited information. Perhaps it was buried in a different gene ID (?) Nonetheless, I would have liked to see immunofluorescence or western blots with early embryos. However, given the current situation, I think reviewers should be willing to settle for textual modifications or appropriate caveats.

A few weaknesses were apparent in the manuscript. No information is provided as to how Dazl might play a role. The assessment of this reviewer is that these studies should be beyond the scope of this initial report at this current time. Another weakness, is that the manuscript is overly long. I found the studies of chk2 and p53 to be extraneous. The premise that these factors could be directly involved was weak. It would have been shocking and completely unexpected if the authors observed epistatic interactions, which they did not. Another issue is that the images are not of uniformly high quality. For example, it was difficult for me to see the perinuclear Vasa granules and it was hard to assess the authors' conclusion that perinuclear Vasa granules were less apparent in the mutant than in the wild type.

A few additional minor points: phalloidin stains cortical actin, not plasma membranes (page 15); Supplemental Figure 10 is incorrectly cited (page 15); the authors spend a lot of time discussing nuclear morphology (page 20), but I am not sure what the ziwi:gfp experiment really tells us; I think the emphasis on when Dazl functions with regard to the meiotic entry decision is misplaced as the germ cells may not progress to the appropriate stage and this may be an indirect consequence; and I think it would be useful to tone down the statement about the actin-rich structures reminiscent of fusomes, perhaps duplicating (p. 21).

David Greenstein

Comments for the author

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David Greenstein

Reviewer 2

Advance summary and potential significance to field

Same as previous review.

Comments for the author

The significance of the manuscript, as written, rest on the ability to unambiguously identified cells that are within cyst from those that are not. Unfortunately, the revisions do not solve this issue. In addition, the revisions raise a few new questions:
1) The polyclonal anti-Dazl antibodies were raised in chickens, as were the anti-Ddx4/Vasa antibodies listed in the M&M (from Blokhina et al., 2019). The new Figure 3 shows double labeling with these two antibodies, yet the M&M does not list how this was accomplished with antibodies from the same species. Perhaps this experiment was instead performed using rabbit anti-Ddx4 antibodies from Knaut et al. This needs to be clarified.
2) Comments state: “The stage (12-14 dpf), when germ cells are lost in dazl mutants occurs before expression of the germline stem cell marker, nanos2 has been reported at day 21 (Beer et al 2013). Thus, Dazl acts upstream of or more conservatively before stem cell specification;” I urge caution when concluding that nanos2 expression is not detectable before 21 dpf based on Beer et al. As I’m sure the authors appreciate, analyzing gene expression by RNA in situ between 10-21 dpf is not trivial.
3) The authors intended use Figure 1 was to define the different states that they are proposing exist in the early gonad: “We felt providing a description and overview was important as these steps have not been previously described.” However, the differences between these proposed steps are not evident to this expert so will unlikely be evident to the general audience. The reason for this are the same as stated in the previous review
(lack of resolution and quantification). The one transition that I do agree with is the transition from the “raisin” shaped germ cell nucleus to the round nucleus containing prominent nucleoli. In addition, the model in 1H is based on data that is not presented in or supported by Figure 1 (e.g. the presence of ring canals between cells at these time points is never established in this paper yet it is presented as a forgone conclusion; in normal gonads prominent nucleoli are present in the nuclei of all premeiotic germ cells, not just those in >2-cell cyst). To conclude, Figure 1 does not serve its intended purpose and instead adds unnecessary confusion.
4) Thanks to the addition of arrows in Figure 1C”’ I am quite convinced that the “irregular cytoplasmic compartments devoid of Vasa and DAPI” are in fact nuclei. This is supported by the DAPI staining in panel C”. Also, while the authors correctly point out that pre-meiotic germ cells generally stain poorly with DAPI, it is possible to obtain significantly better staining and/or images then those shown here (e.g. overnight or longer incubation times in DAPI).

