

Stn1 promotes zebrafish oocyte development via amplifying Wnt/ β -catenin signaling

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As you will see, the referees acknowledge that the findings are potentially interesting, but they also raise a number of concerns and have suggestions how to further strengthen the data. All concerns raised appear to be pertinent and should be addressed. In particular, Stn1's reported role in telomere biology and its potential contribution to germ cell maintenance must be addressed and investigated.

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Martina Rembold, PhD
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Referee #1:

In this interesting manuscript, Zhang et al investigate the role of *stn1* in the regulation Wnt/ β -catenin signaling in zebrafish ovary development. Like in mammals, Wnt signaling was known to play an important role during zebrafish female sex determination, as most *wnt4* and *wnt9b* mutants develop as males. Both *wnt4* and *wnt9b* are expressed in somatic follicle cells that surround the developing germ cells, but it was not known what cells respond to these signals. Using a transgenic Wnt reporter line, they show that the primary cell type in the ovary that appears to be Wnt responsive is germ cells, and in particular premeiotic germ cells and early-stage oocytes. Because *wnt4/wnt9b* are expressed in follicle cells that surround both Wnt responsive and Wnt non-responsive oocytes, they hypothesized that the Wnt responsive oocytes contain a factor that is permissive for activation of downstream responses to Wnt signal reception. They then asked if factors known to interact with β -catenin were also enriched in early-stage germ cells, and identified *Stn1*, which encodes a subunit of the CST complex with known roles in telomere maintenance. *Stn1* had been identified as a potential β -catenin-associated protein by Ehyai et al, 2018, though the evidence for this was weak. In addition, a zebrafish mutant for *stn1* had been made as part of the Zebrafish All Genes KO Consortium for Chromosome 1 (ZAKOC) Chromosome 1 consortium (Sun et al., 2019). Using published scRNA-seq data for the zebrafish ovary they found that *stn1* is primarily expressed in germ cells. Consistent with a function in germ cells development, *stn1* mutants have normal germ cell numbers early, but have lost all germ cells by ~45 dpf. Consistent with a role in Wnt signaling, the germ cells present in late larval *stn1* mutants have slightly reduced expression of the Wnt/ β -catenin reporter transgene. To gain mechanistic insights into the role of *stn1* in germ cell development, they compared the transcriptomes of wild-type and mutants at 19 and 25 dpf, and not surprisingly, found that mutants had reduced expression of genes that are normally expressed in germ cells. Using co-IP experiments they found evidence that *Stn1* interacts with both β -catenin and one of its downstream transcription factors, *Tcf7L2*. Evidence that *Stn1* functions to enhance the output of β -catenin signaling came from showing that it can enhance the transcriptional output of *Tcf7L2* in a transcriptional reporter assay in HEK293 cells. They next identified putative *Tcf/Lef* binding sites in the gene regulatory regions of germ cell-expressed genes that were downregulated in the *stn1* mutants and tested these in transcriptional enhancer assays using *Tcf7L1* and also provide corroborating evidence using ChIP-qPCR that β -catenin associates with these sequences in both ovaries and testes. Finally, they showed that overexpression of *Wnt8a* using a heat inducible transgene system could partially rescue germ cell development, and in particular oocytes, in *stn1* mutants, consistent with *stn1* mutants having a compromised response to endogenous Wnt signals.

Identifying novel regulators of Wnt/ β -catenin signaling would constitute a significant advance in the field, and the data presented in this manuscript makes a compelling argument that Stn functions in Wnt signal amplification. However, there are a few open questions and alternative explanations that need to be addressed before strong conclusions can be drawn. The first major concern is that Stn has known roles in telomere biology as a component of the CST complex that is required for telomere replication, capping, and protection. The authors argue that Stn has a germ cell-specific role, as loss of germ cells is the only major phenotype reported in the *snt1* mutants. However, this reviewer could imagine that germ cells may be more sensitive to loss of the telomere maintenance functions than other cell types and thus telomere defects are the root cause of germ cell loss, not reduced Wnt signaling. The second major concern is that the authors argue that Stn1 function germ cell autonomous, but all evidence is correlative. In supplemental Figure 1E they show that *stn* expression is not restricted to the gonad, but is also expressed in other tissues. While they were able to partial rescue the germ cell defect by transgene overexpression of *stn*, this transgene expressed *stn* in all cells, not just germ cells. A more convincing experiment would be to attempt rescue by expressing *stn* in only germ cells using a germ cell-specific promoter (e.g. *piwl1*).

Minor criticisms/suggestions (in approximate order in text):

1. "In zebrafish, Wnt/ β -catenin signaling plays important roles.." for completeness, Kossack et al., 2019 (PMID:30446521) and Liu et al., 2022 (PMID: 35588359) should also be referenced as these papers more directly demonstrate roles for Wnt signaling (*wnt4* and *wnt9b*) in female development than the two papers that are currently referenced. (Also first add these to first sentence of Results). Also, the accepted nomenclature for *wnt4a* is now simply "*wnt4*" (see updated nomenclature on ZFIN).
2. The experiments using 7X-tcf-Xla-siam:GFP to monitor wnt signaling has the significant caveat that GFP is very stable and may not represent active expression. It would be more accurate to look at *gfp* RNA by in situ hybridization.
3. Fig 1C: Using GFP intensity is not an accurate measure of expression. If the same amount of GFP is present in a small oocyte vs. a large oocyte, the signal will look stronger in the small because the volume is smaller and therefore [GFP] is higher.
4. FigS1a: it is not clear what this data represents. Is the Y-axis oocyte diameter? Is this data from RNA in situ? If so, a representative in situ should be shown.
5. "Since the intrinsic Wnt/ β -catenin signaling activity sharply increased at this stage..." The phrase "...intrinsic Wnt/ β -catenin signaling..." is misleading as it implies that the Wnt signal is intrinsic to the germ cells, yet the lead up to this was describing the role of *wnt4*, which is expressed in somatic cells (follicle cells). Perhaps the authors mean "germ cell-intrinsic response."
6. For completeness, *Wnt4* is not the only Wnt expressed in the ovary. In addition to *wnt4*, *wnt9b* is also expressed in follicle cells and *wnt8a* in oocytes (Liu et al., 2022).
7. Figure 2C. In normal animals, the gonads of males and females at 25-30 dpf are significantly different in size, and therefore have different brightness of GFP (females larger than males; Krovel and Olsen, 2004; Dranow et al., 2016). The wildtype image shown in fig 2C looks to be female, but it would be expected that some gonads looked male-like. Were any WT male-looking gonads present? For completeness, sex ratios should be provided and an male example shown.
8. Fig 2D. Quantifying gonad size from images of gonads that have not been dissected is not an accurate method. Gonads should first be dissected (fixation of fish in PFA overnight aids in dissecting gonads; shrinkage may occur, but it will be proportional to all fish so relative size will still be accurate).
9. Pg6 : "The downregulated genes in the mutants at 19 hpf were not sorted..." should be "...19dpf..."
10. Fig 4b: Bulk RNA seq does not allow you to tell if the reduction of gene expression is due to reduced gene expression in germ cells, or due to mutants having many fewer germ cells relative to wildtype controls.
11. The RNA-seq results do not add anything novel to the story and should be provide as supplemental data. The authors only highlight germ cells expressed genes. Where any non-germ cell expressed genes also affected? Where any gene upregulated?
12. Fig. 4C Why were 4 hpf embryos chosen for IP analysis instead of using germ cells? Is *stn* a maternal transcript? Is there a role for *stn* at 4hpf? What is the relevance of its function at 4 hpf to its function in germ cells? Is Tcf7L2 expressed in germ cells? If so, at what stages?
13. What was the reasoning for only assaying for interactions between Stn1 and Tcf7L2 in Fig. 4C? Where Tcf7 and Tcf7L2 also assayed in pulldowns from fish lysates?
14. Why was Tcf7L1 used instead of Tcf7L2 in figure 4H? Does Stn also interact with Tcf7L1 in pull down assays? Are Tcf7L1a/b expressed in germ cells?

15. BIO assay: Fig4I, J It is not clear why the levels of protein not quantified in this assay? Instead, it is presented as only a qualitative result. Were these assays transient transfections of Stn expression constructs? Were these normalized for transfection efficiency?

16. "Taken together, these data indicate that Stn1 depletion leads to reduced oocyte-intrinsic activity of Wnt/ β -catenin signaling, resulting in decreased expression of germ cell-specific genes and aberrant progression of oocytes." It should be noted that loss of stn1 also affects spermatogenesis as all mutant animals are sterile males.

Referee #2:

Summary:

In this paper by Zhou et al, the authors aim to better understand the role of Wnt/ β -catenin and how they interact with Stn1 during zebrafish oogenesis. They start by measuring Wnt/ β -catenin activity using a transgenic fish model and found that Wnt/ β -catenin signaling is most prominently expressed in oocytes sized 10 to 50um and is dynamic in expression. The authors follow up on this finding using RNA-seq data to identify the Wnt/ β -catenin amplifier Stn1. When Stn1 was ablated, the Stn1 null fish had no gonads and male phenotypes and were infertile. The loss of germ cells was not caused by Caspase3-mediated apoptosis and Stn1 was shown to downregulate germ cells specific genes. Upon further analysis, Stn1 is directly related to Wnt/ β -catenin activity through modulation of the β -catenin/Tcf complex. Lastly, these authors show that Wnt8a is partially sufficient at modulating oocyte loss caused by stn1 loss. These data show the importance of Wnt/ β -catenin in zebrafish oogenesis and support the importance of Stn1 in oogenesis.

Major:

1. Figure 1B,C: 1B) please use indicate oocytes of specific diameters to establish correlation of diameter with GFP brightness. 1C, please clarify that each dot represents a single oocyte. Please include how many animals and ovaries are represented at each time point?
2. Results, paragraph 2, supplementary Figure 1 text. Please rectify the discussion related to the timing of somatic cell Wnt4a, which was reported from a literature source (Kossack et al 2019) to be at 20-25 hours pf, to the data outlined in Supplementary Figure 1, which was focused at 20dayspf (S1A), 40dpf (S1B-D), and adult animals (S1E). It would be helpful to show the images used to calculate data in figure S1A.
3. Supplementary Figure S3: Please define the outcome of breeding images shown in the figure legend of Figure S3C. Figure S3G control appears that it may have the potential to overexpress stn1; was any phenotype noticed in the gonads of these mice?
4. Throughout the manuscript: Please define stn1 +/- genotype; it appears that it represents a sibling control indicating possibly a heterozygote genotype (+/-).
5. Figure 2: the phenotype of the stn mutant zebrafish is very exciting. The results would be strengthened if there was more description to explain the discrepancy in results in mutant animals, many 50% or less representative sections. In addition, germ cell loss did not appear to be caused by a caspase 3-mediated apoptosis pathway, but no quantification is provided. It would also be helpful to investigate other death pathways or proliferation defects.
6. Figure 4: while it is a strength that both fish and in vitro data are used to generate these data, please clarify the rationale for the use of HEK293 cells, it is not clear what a "Wnt-off" cell line means.
7. Figures 5B, 6F, S4, please include a negative control to ensure the effect tested for is specific

Minor:

Throughout: please recheck all hpf and dpf notations, some to appear to be mislabeled (for example last line in the section describing Figure 3).

Referee #3:

The mechanisms of early germline differentiation are not fully understood in vertebrates. Previous studies in mammals and zebrafish have identified canonical Wnts, including Wnt8 and Wnt4 as a regulator of early germline development and germline stem cell renewal, respectively. In this interesting paper from Zhang and colleagues, the authors investigate the role of the Stn1 gene, a component of the CST complex that plays a role in telomere synthesis, replication, and response to replication stress. The authors report multiple loss of function mutant alleles disrupting zebrafish stn1. They provide compelling evidence for an essential Stn1 function development of gonads of both sexes, as all mutants are sterile males. They provide evidence that initial

germ cell numbers are intact; however, all germ cells are lost in juveniles. As anticipated based on loss of the germline, they report reduced expression of germ cell markers. The authors explored the upstream regulatory sequences in several germ cell genes and identified binding sites *in silico* and through CHIP and luciferase assays for TCF/Beta-Catenin and Stn1 in cultured HEK cells and/or from gonadal tissues. They hypothesized and concluded that Wnt signaling is amplified by Stn1 through a cooperative binding mechanism. Using a transgenic approach, they demonstrate that germ cell loss in *stn1* mutant gonads can be suppressed by overexpressing Wnt8a. Based on expression of Wnt4 in the somatic gonad of zebrafish and its roles in mammals, they propose Wnt4 may be the relevant ligand. Overall, the study is well done, and the findings are novel; however, there are several points that should be clarified and some of the conclusions are not fully supported by the data provided. In addition, there are several grammatical errors throughout the manuscript. These are noted below in instances where the meaning or interpretation of the sentences are unclear.

Major:

- 1) Stn1 is not introduced in the introduction so the telomere discussion in the discussion sections comes out of context.
- 2) Although Wnt8a can suppress the mutant phenotype when overexpressed, Wnt4 but not Wnt8a expression in the ovary/testis is discussed. The rescue of germ cell loss by heat shock Wnt8a is convincing; however, what happens to the gonad at later stages is not discussed. Do the gonads develop further, are the rescued males/females fertile or do they arrest in oocyte development? If rescue is incomplete, is it possible that canonical Wnt is only required for the early stage and that noncanonical Wnt might be required later as Wnt4 has been shown to act in both the canonical and noncanonical Wnt pathways?
- 3) Expression of *stn1* transcripts is shown from published single cell data. This data suggests that *stn1* is expressed in germline stem cells and then is off and then is expressed again in early oocytes. Although PCR data are shown for *stn1* expression, it is important to validate the single cell data with HCR or a similar *in situ* hybridization analysis to determine which cells/stages express *stn1*. This might be relevant to the point above, if Wnt8 rescue is incomplete.
- 4) The model suggests that Stn1 amplifies Wnt8, and the cartoon as depicted suggests that there is Wnt- independent Stn1 but also shows Wnt8 upstream of Stn1 abundance. This seems inconsistent with the rescue data, which seem to place Wnt downstream or in a parallel pathway to Stn1. It would help to clarify this in the model. Also, given that Stn1 expression also appears to be dynamic it would help to compare its expression to Wnt expression and to some of the targets indicated, and also to the Wnt receptors since they would likely be an intermediate between an activating Wnt and downstream signaling.
- 5) Related to *stn1* expression, it is unclear how the cells/stages labeled by the Wnt reporter relate to the dynamic expression pattern of *stn1*.
- 6) "Conversely, in the *stn1* mutants, the oocytes gradually diminished, ultimately leaving only fibrous tissue in the gonads." What was the evidence that the tissue is fibrous tissue as opposed to somatic gonad tissue?
- 7) In several places, the germ cell genes are described as downregulated. However, it is likely that the reduced expression is due to the reduced number and less advanced stages of germ cells in the mutant gonads. To distinguish between downregulation and less expression because there are fewer germ cells one would need to look at single cells, either with quantitative HCR (normalized to a "housekeeping" gene or single cell analysis).
- 8) "Wildtype larval zebrafish were treated from 19 to 25 dpf with XAV939 or PNU74654, which are Wnt/ β -catenin signaling specific inhibitors that activate Axin or inhibit the β -catenin/TCF complex, respectively (Huang et al, 2009; Trosset et al, 2006). Subsequently, the transcript levels of *ddx4*, *dnd1*, *piwil1*, *piwil2*, *tldr9*, *tldr1*, and *tldr7a* were evaluated. evaluated. All transcripts were decreased following XAV939 or PNU74654 treatment, as indicated by qRT-PCR analysis (Fig. S4A, B)." Did these treatments lead to loss of germ cells or phenocopy *stn1* mutants? Were there any other phenotypes observed in treated fish? It is important to distinguish whether reduced expression was due to regulation, loss of germ cells, or other developmental delay phenotypes, and to determine the extent to which the phenotypes resemble *stn1* mutants. Examination of transcripts by PCR is not sufficient to distinguish between these possibilities.
- 9) "Stn1 depletion weakened the association between β -catenin and the promoter regions of germ cell-specific genes at 25 dpf (Fig. 6B)." In a later sentence "strengthens" is used. The problem with these statements is that the stages of activation appear not to be achieved in the mutant and there are fewer germ cells, thus it is difficult to claim weaker association is directly due to loss of *stn1* rather than due to loss of the germ cells or stage differences between mutant and control gonads. This should be qualified.
- 10) "which acts as a novel binding partner of Tcf/Lef and cooperates with the β -catenin/Tcf complex to amplify Wnt/ β -catenin signaling in oocytes." The mutant data provided are not sufficient to support the statement that there is cooperative activity in oocytes.
- 11) In Figure 4A, it appears that the mutant germ cells do not reach the stage when robust activation of the reporter is activated. Failure to reach the stage makes it challenging to establish a regulatory relationship. Initial expression/reporter expression in early GCs of *Ddx4* seems independent of *stn1*. Plus, as mentioned above, expression data indicates Stn1 is on in GSCs, then off then on in oocytes? How does reporter activation align with Stn1 expression? Stn1 expression should be validated.
- 12) Figure 4G, in the IP, all of the proteins also appear to come down with just GST.
- 13) For the luciferase assays, empty vector may not be the right control given how low the activity is for the germ cell genes compared to TOPFlash.
- 14) Fig. 6F, it seems challenging to make these comparisons since there are different numbers and stages of germ cells and there is high variation from gonad to gonad, even in the controls. Histological evaluation is likely to provide more meaningful comparisons as the stages can be directly compared.