5) Along these lines, in subsequent figures there are large vacuole-looking spaces in or between germ cells that are devoid of Vasa and DAPI staining (e.g. in Figs. 4D and 5C, 6 7). Might these be artifacts of the fixation and/or rehydration methods?
6) The size of the images in Figure 1 are too small. Please consider that this figure is not likely to be reproduced at its current size so adding small arrows to an already small panel that will later be reduced even smaller does not help the reader (or at least old readers!).
Also, while arrows were added there is no mention of what they are pointing to in the figure legend. In addition, the legend for 1D states that "…perinuclear cytoplasm becomes round…" I am unclear what point is being made.
7) Panels 1E shows the same cell cluster as panels 1G, though the images in 1G are at a higher magnification. First, this should be noted in the figure legend. Second, the nucleolus that is indicated, though barely visible, in E" is not seen in the higher magnification view in G. Were these two images taken at a different focal plane?
8) New text: "ß-Catenin immunostaining revealed some partitioning…" What is meant by "partitioning" and how would it provide evidence that the cells shown are in a cyst? "…but it was unclear if cyst cells had a shared cytoplasm or were interconnected by cytoplasmic bridges." As written this statement could be interpreted to suggest that the authors are entertaining the possibility that these germ cells are multinucleated, which is fundamentally different from a cyst that is connected by only a cytoplasmic bridge.
Multinucleated cells would imply cell fusion. This should be clarified.
9) New Figure 8: I am very uneasy concluding that the arrows in this figure are pointing to ring canals. In both flies and mice the ring canals are a very uniform size, yet these are not a uniform size. How do they compare to the size identified previously by TEM (Marlow and Mullins)? They instead look to be of similar size and distribution to the vacuoles seen in other images (e.g. Figure 8A', B' and D'). Without a specific marker for ring canals, the best definitive assay for determining when they first appear during development would be TEM.
10) Additional supplemental figures have been added (Sup Figs 11-13) but they are not mentioned anywhere in the text.

Reviewer 3

Advance summary and potential significance to field

In this revision Bertho et al add additional characterization of observed phenotypes, for example lamin labeling to better observe nuclear morphology, quantification and clarification, including a helpful introduction diagram in figure 1. This was to what was already an interesting manuscript with novel findings regarding the transition of germ cells from mitotic to meiotic control and the role and cellular distribution of key factors such as Dazl and Vasa.

Comments for the author

Several of the related points below on whether dazl mutants indeed form cysts are important to clarify:
- In figure 5 and more generally, are there phenotypic differences between the early individual cell morphology (white color code) and the defective cyst formation (black color code) categories? From the text it seems that the latter is a regressed state into the former. If this is the case then the distinction of the two categories, though reasonable, is a matter of interpretation of the phenotype in a temporal context rather than the presence to different phenotypic categories, and having a the same color code or a note to state this interpretation would help present the findings more objectively. If the two phenotypes have differences then highlighting those would help.
- The text largely states that the transition into cysts in dazl mutant GCs is normal, but the quantification in figure 5 K appears to suggest a reduction in that transition in the mutant. Have the authors considered other interpretations of the phenotype, for example that there is indeed a defect in cyst formation in dazl mutants, and that the minority of formed mutant cysts rapidly degrade leaving only the un-transitioned, individual (initial cell state) cells behind? This is related to the above comment on treating "individual cells" and "defective cyst formation" categories separate, since under a scenario in which cysts do not form, cells would never have transitioned into cyst state and back.

- Why does the number of cells in mutant cysts in figure 5L (all time points) appear to be “1”? if there are cysts that formed one would expect multiple cells present at least in some time points. This is again related to the interpretation of the phenotype as in the two previous comments and additionally is difficult to understand given images such as 5D’ (which appears as if there were multiple cells in a cyst)

Other minor concerns:
- In the description of the transition, it would be helpful to clearly state that somatic cells are enclosing single cyst cells, leading to enclosed cysts with smaller numbers. This seems to be the case from the description in the text as well as the quantification of cell number in figure 5 but it still seemed somewhat unclear at least to this reader.
- what is the significance of vasa+ cells being vacuolated in dazl;chk2/+ GCs? Is vacuolation a property of dazl or zili mutants as seems to be the case (from the Discussion) or is it only found under conditions of reduced chk2 function?
- In figure 1H (introductory diagram) at 7-10 dpf, these are presumably separate GCs within a cyst, yet as drawn nuclei seem to be lacking surrounding membranes and be cysts appear syncytial. Dashed lines or other would help to convey that there are multiple cells. The same would be the case in 12-14 dpf cysts, if indeed there is membrane between the cells other than the ring canals.

Second revision

Author response to reviewers' comments

We are grateful to the reviewers for their time and thoughtful comments. We have carefully reviewed the reviewer’s comments, and we are delighted that they appreciated the extent to which we were able to revise the manuscript while the labs were shut down. Soon after receiving the reviewer comments, our lab was partially reopened and therefore we elected to address each of the remaining concerns by performing the experiments that we planned to but were unable to perform while our lab was shut down. Specifically, we have been able to examine *nanos2* expression using RNAscope and we have performed the TEM analysis requested by the reviewers. We hope that the reviewers agree that these new data provide support for our conclusions and strengthen our manuscript. Our point by point responses to each of the reviewer concerns are detailed below.

Reviewer 1 Advance Summary and Potential Significance to Field...

The authors have done a commendable job in revising their prior submission given the constraints of the current public health crisis. The manuscript has several strengths. The authors generate mutant alleles of the zebrafish Dazl gene and show that they result in recessive sterility. The expected effect on sex determination—masculinization—is observed. Although the authors did not conduct transgenic rescue experiments, they can exclude off-target effects because independently generated alleles behave similarly and mutant alleles fail to complement as expected. The Dazl alleles are a good contribution to the field and contribute to our understanding of the roles for this gene in germline development of vertebrates and invertebrates. A key contribution of this manuscript is its documentation of the process of cystogenesis in zebrafish germline development and the finding that Dazl plays a key role. No doubt it will be important to examine this process at an ultrastructural level and in real time, but the videos provided by the authors clearly document that ring canals can form in the mutant but are likely not maintained for some reason. The manuscript also reports specific antibodies to Dazl and its specificity for the germline. The authors mention that Dazl protein was not detectable in germ cells at the 30 hpf stage and thus any maternally contributed protein is likely to be removed before germline defects are first observed.

It would have been nice for the authors to stain early embryos to determine what extent if any maternally contributed Dazl might play. I was confused by the citation of the Giraldez et al. (2006) paper saying that Dazl mRNA is removed from early embryos in a Dicer-dependent fashion.

I waded through the main text and the supplemental data of that paper and couldn't find the cited information. Perhaps it was buried in a different gene ID (?) Nonetheless, I would have liked to see immunofluorescence or western blots with early embryos. However, given the current situation, I think reviewers should be willing to settle for textual modifications or appropriate caveats.

We also were interested in and indeed expected potential maternal Dazl contributions, and so immunostained early embryos at several stages when all the RNAs and proteins are maternal (1-cell stage, 2 cell stage, 4-cell stage), and at early stages when maternal and zygotic products overlap: shortly after genome activation (sphere stage) and early gastrula (shield). To visualize the germ plasm and germ cells we co-immunostained with Buc and Vasa antibodies at 1-4-cell stage and with Vasa at sphere and shield stage. We did not detect Dazl protein at the vegetal pole at 1-cell stage, at the cleavage furrows (2-4 cell stage), or in presumptive primordial germ cells at sphere stage.

Mishima (current biol, 2006 and Bazzini, science, 2012 showed that *dazl* RNA has miR430 sites in the 3'UTR suggesting that *dazl* RNA would be degraded by miR430. In that work they overexpressed GFP-Dazl, and showed that Dazl protein binds to the miR430 site and increases translation of its own RNAs and other germline RNAs; therefore, we anticipated Dazl protein would be present at this time. However, we did not detect Dazl protein at 30 hpf in the germ cells, suggesting that either maternal *dazl* RNA is degraded, is not translated, or our antibody does not detect Dazl in germ cells at that stage. Although we cannot exclude the possibility that there is a distinct splice variant that our antibodies do not recognize, multiple lab members repeated these experiments (at least 5 independent experiments) and we tested 4 distinct batches of antibody, including a C-terminal antibody, all of which detected Dazl protein in wild- type but not mutant gonads at later stages.

A few weaknesses were apparent in the manuscript.

No information is provided as to how Dazl might play a role. The assessment of this reviewer is that these studies should be beyond the scope of this initial report at this current time.

We are keen to understand how Dazl regulates this process. In the revised results and discussion we suggest potential Dazl mechanisms based on known interacting partners and previous in vitro and overexpression assays. We agree with the reviewer that this additional analysis is beyond the scope of this initial report.

Another weakness, is that the manuscript is overly long. I found the studies of chk2 and p53 to be extraneous. The premise that these factors could be directly involved was weak. It would have been shocking and completely unexpected if the authors observed epistatic interactions, which they did not.