Minor:

- 1) In Fig. 4H, there appears to be an extra plus sign in the line for Stn1-Flag.

- 2) In the abstract the following sentence needs context. It reads like this is already known as written. "The dynamically intrinsic activity. "However, the dynamically intrinsic activity of Wnt/ β -catenin signaling in the progression of oocytes is poorly understood." Maybe "the dynamics of Wnt signaling in oocyte progression has not been reported".
- 3) Stn1 should be spelled out and introduced the first time it is used.
- 4) In the abstract, "is enrichingly expressed in germ cells" should instead be "expression is enriched in..."
- 5) Also in the abstract, "the" is needed before transcription factor.
- 6) Abstract: "stn1 leads to loss of massive oocytes prior to or during their development to the IB stage," "should instead be leads to massive loss of oocytes."
- 7) Abstract: By "male-like phenotype" I assume the authors mean "phenotypically male" or "sterile male".
- 8) Abstract: The following sentence is unclear as written. "Temporally induced activation of the Wnt/ β -catenin signaling partially reverses Stn1 depletion induced loss of germ cells and eventually promotes development of oocytes to the stage IB."
- 9) Abstract: I believe this is a typo and that "facilitating" should be "facilitating".
- 10) Introduction: The following sentence is unclear as written. "In ovaries, a key germ cell-intrinsic activity of signaling pathway, Wnt/ β -catenin signaling that govern their development is evolutionarily conserved across the animal kingdom(Le Rolle et al, 2021; Takase & Nusse, 2016)."
- 11) Introduction: "is crucial for its pluripotent maintenance." "its" is not needed.
- 12) Introduction: "meiosis entry" is typically written as "meiotic entry"
- 13) Introduction: "regulated in progression". Consider instead "regulated during the progression".
- 14) The conclusion "These results imply that Stn1 may regulate Wnt/ β -catenin signaling pathway and play key roles in germ cell development." Isn't really supported by the data presented immediately before it is made.
- 15) The following is a complex sentence. Consider splitting it into two sentences. Hence, an anti-active-caspase-3 antibody was employed to immunostain stn1 mutant embryos with a Tg(piwil1:EGFP-UTRnanos3) genetic background at 25 dpf. In agreement with phenomenon in vasa mutants (Hartung et al, 2014), the abundance of active-caspase-3-positive oocytes in mutants was not higher than in siblings despite oocyte sin stn1 mutants are apparent loss (Fig. 2F)."
- 16) In the section titled "Loss of Stn1 reduces the expression abundance of germ cell-specific genes" hpf is used several times to refer to the stage, but I think these are typos and should be dpf. - also, there is a typo in this title "abundance".
- 17) The following sentence is unclear as written. "(Fig. 3E). Taken together, these results suggest that loss of Stn1 leads to the germ cell numbers are greatly reduced in mutant gonads at 25 while not 19 hpf.
- 18) The first "the" is not needed in this sentence "The Stn1 bound with the Tcf7l2 fragment spanning residues 63-327 (Fig. 4F and G).
- 19) The basis for describing HEK cells as a "wnt off" line should be discussed briefly.
- 20) There is a typo in the following sentence. "Thus, Stn1 likely functions by binding with Tcf/Lef at target promoters and promoting the association of β -catenin/Tcf complexe with the target gene promoters.
- 21) "And its genetic deletion in zebrafish leads to loss of oocytes and results in a male-like phenotype and infertility." It appears to lead to loss of the stem cells too.
- 22) "its actin in oogenesis." Should "actin" be "action".
- 23) "In zebrafish, Ddx4 is required for germ cell differentiation and maintenance, while its disruption results in sterile adult male-like fish (Hartung et al., 2014). Similarly, Dnd1 is implicated in germ cell fate maintenance; its depletion in zebrafish leads to complete loss of germ cells (Chu et al, 2023; Weidinger et al, 2003)." The Ddx4 and Dnd1 phenotypes both lead to loss of the germline but are very different phenotypes from one another. In Dnd1 loss, the primordial germ cells are lost within the first few days of development and in ddx4 mutants they are lost much later in gonad development. Thus, they should not be described as "similar"
- 24) For the genotypes +/- is used throughout the figures. Instead, label these, which I assume to be either heterozygotes or homozygous WT, as "wild type", and assuming this is the case, in the figure state that no differences were observed between heterozygotes and homozygous WT, thus both genotypes are referred to as wild type.

Point-to-point response:

We thank the reviewers for their comments and suggestions. In response to these comments and suggestions, the following changes have been made. Also, a formatted pdf of the response was attached as supplementary information.

Referee #1:

In this interesting manuscript, Zhang et al investigate the role of *stn1* in the regulation Wnt/ β -catenin signaling in zebrafish ovary development. Like in mammals, Wnt signaling was known to play an important role during zebrafish female sex determination, as most *wnt4* and *wnt9b* mutants develop as males. Both *wnt4* and *wnt9b* are expressed in somatic follicle cells that surround the developing germ cells, but it was not known what cells respond to these signals. Using a transgenic Wnt reporter line, they show that the primary cell type in the ovary that appears to be Wnt responsive is germ cells, and in particular premeiotic germ cells and early-stage oocytes. Because *wnt4/wnt9b* are expressed in follicle cells that surround both Wnt responsive and Wnt non-responsive oocytes, they hypothesized that the Wnt responsive oocytes contain a factor that is permissive for activation of downstream responses to Wnt signal reception. They then asked if factors known to interact with β -catenin were also enriched in early-stage germ cells, and identified *Stn1*, which encodes a subunit of the CST complex with known roles in telomere maintenance. *Stn1* had been identified as a potential β -catenin-associated protein by Ehyai et al, 2018, though the evidence for this was weak. In addition, a zebrafish mutant for *stn1* had been made as part of the Zebrafish All Genes KO Consortium for Chromosome 1 (ZAKOC) Chromosome 1 consortium (Sun et al., 2019). Using published scRNA-seq data for the zebrafish ovary they found that *stn1* is primarily expressed in germ cells. Consistent with a function in germ cells development, *stn1* mutants have normal germ cell numbers early, but have lost all germ cells by ~45 dpf. Consistent with a role in Wnt signaling, the germ cells present in late larval *stn1* mutants have slightly reduced expression of the Wnt/ β -catenin reporter transgene. To gain mechanistic insights into the role of *stn1* in germ cell development, they compared the transcriptomes of wild-type and mutants at 19 and 25 dpf, and not surprisingly, found that mutants had reduced expression of genes that are normally expressed in germ cells. Using co-IP experiments they found evidence that *Stn1* interacts with both β -catenin and one of its downstream transcription factors, *Tcf7L2*. Evidence that *Stn1* functions to enhance the output of β -catenin signaling came from showing that it can enhance the transcriptional output of *Tcf7L2* in a transcriptional reporter assay in HEK293 cells. They next identified putative Tcf/Lef binding sites in the gene regulatory regions of germ cell-expressed genes that were downregulated in the *stn1* mutants and tested these in transcriptional enhancer assays using *Tcf7L1* and also provide corroborating evidence using ChIP-qPCR that β -catenin associates with these sequences in both ovaries and testes. Finally, they showed that overexpression of *Wnt8a* using a heat inducible transgene system could partially rescue germ cell development, and in particular oocytes, in *stn1* mutants, consistent with *stn1* mutants having a compromised response to endogenous Wnt signals.