We respectfully disagree as p53 has been shown to suppress loss of the germline in other zebrafish fertility mutants. Nonetheless, we have shortened this section and moved these results to the supplemental data section.

Another issue is that the images are not of uniformly high quality. For example, it was difficult for me to see the perinuclear Vasa granules and it was hard to assess the authors' conclusion that perinuclear Vasa granules were less apparent in the mutant than in the wild type.

We have toned down this conclusion in the revised manuscript.

A few additional minor points: phalloidin stains cortical actin, not plasma membranes (page 15);

We thank the reviewer for this feedback. "Plasma membranes" has been replaced with "cortical actin" (page 17), as suggested.

Supplemental Figure 10 is incorrectly cited (page 15);

This has been corrected.

the authors spend a lot of time discussing nuclear morphology (page 20), but I am not sure what the ziwi:gfp experiment really tells us; I think the emphasis on when Dazl functions with regard to the meiotic entry decision is misplaced as the germ cells may not progress to the appropriate stage and this may be an indirect consequence;

We have revised the text to better explain why we examined *ziwi:GFP*. We used this germ cell specific zygotic reporter to determine if the *dazl* mutant germ cells activate the zygotic germ cell program. We found that wild type and mutants cells activate and express *ziwi* at germ cells stages before and after nuclear membrane morphology changes. Thus, zygotic transcription is initiated independent of *dazl* because we detect the GFP reporter in the mutant cells.

"To determine if germline specific zygotic programs are activated in the absence of Dazl, we examined activation of *ziwi*, a germ cell marker that is known to be expressed zygotically, by crossing each allele onto the established *ziwi:GFP* reporter background (Leu and Draper, 2010)."

and I think it would be useful to tone down the statement about the actin-rich structures, reminiscent of fusomes, perhaps duplicating (p. 21).

The sentence has been revised as follows:

"Close inspection of actin (labeled with Phalloidin) in gonads with wild-type genotypes at 8 and 10d revealed an actin rich density within GCs (Figure 8A-A'; C-C')."

David Greenstein

Reviewer 2 Comments for the Author...

The significance of the manuscript, as written, rest on the ability to unambiguously identified cells that are within cyst from those that are not. Unfortunately, the revisions do not solve this issue. In addition, the revisions raise a few new questions:
1) The polyclonal anti-Dazl antibodies were raised in chickens, as were the anti-Ddx4/Vasa antibodies listed in the M&M (from Blokhina et al., 2019). The new Figure 3 shows double labeling with these two antibodies, yet the M&M does not list how this was accomplished with antibodies from the same species. Perhaps this experiment was instead performed using rabbit anti-Ddx4 antibodies from Knaut et al. This needs to be clarified.

The reviewer is correct. We performed the experiment with an anti-Vasa (Ddx4) rabbit antibody that was inadvertently left out of the Materials and Methods section. This M&M has been revised as follows: "Chicken anti-Vasa antibody (Blokhina et al., 2019) was used at a 1:5000 dilution or rabbit anti-Vasa (SAB2702444, Millipore-sigma,1:500)."

2) Comments state: "The stage (12-14 dpf), when germ cells are lost in dazl mutants, occurs before expression of the germline stem cell marker, nanos2 has been reported at day 21 (Beer et al 2013). Thus, Dazl acts upstream of or more conservatively before stem cell specification;" I urge caution when concluding that nanos2 expression is not detectable before 21 dpf based on Beer et al. As I'm sure the authors appreciate, analyzing gene expression by RNA in situ between 10-21 dpf is not trivial.

We performed RNAscope experiments (fluorescent *in situ* hybridization) to confirm that Dazl acts upstream of *nanos2*. For this purpose, we probed *dazl* and *nanos2* RNA in 'individual/cluster' germ cells at d7, during the transition/amplification step at d10 and when the cyst has formed at d14 in wild-type and *dazl* mutant siblings. We first detected *dazl* and *nanos2* RNAs at d7. Beginning after change in nuclear morphology and during the transition stage, all germ cells activate *nanos2*. During the transition/amplification stage some cells had more *nanos2* foci than others, whereas *dazl* was expressed in all germ cells. At d14, a limited number of cells highly expressed *nanos2* (*nanos2*^{high} expressing cells) and had low expression of *dazl*. Based on their high levels of *nanos2*, we consider these to be the germline stem cells. In *dazl* mutant cells, we only observed *nanos2*^{low} expressing cells. These results suggest that Dazl acts upstream of germline stem cell specification. The results and discussion sections have been revised to include these new data images and

quantification (Figure 10).

3) The authors intended use Figure 1 was to define the different states that they are proposing exist in the early gonad: “We felt providing a description and overview was important as these steps have not been previously described.” However, the differences between these proposed steps are not evident to this expert so will unlikely be evident to the general audience. The reason for this are the same as stated in the previous review (lack of resolution and quantification). The one transition that I do agree with is the transition from the “raisin” shaped germ cell nucleus to the round nucleus containing prominent nucleoli. In addition, the model in 1H is based on data that is not presented in or supported by Figure 1 (e.g. the presence of ring canals between cells at these time points is never established in this paper yet it is presented as a forgone conclusion; in normal gonads prominent nucleoli are present in the nuclei of all premeiotic germ cells, not just those in >2-cell cyst). To conclude, Figure 1 does not serve its intended purpose and instead adds unnecessary confusion.

We have revised the figure and moved it to the supplemental data.

4) Thanks to the addition of arrows in Figure 1C”’ I am quite convinced that the “irregular cytoplasmic compartments devoid of Vasa and DAPI” are in fact nuclei. This is supported by the DAPI staining in panel C”. Also, while the authors correctly point out that pre-meiotic germ cells generally stain poorly with DAPI, it is possible to obtain significantly better staining and/or images then those shown here (e.g. overnight or longer incubation times in DAPI).

We thank the reviewer for his advice. In fact, the samples were incubated overnight with DAPI (Vectashield DAPI), and we agree that if nuclei were in those regions, we would have detected them. Although further characterization is required, we are quite convinced that there are no nuclei in those regions. We intend to examine this further by EM in the future, but including this description here would significantly lengthen the manuscript and is beyond the scope of this study, particularly since there is not a difference between wild-type and mutants at that phase.

5) Along these lines, in subsequent figures there are large vacuole-looking spaces in or between germ cells that are devoid of Vasa and DAPI staining (e.g. in Figs. 4D and 5C, 6, 7). Might these be artifacts of the fixation and/or rehydration methods?

The large vacuole-looking space are corresponding to a biological state, the transit-amplification state. (cell in division forming a syncytium). We do not think that fixation or rehydration are an artifact because if this were the case, we would see ‘vacuole-looking spaces’ everywhere in all the samples. However, this was not the case. As mentioned above, we intend to more fully describe this phase based on additional TEM analysis in the future.

6) The size of the images in Figure 1 are too small. Please consider that this figure is not likely to be reproduced at its current size so adding small arrows to an already small panel that will later be reduced even smaller does not help the reader (or at least old readers!).
Also, while arrows were added there is no mention of what they are pointing to in the figure legend. In addition, the legend for 1D states that “…perinuclear cytoplasm becomes round…” I am unclear what point is being made.

The legend has been modified and the figure has been moved to the supplemental data section. Huge smiles for old readers J!

7) Panels 1E shows the same cell cluster as panels 1G, though the images in 1G are at a higher magnification.
First, this should be noted in the figure legend.

We confirmed that the panel 1E and panel 1G show the same cluster. The figure legend has been updated as follows:
“An early cyst labeled with ß-Catenin (Magenta) from Figure 1E-E’’’ (above)”

Second, the nucleolus that is indicated, though barely visible, in E” is not seen in the higher magnification view in

G. Were these two images taken at a different focal plane?

The two images represent different focal planes.

8) New text: “ß-Catenin immunostaining revealed some partitioning…” What is meant by “partitioning” and how would it provide evidence that the cells shown are in a cyst? “…but it was unclear if cyst cells had a shared cytoplasm or were interconnected by cytoplasmic bridges.” As written this statement could be interpreted to suggest that the authors are entertaining the possibility that these germ cells are multinucleated, which is fundamentally different from a cyst that is connected by only a cytoplasmic bridge. Multinucleated cells would imply cell fusion. This should be clarified.