Response: We highly appreciate the positive feedback from this referee.

Identifying novel regulators of Wnt/ β -catenin signaling would constitute a significant advance in the field, and the data presented in this manuscript makes a compelling argument that Stn functions in Wnt signal amplification. However, there are a few open questions and alternative explanations that need to be addressed before strong conclusions can be drawn. The first major concern is that Stn has known roles in telomere biology as a component of the CST complex that is required for telomere replication, capping, and protection. The authors argue that Stn has a germ cell-specific role, as loss of germ cells is the only major phenotype reported in the *snt1* mutants. However, this reviewer could imagine that germ cells may be more sensitive to loss of the telomere maintenance functions than other cell types and thus telomere defects are the root cause of germ cell loss, not reduced Wnt signaling. The second major concern is that the authors argue that Stn1 function germ cell autonomous, but all evidence is correlative. In supplemental Figure 1E they show that *stn* expression is not restricted to the gonad, but is also expressed in other tissues. While they were able to partial rescue the germ cell defect by transgene overexpression of *stn*, this transgene expressed *stn* in all cells, not just germ cells. A more convincing experiment would be to attempt rescue by expressing *stn* in only germ cells using a germ cell-specific promoter (e.g. *piwl1*).

Response: We sincerely appreciate your valuable comments and constructive suggestions. In response to the two major concerns raised, we performed additional experiments. The new results have been incorporated into the revised manuscript as Appendix Figure S5 and Appendix Figure S3I-K. Furthermore, we have added the corresponding results and discussion for these two sections to enhance the clarity and readability of the manuscript.

1) The role of telomere defects in germ cell loss after Stn1 depletion

Stn1 is a component of CST complex. CST depletion results in excessive telomerase activity and unleashed telomere length control (Chen et al, 2012, Nature 23; 488:540-4. doi: 10.1038/nature11269; Zaug et al., 2021, Nucleic Acids Res 49:11653-11665. DOI: 10.1093/nar/gkab942). As the reviewer pointed out, the role of telomere defects in germ cell loss after Stn1 depletion should be investigated. Adult telomerase-depleted zebrafish mutants (*tert*^{-/-}) exhibit both female and male phenotypes, and both sexes are capable of producing fertilized eggs and functional sperm (Anchelin et al., 2013 Dis Model Mech 6: 1101-1112 doi: 10.1242/dmm.011635; Henriques et al., 2013 PLoS Genet 9: e1003214 doi: 10.1371/journal.pgen.1003214). If excessive telomerase activity is a major contributor to germ cell loss after Stn1 depletion, inhibition of telomerase activity would be expected to restore germ cell development. To investigate this hypothesis, we treated *stn1* mutant zebrafish with specific telomerase inhibitors from 19 to 33 dpf to assess whether gonadal defects could be ameliorated. Small-molecule compounds BIBR1532 and RHPS4 are structurally distinct telomerase inhibitors. Both selectively inhibit telomerase activity and promote telomere shortening (Damm et al., 2001 EMBO J doi: 10.1093/emboj/20.24.6958; Chen et al, 2012, Nature 23; 488:540-4. doi: 10.1038/nature11269; Cookson et al., 2005, Mol. Pharmacol doi:10.1124/mol.105.013300; Gowan et al., Mol. Pharmacol doi:

10.1124/mol.60.5.981). Addition of BIBR1532 or RHPS4 did not ameliorate germ cell loss caused by *Stn1* depletion. Therefore, telomerase dysfunction is unlikely to be a major contributor to germ cell loss after *Stn1* depletion. This result has been included as Appendix Figure S5 in the revised manuscript and discussed in page 7-8, lines 294-309, and page 13, lines 539-556.

2) *Stn1* functions in a germ cell-autonomous manner

We agree with your opinion that the current evidence regarding *Stn1* function in a germ cell-autonomous manner remains correlative, and that this issue warrants further investigation. Following your suggestion, we generated a germ cell-specific transgenic line, *Tg(piwil1:stn1-Flag)*, expressing *Stn1*-Flag, for use in rescue analysis. Adult *stn1*^{-/-}; *Tg(piwil1:stn1-Flag)* zebrafish exhibit both female and male, and both are capable of producing fertilized eggs when crossed with wild-type males and females, respectively. Thus, we concluded that *Stn1* functions in a germ cell-autonomous manner. This result has been included as Appendix Figure S3I-K in the revised manuscript and discussed in page 5-6, lines 216-224.

Minor criticisms/suggestions (in approximate order in text):

1. "In zebrafish, Wnt/ β -catenin signaling plays important roles." for completeness, Kossack et al., 2019 (PMID:30446521) and Liu et al., 2022 (PMID: 35588359) should also be referenced as these papers more directly demonstrate roles for Wnt signaling (*wnt4* and *wnt9b*) in female development than the two papers that are currently referenced. (Also first add these to first sentence of Results). Also, the accepted nomenclature for *wnt4a* is now simply "*wnt4*" (see updated nomenclature on ZFIN).

Thank you for pointing out these issues. In accordance with your suggestion, the two papers have been cited in the revised manuscript as you indicated in both *Introduction* section and *Result* section. Additionally, *wnt4a* has been corrected to *wnt4* throughout the manuscript.

2. The experiments using 7X-tcf-Xla-siam:GFP to monitor wnt signaling has the significant caveat that GFP is very stable and may not represent active expression. It would be more accurate to look at *gfp* RNA by *in situ* hybridization.

Thanks for this comment and helpful suggestion. We agree with your opinion that the GFP in this transgenic line is highly stable and may not accurately reflect active gene expression. Following your suggestion, we performed fluorescence *in situ* hybridization to examine *gfp* mRNA expression levels in germ cells of different sizes, and quantified the fluorescence intensity of *gfp* mRNA. The results showed a similar pattern to total GFP fluorescence, with the size range shifting from 10- 45 μ m to 10-35 μ m when comparing GFP protein to *gfp* mRNA. The slight discrepancy in the oocyte size range between GFP mRNA and protein expression is likely attributable to the greater stability of the GFP protein. These new data have been included as Figure EV1 in the revised manuscript.

3. Fig 1C: Using GFP intensity is not an accurate measure of expression. If the same amount of GFP is present in a small oocyte vs. a large oocyte, the signal will look stronger in the small because the volume is smaller and therefore [GFP] is higher.

Thanks for this comment. We totally agree with your opinion that using GFP intensity as a measure of expression is not entirely accurate. To address the question, we re-quantified the total fluorescence intensity per cell in oocytes of specific diameters and established a correlation between oocyte diameter and total GFP fluorescence intensity. The new data has been included as Figure 1B, C in the revised manuscript. Both the new data and the fluorescence intensity of *gfp* mRNA support that Wnt/ β -catenin signaling activation undergoes rapid up-and-down fluctuations in developing oocytes during the early primary growth stage.

4. FigS1a: it is not clear what this data represents. Is the Y-axis oocyte diameter? Is this data from RNA in situ? If so, a representative in situ should be shown.

Thank you for pointing out this issue. We regret that the data were not presented clearly. In addition, the citation was unintentionally omitted in the Figure legend section, as all authors were using the "Track Changes" feature in Microsoft Word during manuscript revision. The Y-axis represents the diameter of oocytes surrounded by *wnt4*⁺ somatic cells. These raw data were extracted from confocal images of gonads at different developmental stages, which were stained for *wnt4* mRNA and are presented in Figure 2 of Kossack et al., 2019 (DOI: 10.1534/genetics.118.301620). We measured the oocyte diameter surrounded by *wnt4*⁺ somatic cells and qualified the data. To present the data more clearly, Appendix Figure S1A has been revised, and the citation to Figure 2 of Kossack et al., 2019 has been added to the revised figure legend.

5. "Since the intrinsic Wnt/ β -catenin signaling activity sharply increased at this stage..." The phrase "...intrinsic Wnt/ β -catenin signaling..." is misleading as it implies that the Wnt signal is intrinsic to the germ cells, yet the lead up to this was describing the role of *wnt4*, which is expressed in somatic cells (follicle cells). Perhaps the authors mean "germ cell-intrinsic response."

Thank you for pointing out this issue. This sentence has been revised to state: "Since the germ cell-intrinsic response of Wnt/ β -catenin signaling sharply increases at this stage".

6. For completeness, *Wnt4* is not the only Wnt expressed in the ovary. In addition to *wnt4*, *wnt9b* is also expressed in follicle cells and *wnt8a* in oocytes (Liu et al., 2022).

Thank you for pointing out this issue. The citation to Liu et al., 2022 has been added, and the information regarding *Wnt9b* and *Wnt8a* has been included in the revised *Results* and *Discussion* sections. Thank you again for your helpful suggestion.

7. Figure 2C. In normal animals, the gonads of males and females at 25-30 dpf are significantly different in size, and therefore have different brightness of GFP (females larger than males; Krovel and Olsen, 2004; Dranow et al., 2016). The wildtype image shown in fig 2C looks to be female, but it would be expected that some gonads looked male-like. Were any WT male-looking gonads present? For completeness, sex ratios should be provided and a male example shown.

Thank you for your helpful suggestion. Some wide-type gonads exhibited “male-like” morphology. Following your suggestion, an image of a representative male image and the corresponding sex ratios have been added to the revised Figure 2C to ensure completeness.

8. Fig 2D. Quantifying gonad size from images of gonads that have not been dissected is not an accurate method. Gonads should first be dissected (fixation of fish in PFA overnight aids in dissecting gonads; shrinkage may occur, but it will be proportional to all fish so relative size will still be accurate).

Thanks for this comment and helpful suggestion. Following your suggestion, we performed additional experiments to dissect PFA-fixed gonads and compare gonadal size between sibling and mutant fish at 19, 25, and 33 dpf. These new data have been incorporated into the revised manuscript as Figure EV2. These results are consistent with the observations from gonadal images. Hence, they further indicate that genetic loss of *Stn1* leads to severely underdeveloped gonads starting at approximately 25 dpf.

9. Pg6 : "The downregulated genes in the mutants at 19 hpf were not sorted..." should be "...19dpf...."

Changed. Thank you.

10. Fig 4b: Bulk RNA seq does not allow you to tell if the reduction of gene expression is due to reduced gene expression in germ cells, or due to mutants having many fewer germ cells relative to wildtype controls.

Thank you for your comment. We totally agree with this opinion that it is difficult to determine whether the reduction in gene expression is attributable to decreased expression levels in germ cells or to mutants possessing significantly fewer germ cells. We have revised the sentence to read: “In contrast, 376 reduced transcripts were specifically identified in the mutants at 25 dpf (Appendix Fig. S4B, Table S1).”

11. The RNA-seq results do not add anything novel to the story and should be provide as supplemental data. The authors only highlight germ cells expressed genes. Where any non-germ cell expressed genes also affected? Where any gene upregulated?

Thank you for your comments and helpful suggestions. The bulk RNA-seq data derived from the trunk region (including the gonads) make it difficult to identify non-germ cell-specific

genes in the gonads that are affected, apart from the previously reported germ cell-specific genes. The increased transcripts have not been annotated to any "biological process" category, and no specific germ cell-expressed genes have been reported. Therefore, the reduced transcripts that are not germ cell-specific, along with the increased transcripts, were excluded from further analysis. Following this suggestion, the affected non-germ cell-specific expressed genes have been included in the revised Supplementary Table to ensure completeness of the dataset presentation. The RNA-seq results have been relocated to the supplementary data as Appendix Figure S4 to improve clarity of presentation.

12. Fig. 4C Why were 4 hpf embryos chosen for IP analysis instead of using germ cells? Is *stn* a maternal transcript? Is there a role for *stn* at 4hpf? What is the relevance of its function at 4 hpf to its function in germ cells? Is *Tcf7L2* expressed in germ cells? If so, at what stages?

Thanks for this comment. We regret that our initial rationale for selecting 4 hpf embryos for IP analysis, rather than using germ cells, was not adequately explained. As mentioned in the manuscript, STN1 was identified as a potential β -catenin-associated protein from cultured cells through IP-MS analysis. However, the interaction between β -catenin and STN1 has not yet been validated by Co-IP or pull-down assays. Hence, we first investigated whether STN1 forms a complex with β -catenin and/or TCF. However, suitable antibodies against zebrafish *Stn1* were not available. A *stn1*^{-/-};Tg (*β -actin:stn1-Flag*) genetic background was utilized in combination with an anti-Flag antibody for the Co-IP assay. The *β -actin* promoter is ubiquitously active throughout development and into adulthood. To minimize the sacrifice of adult female fish while ensuring sufficient cell yield for Co-IP analysis, we used embryos from a *stn1*^{-/-};Tg (*β -actin:stn1-Flag*) line to examine the interaction between Flag-Stn1 and endogenous β -catenin and/or Tcf. A recent study by He et al. (2020, PLoS Biol 18: e3000561) demonstrated that β -catenin interacts with Tcf7 or Tcf7l2 to induce expression of maternal β -catenin target genes at 4 hpf. Given that Flag-Stn1 is ubiquitously expressed in the *stn1*^{-/-};Tg(*β -actin:stn1-Flag*) line, embryos at 4 hpf were selected for Co-IP analysis to investigate whether Flag-Stn1 forms a complex with endogenous β -catenin and/or Tcf7l2. To ensure a clearer description, we revised the corresponding section in the *Results* (page 8, lines 319-335).

The *stn1* mRNA is indeed maternally deposited, as shown in the Figure 1-12, which was generated from previously published RNA-seq data (White et al., 2017, Elife <https://doi.org/10.7554/elife.30860> PMID: 29144233; <https://www.ebi.ac.uk/gxa/experiments/E-ERAD-475/>). Since the *stn1* zygotic mutant fish exhibits male-like phenotype associated with infertility, we could not obtain maternal mutant embryos at 4 hpf to eliminate maternally derived mRNA or protein for phenotypic analysis. Therefore, investigating the function of maternally deposited Stn1 and comparing its functional significance at 4 hpf with that in germ cells remains challenging. In future studies, we plan to employ translation-blocking morpholinos to perform knockdown (KD) analysis for elucidating the additional role of maternal Stn1 in embryos at 4 hpf.

He et al. (2020) has examined the expression of *tcf7l2* during oogenesis. *tcf7l2* exhibited strong expression in developing oocytes (Figure S9, PLoS Biol 18: e3000561). Additionally, the scRNA-seq dataset generated by Liu et al. (2022) revealed that *tcf7l2* mRNA is expressed

in subclusters from germline stem cells to early oocytes (Figure 1-13). Given that *stn1* and *tcf7l2* are expressed during the same developmental stage, their interaction is likely to occur.