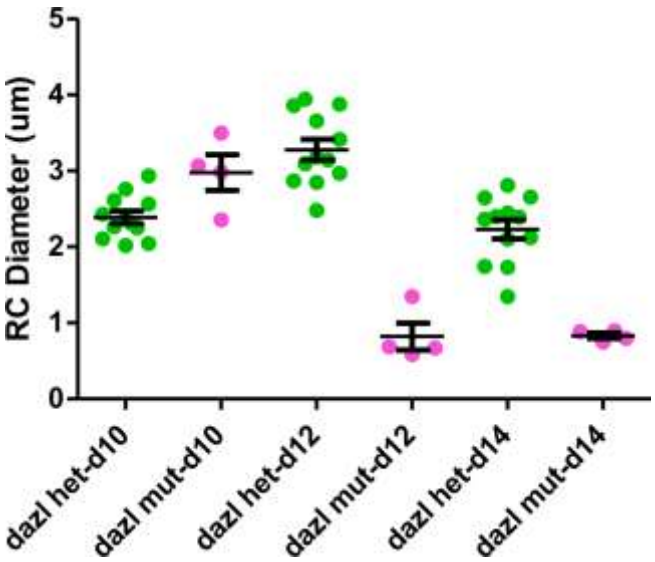
We have revised the sentence as follows:
“ß-Catenin immunostaining revealed an incomplete boundary between cells within a cyst (Figure 1G);”

9) New Figure 8: I am very uneasy concluding that the arrows in this figure are pointing to ring canals. In both flies and mice the ring canals are a very uniform size, yet these are not a uniform size. How do they compare to the size identified previously by TEM (Marlow and Mullins)? They instead look to be of similar size and distribution to the vacuoles seen in other images (e.g. Figure 8A’, B’ and D’). Without a specific marker for ring canals, the best definitive assay for determining when they first appear during development would be TEM.

We provide TEM evidence for ring canal ultrastructure in the revised Figure 9 G-J.

In addition, as requested by the reviewer, we roughly measured the diameter of each actin ring structure and provide the data as a reviewer figure below. Because these rings are much larger in diameter than ring canals observed in the juvenile gonad (~700nm) (Marlow and Mullins), we refer to these structures as “actin rings” throughout the revised text, and refer to the structures confirmed by TEM as ring canals.

d10-*dazl*$^{ae57/+}$ - Actin Ring $_{diameter}$ = 2.39 µm +/- 0.4 (3 samples)
d10-*dazl*$^{ae57/ae57}$ – Actin Ring $_{diameter}$ = 2.98 µm +/- 0.4 (1 sample)
d12-*dazl*$^{ae57/+}$ - Actin Ring $_{diameter}$ = 3.28 µm +/- 0.4 (3 samples)
d12-*dazl*$^{ae57/ae57}$ – Actin Ring $_{diameter}$ = 0.82 µm +/- 0.4 (1 sample)
d14-*dazl*$^{ae57/+}$- Actin Ring $_{diameter}$ = 2.23 µm +/- 0.4 (3 samples)
d14-*dazl*$^{ae57/ae57}$ – Actin Ring $_{diameter}$ = 0.83 µm +/- 0.0 (1 sample)



10) Additional supplemental figures have been added (Sup Figs 11-13) but they are not mentioned anywhere in the text.

The corresponding supplemental figures have been indicated in the revised text.

Reviewer 3 Advance Summary and Potential Significance to Field...

In this revision Bertho et al add additional characterization of observed phenotypes, for example lamin labeling to better observe nuclear morphology, quantification and clarification, including a helpful introduction diagram in figure 1. This was to what was already an interesting manuscript with novel findings regarding the transition of germ cells from mitotic to meiotic control and the role and cellular distribution of key factors such as Dazl and Vasa.

Several of the related points below on whether dazl mutants indeed form cysts are important to clarify:

- In figure 5 and more generally, are there phenotypic differences between the early individual cell morphology (white color code) and the defective cyst formation (black color code) categories? From the text it seems that the latter is a regressed state into the former. If this is the case then the distinction of the two categories, though reasonable, is a matter of interpretation of the phenotype in a temporal context rather than the presence to different phenotypic categories, and having a the same color code or a note to state this interpretation would help present the findings more objectively. If the two phenotypes have differences, then highlighting those would help.

This is a great comment and suggestion. It is true that temporally one can see *dazl* mutant cells with apparently equivalent gross level phenotypes between d8/d10 and d12/d14. At d8 and d10, mutant cells look small, have a high nucleus to cytoplasm ratio and a very compact DNA morphology. At d10, some of the *dazl* mutant cells enter the transition/amplification stage. Thereafter, we only observed mutant cells with overall phenotypes resembling those observed at d8/d10; however, mutant cells at the later stages show abnormalities not apparent at the early stages. Based on these observations, we think that the mutant attempt to form a cyst but fail to do so, and thus we interpreted this as a failed cystogenesis. Nonetheless we agree that it the phenotype is equally apparent if we categorize these cells as individuals and describe the observed abnormalities. We have modified figures Fig. 5 and Supp Fig. 6 and the text accordingly.

- The text largely states that the transition into cysts in dazl mutant GCs is normal, but the quantification in figure 5 K appears to suggest a reduction in that transition in the mutant.

Since early gonad development is not synchronous and varies from individual to individual even in wild-type, this apparent difference is likely due to timing as indicated in the text "*dazl* mutant GCs remained as individuals indicating a failure of or severe delay in cystogenesis (Figure 5H, J-L; Supplemental Figures 5, 6, 7)." Nonetheless, mutant cells are able to initiate this transition even without Dazl.

Have the authors considered other interpretations of the phenotype, for example that there is indeed a defect in cyst formation in dazl mutants, and that the minority of formed mutant cysts rapidly degrade leaving only the un-transitioned, individual (initial cell state) cells behind? This is related to the above comment on treating "individual cells" and "defective cyst formation"

categories separate, since under a scenario in which cysts do not form, cells would never have transitioned into cyst state and back.

The text has been revised to more clearly state this possibility.

"It is unclear if failed cysts return to the individual state, or if the germ cells are quickly eliminated such that only pre-cystogenic cells remain. In either case, mutant cells ultimately remain as individuals that fail to differentiate as meiocytes."

- Why does the number of cells in mutant cysts in figure 5L (all time points) appear to be "1"? if there are cysts that formed one would expect multiple cells present at least in some time points. This is again related to the interpretation of the phenotype as in the two previous comments and additionally is difficult to understand given images such as 5D' (which appears as if there were multiple cells in a cyst)

We apologize for the confusion. The transition state gonads are not included in cyst quantification in wild-type or mutants because there are no clear cyst boundaries during this phase. Other than the transition state, we did not observe multicellular cysts in mutant gonads. This has been clarified in the text.

Other minor concerns:

- In the description of the transition, it would be helpful to clearly state that somatic cells are enclosing single cyst cells, leading to enclosed cysts with smaller numbers. This seems to be the case from the description in the text as well as the quantification of cell number in figure 5 but it still seemed somewhat unclear at least to this reader.
We have revised the text as follows:

"During the transition-amplification step somatic gonadal cells enclosed the germ cells of both wild-type and mutants."

- what is the significance of vasa+ cells being vacuolated in dazl;chk2/+ GCs? Is vacuolation a property of dazl or zili mutants as seems to be the case (from the Discussion) or is it only found under conditions of reduced chk2 function?

The vacuolation is observed in *dazl* (this study), *vasa* (Hartung et al., 2014), *zili* (Houwing et al, 2007) mutant cells when the cells are dying. The vacuolation appears to be a common feature, and suggests that germ cell death at these stages involves vacuolation. This is also confirmed by our TEM analysis. Additional investigation is required to understand the regulators and mechanism of germ cell elimination at this stage, but is beyond the scope of this study. Loss of *chk2* does not cause germ cell death or vacuole formation as *chk2* mutant gonads are indistinguishable from wild-type.

- In figure 1H (introductory diagram) at 7-10 dpf, these are presumably separate GCs within a cyst, yet as drawn nuclei seem to be lacking surrounding membranes and be cysts appear syncytial. Dashed lines or other would help to convey that there are multiple cells. The same

would be the case in 12-14 dpf cysts, if indeed there is membrane between the cells other than the ring canals.

This has been addressed and the figure is now Supp. Fig.1

Third decision letter

MS ID#: DEVELOP/2019/187773

MS TITLE: Zebrafish *dazl* regulates cystogenesis and germline stem cell specification during the primordial germ cell to germline stem cell transition.