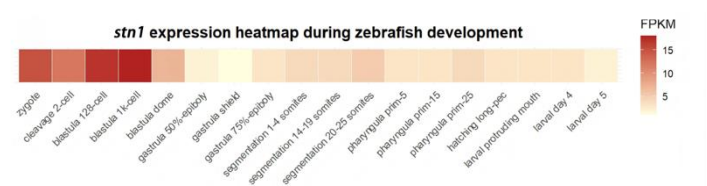


Figure 1-12 The expression of *stn1* during embryogenesis.

13. What was the reasoning for only assaying for interactions between Stn1 and Tcf7L2 in Fig. 4C? Where Tcf7 and Tcf7L2 also assayed in pulldowns from fish lysates?

Thank you for your questions. He et al. (2020, PLoS Biol 18: e3000561) examined the expression of *tcf7*, *tcf7l2*, *tcf7l1a*, and *tcf7l1b* during early developmental stages. Their results showed that *tcf7* and *tcf7l2* exhibited strong maternal expression in pre-ZGA embryos, whereas *tcf7l1a* and *tcf7l1b* showed very weak expression at these stages. Due to an antibody against zebrafish Tcf7l2 was already validated by knockout analysis, we therefore detected the interaction between Stn1 and Tcf7l2 in the Co-IP assay. However, due to the absence of antibodies specific to zebrafish Tcf7 and the failure to obtain suitable commercial antibodies against zebrafish Tcf7l1, we were unable to detect these two Tcf family members in the fish lysates. To address these questions raised by the reviewer, we alternatively performed additional GST pull-down assays using HEK293T cells expressing all tagged members of the Tcf/Lef family. The GST pull-down assays showed that Stn1 binds to all tagged members of the Tcf/Lef family. These results have been included as Figure 3D in the revised manuscript. Additionally, given that *stn1* and each member of the *tcf/lef* family are co-expressed in germ cells based on the scRNA-Seq dataset (Figure 1-13), it is likely that Stn1 interacts with members of the Tcf/Lef family in these cells.

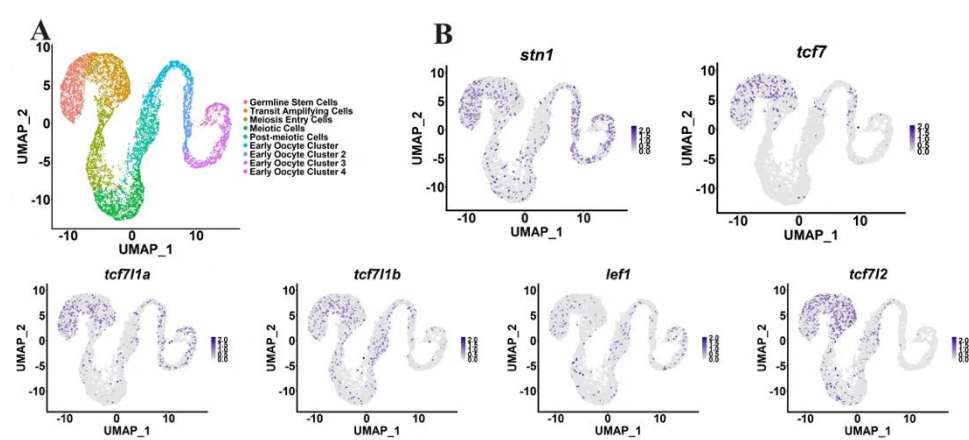


Figure 1-13 Gene expression UMAP plots of the *tcf/lef* gene family. (A) UMAP plot of germ cell subclusters, with cells colored according to computationally inferred subtypes. (B) UMAP plots showing gene expression patterns for the indicated genes.

14. Why was Tcf7L1 used instead of Tcf7L2 in figure 4H? Does Stn also interact with Tcf7L1 in pull down assays? Are Tcf7L1a/b expressed in germ cells?

In the current version of the manuscript, only Tcf7l2 is included in Figure 4D to maintain consistency with Figure 4C. To investigate whether Stn1 interacts with Tcf/Lef family members, we performed GST pull-down experiments using all members of the Tcf/Lef family. The results showed that Stn1 binds to all Tcf/Lef family members. From these questions raised by the reviewer, we realized the complete dataset should be incorporated into the Figure to make the manuscript easily to read. Hence, the complete dataset has been incorporated into the revised manuscript as Figure 3D. Given that Stn1 interacts with all Tcf/Lef family members and that the constitutively active VP16-Tcf7l1 Δ N construct was exactly in our hands, we used this construct together with the Flag-Stn1 construct to perform co-transfection experiments. Indeed, both *tcf7l1a* and *tcf7l1b* mRNAs were detected in germ cells from single-cell RNA-Seq data of ovaries at 40 dpf (Figure 1-13) (Liu et al., 2022, eLife 11: e76014) as well as by *in situ* hybridization on cryosections of adult ovaries (Fig. S9 in He et al., 2020, PLoS Biology 18: e3000561).

15. BIO assay: Fig4I, J It is not clear why the levels of protein not quantified in this assay? Instead, it is presented as only a qualitative result. Were these assays transient transfections of Stn expression constructs? Were these normalized for transfection efficiency?

Thank you for your comments and helpful suggestions. All experiments were performed in at least three independent replicates, and a representative result is presented in Figure 4I, J. These assays were performed using transient transfections of Flag-Stn1 expression constructs co-transfected with GFP expression constructs. The expression of GFP was used as a control to monitor transfection efficiency. Following this suggestion, we quantified the relative Stn1 protein levels normalized to GFP, and the new data have been included in the revised Figure 4I.

16. "Taken together, these data indicate that Stn1 depletion leads to reduced oocyte-intrinsic activity of Wnt/ β -catenin signaling, resulting in decreased expression of germ cell-specific genes and aberrant progression of oocytes." It should be noted that loss of *stn1* also affects spermatogenesis as all mutant animals are sterile males.

Thank you for your valuable comments. We have revised this sentence to state: "Taken together, these data indicate that Stn1 depletion leads to reduced germ cell-intrinsic Wnt/ β -catenin signaling activity, resulting in decreased expression of germ cell-specific genes and aberrant progression of germ cell development".

Referee #2:

Summary:

In this paper by Zhou et al, the authors aim to better understand the role of Wnt/ β -catenin and how they interact with Stn1 during zebrafish oogenesis. They start by measuring Wnt/ β -catenin activity using a transgenic fish model and found that Wnt/ β -catenin signaling is most prominently expressed in oocytes sized 10 to 50um and is dynamic in expression. The authors follow up on this finding using RNA-seq data to identify the Wnt/ β -catenin amplifier Stn1. When Stn1 was ablated, the Stn1 null fish had no gonads and male phenotypes and were infertile. The loss of germ cells was not caused by Caspase3-mediated apoptosis and Stn1 was shown to downregulate germ cells specific genes. Upon further analysis, Stn1 is directly related to Wnt/ β -catenin activity through modulation of the β -catenin/Tcf complex. Lastly, these authors show that Wnt8a is partially sufficient at modulating oocyte loss caused by stn1 loss. These data show the importance of Wnt/ β -catenin in zebrafish oogenesis and support the importance of Stn1 in oogenesis.

Major:

1. Figure 1B, C: 1B) please use indicate oocytes of specific diameters to establish correlation of diameter with GFP brightness. 1C, please clarify that each dot represents a single oocyte. Please include how many animals and ovaries are represented at each time point?

Thank you for your valuable suggestions. Following your suggestion, we re-analyzed the data and calculated the correlation between diameters of oocyte and total GFP fluorescence intensity per oocyte. The updated data have been incorporated into revised Figure 1C. Additionally, the statement "each data point represents an individual oocyte" has been added to the figure legend for panel 1C. The number of zebrafish analyzed at each time point is indicated in the figure legend.

1. Results, paragraph 2, supplementary Figure 1 text. Please rectify the discussion related to the timing of somatic cell Wnt4a, which was reported from a literature source (Kossack et al 2019) to be at 20-25 hours pf, to the data outlined in Supplementary Figure 1, which was focused at 20days pf (S1A), 40dpf (S1B-D), and adult animals (S1E). It would be helpful to show the images used to calculate data in figure S1A.

Thank you for your valuable suggestion. Following this suggestion, we revised the *Result* section pertaining to Supplementary Figure 1 in paragraph 2 as follows: "We noted that *wnt4*, a Wnt ligand, is specifically expressed in the somatic cells surrounding oocytes measuring 20 to 50 μ m in zebrafish gonads at 20 to 25 dpf (Appendix Fig. S1A) (Kossack et al., 2019)," to highlight the oocytes surrounded by *wnt4*⁺ somatic cells within this size range. The data presented in Appendix Fig. S1A were re-analyzed from confocal images shown in Figure 2E–G of Kossack et al., 2019. Since these images have already been published in the journal *Genetics*, we have included a citation to "Figure 2E–G, Kossack et al., 2019" in the legend for Appendix Fig. S1A to ensure clarity and proper attribution.

2. Supplementary Figure S3: Please define the outcome of breeding images shown in the figure legend of Figure S3C. Figure S3G control appears that it may have the potential to overexpress *stn1*; was any phenotype noticed in the gonads of these mice?

Thank you for this valuable suggestion. We determined the fertilization rate based on whether embryos reached the shield stage by 6 hpf. The “Representative images of embryos at 6 hpf from different mating combination are shown” has been included in the figure legend for S3C to ensure clarity in the description. For the control in Appendix Figure S3G with *Stn1* overexpression, no morphological phenotypic differences in the gonads were observed between *Stn1*-overexpressing WT and control WT.

3. Throughout the manuscript: Please define *stn1*^{+/?} genotype; it appears that it represents a sibling control indicating possibly a heterozygote genotype (+/-).

Thank you for pointing out this issue. We regret that we did not present this clearly. In the revised manuscript, the *stn1*^{+/?} genotype was designated as the WT sibling to ensure clarity in the description.

4. Figure 2: the phenotype of the *stn* mutant zebrafish is very exciting. The results would be strengthened if there was more description to explain the discrepancy in results in mutant animals, many 50% or less representative sections. In addition, germ cell loss did not appear to be caused by a caspase 3-mediated apoptosis pathway, but no quantification is provided. It would also be helpful to investigate other death pathways or proliferation defects.

Thanks for your comments and helpful suggestions. Indeed, the proportion of representative sections corresponds to that observed in WT siblings, rather than in mutants. Representative sections with 50% or less were in the sibling group to show the sex ratios.

Following this suggestion, we quantified the cleaved caspase-3 immunostaining data presented in Figure 2F, and the quantitative analysis has been included as Figure 2G in the revised manuscript. The results show that the number of active caspase-3-positive oocytes in the mutants was not significantly increased compared to sibling controls. Therefore, germ cell loss in *stn1* mutants does not primarily occur via caspase-mediated apoptosis.

Germ cell loss may involve non-caspase-mediated cell death pathways or defects in proliferation. It appears unlikely that proliferative defects occur in *stn1* mutants, as germ cells are gradually lost over time from the H.E. sections. Our new results from germ cell-specific rescue experiment by *Tg(piwil1:stn1-Flag)* showed that *Stn1* functions in a germ cell-autonomous manner. *Stn1* depletion leads to a reduction in the expression of various germ cell-specific genes, including *ddx4*, *piwil1*, and *piwil2*. Mutants of *ddx4* and *piwil2* exhibit germ cell loss through non-caspase-mediated mechanisms (Hartung et al., 2014, Molecular Reproduction and Development, 81: 946–961, [doi: 10.1002/mrd.22414](https://doi.org/10.1002/mrd.22414) ; Houwing et al., 2008, The EMBO Journal DOI: 10.1038/emboj.2008.204). In contrast, *piwil1* mutants exhibit germ cell loss through caspase-mediated mechanisms (Houwing et al., 2007, Cell, [doi: 10.1016/j.cell.2007.03.026](https://doi.org/10.1016/j.cell.2007.03.026)). However, the mechanisms underlying germ cell loss through

non-caspase-mediated pathways remain poorly understood. Given the complexity of the process, a comprehensive understanding of the molecular mechanisms driving germ cell loss in *stn1* mutants within a short timeframe is challenging. As the reviewer pointed out, this represents an important issue that warrants further investigation. Additional studies are therefore required to fully elucidate these mechanisms. This issue has been discussed in the revised manuscript page 13, line 530-538.

5. Figure 4: while it is a strength that both fish and in vitro data are used to generate these data, please clarify the rationale for the use of HEK293 cells, it is not clear what a "Wnt-off" cell line means.

Thank you for your suggestion. We regret that we did not clarify the rationale for using HEK293T cells or provide sufficient information regarding the "Wnt-off" cell line HEK293T. HEK293T cells exhibit a functional Wnt signaling system with basal Wnt/ β -catenin activity and are commonly regarded as a Wnt-off cell line. This cell line is highly amenable to induction of Wnt/ β -catenin signaling activity through modulation of Wnt activators at multiple levels. Therefore, HEK293T cells were transfected with a low dose of VP16-Tcf7l1 Δ N) along with different dose of Stn1 to examine whether Stn1 facilitated VP16-Tcf7l1 Δ N-induced Wnt activity in a dose-dependent manner. This paragraph has been revised in the new version according to your suggestion.

7. Figures 5B, 6F, S4, please include a negative control to ensure the effect tested for is specific

Thank you for your helpful suggestion. For Figure 5B, we repeated the experiments using the PopFlash plasmid (a mutant version of TopFlash containing mutated TCF/LEF binding sites upstream of a luciferase reporter, serving as a control for Wnt reporter activity) as a negative control, and the promoter of the Wnt target gene *lef1*-driven luciferase expression plasmid as a positive control. The new result has been included as revised Figure 4B to ensure that the effect being tested is specific. For Figures 6F and S4, the original results have been removed and replaced with fluorescence *in situ* hybridization data from ovarian cryosections as revised Figure EV4 and Appendix Figure S6, 7. These updated data help avoid potential misinterpretation due to the reduced number of germ cells in mutants compared to WT siblings.

Minor:

Throughout: please recheck all hpf and dpf notations, some to appear to be mislabeled (for example last line in the section describing Figure 3).

We carefully checked the entire manuscript and changed all mislabeled hpf to dpf. Thank you.

Referee #3:

The mechanisms of early germline differentiation are not fully understood in vertebrates.

Previous studies in mammals and zebrafish have identified canonical Wnts, including Wnt8 and Wnt4 as a regulator of early germline development and germline stem cell renewal, respectively. In this interesting paper from Zhang and colleagues, the authors investigate the role of the *Stn1* gene, a component of the CST complex that plays a role in telomere synthesis, replication, and response to replication stress. The authors report multiple loss of function mutant alleles disrupting zebrafish *stn1*. They provide compelling evidence for an essential *Stn1* function development of gonads of both sexes, as all mutants are sterile males. They provide evidence that initial germ cell numbers are intact; however, all germ cells are lost in juveniles. As anticipated based on loss of the germline, they report reduced expression of germ cell markers. The authors explored the upstream regulatory sequences in several germ cell genes and identified binding sites in silico and through CHIP and luciferase assays for TCF/Beta-Catenin and *Stn1* in cultured HEK cells and/or from gonadal tissues. They hypothesized and concluded that Wnt signaling is amplified by *Stn1* through a cooperative binding mechanism. Using a transgenic approach, they demonstrate that germ cell loss in *stn1* mutant gonads can be suppressed by overexpressing Wnt8a. Based on expression of Wnt4 in the somatic gonad of zebrafish and its roles in mammals, they propose Wnt4 may be the relevant ligand. Overall, the study is well done, and the findings are novel; however, there are several points that should be clarified and some of the conclusions are not fully supported by the data provided. In addition, there are several grammatical errors throughout the manuscript. These are noted below in instances where the meaning or interpretation of the sentences are unclear.

Major:

- 1) *Stn1* is not introduced in the introduction so the telomere discussion in the discussion sections comes out of context.

Thank you for your helpful suggestion. Following this suggestion, a paragraph introducing *Stn1* has been added to the *Introduction* section to improve the logical coherence of the manuscript.