AUTHORS: Sylvain Bertho, Mara Clapp, Torsten U Banisch, Jan Bandemer, Erez Raz, and Florence L. Marlow

I hope that all is well. Apologies for the delay in handling your manuscript. I have now received all the referees reports on the above manuscript. As you will see the referees are happy with your

revisions and there are just some minor scientific issues to address before publication. However, I have just noticed that the system-generated manuscript word count is 9034 which is way over the word limit of 7000-7500 - I have some flexibility to go a bit higher than this but not much so assuming the word count is correct, you will need to trim the text - sorry.

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.

Reviewer 1

Advance summary and potential significance to field

The authors have done a terrific job in addressing the critiques from the prior rounds of review. This manuscript is a great contribution to the field for its analysis of early germline cyst formation in zebrafish and the role of Dazl. I hope the manuscript can be published without delay. The authors may wish to address a few minor points listed in the box below. Congratulations on this beautiful work under difficult circumstances.

David Greenstein

Comments for the author

Minor Points
1. Page 3, line 8. The authors might consider citing Extavour and Akam (2003).
2. Page 5, line 5. The authors should cite Wolke et al. (2007).
3. Page 8, last paragraph of Introduction. The rationale is not well set up or motivated or tightly linked to what comes before. The presentation dilutes the impacts of the chief findings and contributions of the manuscript.
4. Page 11. The dose of tricaine was not specified.
5. Page 16. Refer the reader to individual panels of Supplemental Figure 1.
6. Page 18. Remind the reader what the epitope was (e.g., an N-terminal peptide).
7. Page 23, line 3. elegans 8. Page 24. Please consider putting unannotated TEMs that lack false coloring in the supplemental information.
9. Page 25, line 17. The "male bias" wasn't compelling because control data of heterozygotes was not presented for comparison.
10. I would encourage the authors to tone done the conclusions about entry into the meiotic pathway of development because examination of the expression of meiotic markers and meiotic events are not presented.

Reviewer 2

Advance summary and potential significance to field

See previous reviews for summary of advances.

Comments for the author

The authors have satisfactorily addressed the concerns I had with the previous manuscript.

Reviewer 3

Advance summary and potential significance to field

In this second revision Bertho et al revise the article for clarity and add additional data on the expression of germ line genes. This was to what was already an interesting manuscript with novel findings regarding the transition of germ cells from mitotic to meiotic control and the role and cellular distribution of key factors such as Dazl and Vasa.

Comments for the author

Minor comments for revision at the discretion of authors
- bottom of page 5: it is unclear what "functional ploidy" is. A brief sentence or parenthesis would help understand this
-bottom of page 18: "maternal contribution is likely removed during the MZT"; this is indeed likely the case in somatic tissues as stated by the authors. However maternal contribution of dazl in PGCs could well continue to be present and contribute to processes between MZT and 30 hpf, which is when it no longer is observed. This is also consistent with the author's finding that many early PGC processes like PGC viability are unaffected
-bottom of page 21:" raising-like nuclear membrane like the GCs at 10d" Do the authors mean at 8d here? (and are these GCs wild-type?)
- middle of p. 23: "also acts via tp63". Is this meant to be tp53?
- end of Results "nanos2 low cells detected earlier and nanos2 high cells in wild type". The language seems to be jargon or at least undefined terms.
- the model figure seems to suggest that actin bridge formation is initiated by degradation of completed cell walls to result in inter-cellular canals. However, at least in Drosophila, ring canals are formed during cell division, where canals are formed by incomplete cytokinesis. Adjusting the early part of the model diagram or at least clarifying this in the figure legend would help at least not rule out the second option.

Third revision

Author response to reviewers' comments

Thank you for this exciting news. We are thrilled that the reviewers are happy with our revisions and are grateful to each of them for their time and thoughtful review. We have shortened the manuscript to conform to journal guidelines and have addressed the minor comments and suggestions from the reviewers.

With many thanks and best wishes to all.

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This reference has been added as recommended.
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400mg/L has been indicated.
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Added as recommended. Supp Figure 13.
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The sentence has been revised as recommended.

-bottom of page 21:" raising-like nuclear membrane like the GCs at 10d" Do the authors mean at 8d here? (and are these GCs wild-type?).
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It acts through both. This has been revised to clarify.
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The legend has been revised as recommended.

Fourth decision letter

MS ID#: DEVELOP/2019/187773

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AUTHORS: Sylvain Bertho, Mara Clapp, Torsten U Banisch, Jan Bandemer, Erez Raz, and Florence L. Marlow
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.