- 2) Although Wnt8a can suppress the mutant phenotype when overexpressed, Wnt4 but not Wnt8a expression in the ovary/testis is discussed. The rescue of germ cell loss by heat shock Wnt8a is convincing; however, what happens to the gonad at later stages is not discussed. Do the gonads develop further, are the rescued males/females fertile or do they arrest in oocyte development? If rescue is incomplete, is it possible that canonical Wnt is only required for the early stage and that noncanonical Wnt might be required later as Wnt4 has been shown to act in both the canonical and noncanonical Wnt pathways?

Thank you for your comments. In addition to *wnt4*, *wnt9b* was expressed in follicle cells, and *wnt8a* was expressed in oocytes at this stage. This information has been added and discussed in the revised manuscript in accordance with the suggestion of Reviewer 1. The *stn1* mutants with *Tg(hsp70l:wnt8a-GFP)* transgenic background were heated shock daily from 19 to 45 dpf, and oocyte development was restored by heat shock-induced Wnt8a expression to stage IB with diameters ranging from 20 to 60 μm ; however, further development did not proceed.

These results suggest that activation of Wnt/ β -catenin signaling maintains oocyte development in *Stn1*-deficient individuals up to 20-60 μ m (Figure 5D, E). This result is consistent with the observation that Wnt activity sharply increases in developing oocytes ranging in size from 10 to 45 μ m and then rapidly declines (Figure 1B, C; Figure EV1). We also performed additional experiments. The *stn1* mutants with *Tg(hsp70l:wnt8a-GFP)* transgenic background were subjected to daily heat shock from 19 to 90 dpf; however, neither adult females nor males were obtained, and oocyte development failed to progress. When heat-shocked adult *stn1*^{-/-};*Tg(hsp70l:wnt8a-GFP)* male-like fish crossed with wild-type females, these fish induced the females to lay unfertilized eggs. Therefore, the restoration is incomplete. There are several possible explanations for this observation. First, as mentioned by the reviewer, Wnt4 and Wnt9b have been shown to function in both canonical and noncanonical Wnt signaling pathways. Canonical Wnt signaling may be required only at early stages, whereas noncanonical Wnt signaling might be necessary at later stages. Second, *Stn1* is a component of the CST complex, which plays an essential role in the suppression of telomerase activity and telomere length control. The function of the CST complex may be important during later stages of development. Further studies are required to elucidate these underlying mechanisms. This issue has been discussed in the revised manuscript, page 13, line 539-556. Thank you again for your valuable suggestion.

- 3) Expression of *stn1* transcripts is shown from published single cell data. This data suggests that *stn1* is expressed in germline stem cells and then is off and then is expressed again in early oocytes. Although PCR data are shown for *stn1* expression, it is important to validate the single cell data with HCR or a similar in situ hybridization analysis to determine which cells/stages express *stn1*. This might be relevant to the point above, if Wnt8 rescue is incomplete.

Thank you for your valuable suggestion. The *stn1*^{-/-};*Tg(hsp70l:wnt8a-GFP)* were heated shock daily from 19 to 45 dpf, and oocyte size was restored to the range of 20–60 μ m without further developmental progression (Figure 5D, E). Additionally, we generated a transgenic line with the germ cell-specific *piwil1* promoter driving *Stn1*-Flag expression, designated *Tg(piwil1:stn1-Flag)*, and performed rescue analysis. *Tg(piwil1:stn1-Flag)* was able to rescue the germ cell deficiency in *stn1* mutants, restoring to both adult female and male associated with fertility (Appendix Figure S3I-K). Given distinct expression patterns between *piwil1* and *stn1* during germ cell development, as shown in Figure 3-4a, and considering that *Stn1* protein stability is regulated by Wnt/ β -catenin signaling activity at the post-translational level according to our biochemical analysis, it is likely that post-translational regulation of the germ cell-specifically expressed *Stn1* plays a more critical role in its function during germ cell development than its mRNA level. Additionally, as the reviewer suggested in Question 2, alternative explanations may exist for the incomplete rescue observed with Wnt8a. Following this suggestion, we also performed fluorescence *in situ* hybridization on ovarian cryosections to stain *stn1* mRNA for validating the single-cell data. The new data has been included as Appendix Figure S1E in the revised manuscript. We observed that *stn1* mRNA expression is enriched at different developmental stages, including oogonia and oocytes at stages IA and IB, which are characterized by Wnt activation-driven GFP protein or mRNA expression as shown

in the revised Figure 1B and EV1. These results validated the single-cell data and further confirms the high expression of *stn1* across various developmental stages of female germ cells.

- 4) The model suggests that *Stn1* amplifies *Wnt8*, and the cartoon as depicted suggests that there is *Wnt*- independent *Stn1* but also shows *Wnt8* upstream of *Stn1* abundance. This seems inconsistent with the rescue data, which seem to place *Wnt* downstream or in a parallel pathway to *Stn1*. It would help to clarify this in the model. Also, given that *Stn1* expression also appears to be dynamic it would help to compare its expression to *Wnt* expression and to some of the targets indicated, and also to the *Wnt* receptors since they would likely be an intermediate between an activating *Wnt* and downstream signaling.

Thank you for your comments and valuable suggestions. We regret that we did not present the last paragraph of the *Results* section clearly and provide a schematic illustration sufficient to summarize our findings. Our results showed that *Wnt*/ β -catenin signaling activity sharply rises in the oocytes with size from 10 to 45 μm and rapidly declines later. Additionally, *Wnt* activity promotes the stability of *Stn1*, which in turn binds to *Tcf* and facilitates the association of the β -catenin/*Tcf* transcriptional complex with the promoter regions of germ cell-specific *Wnt* target genes, thereby amplifying *Wnt* activity. Depletion of *Stn1* fails to amplify *Wnt* signaling in zebrafish germ cells, resulting in reduced *Wnt* activity and impaired oocyte development, with size of most oocytes failing to reach 20 μm . Therefore, forced activation of *Wnt* signaling in *stn1*^{-/-} mutant fish with the *Tg(hsp70l:wnt8a-GFP)* transgenic background, achieved through daily heat shock from 19 to 45 dpf, was used to determine whether it could promote oocyte development in these mutants. Indeed, forced activation of *Wnt* signaling in *stn1*^{-/-} mutant fish promotes progression of oocyte development to a size within the 20–60 μm range, which corresponds to the stage characterized by *Wnt* activation. These results suggest that *Stn1*-mediated amplification of *Wnt* signaling is essential for oocyte development to attain a size within the 20–60 μm range. Indeed, the transgenic line was used to induce ectopic expression of *Wnt8a* in order to enhance *Wnt* signaling in *stn1*^{-/-} mutant fish. In our opinion, this approach should not be considered a phenotypic rescue but rather an experimental manipulation involving forced gene expression. Therefore, the last paragraph of the *Result* section has been revised for clarity. The final paragraph of the *Discussion* section has been revised to enhance the readability of the accompanying schematic. Additionally, the cartoon has been modified to enhance its clarity and facilitate understanding (Figure 5F).

Following this suggestion, we used UMAP analysis of the developmental trajectory of female germ cells from a single-cell RNA sequencing dataset (in which germ cells with a diameter less than 40 μm) to compare *stn1* expression with the *Wnt* germ cell-specific targets that we identified (Liu et al., 2022). As shown in Figures 3–4a, *stn1* expression is abundant in subpopulations of germline stem cells and early oocytes, and relatively lower in subpopulations located at intermediate stages. The expression of the *Wnt* germ cell-specific targets that we identified spans across the developmental stages of germ cells, albeit with varying abundance. The overlapping expression subpopulations between these *Wnt* targets and *stn1* suggest that the expression of the germ cell-specific targets are likely regulated by

Stn1. The *stn1* mRNA levels may not accurately reflect its protein abundance in germ cells, as our biochemical analysis showed that Stn1 stability is regulated by Wnt/ β -catenin signaling activity at the post-translational level. Additionally, as mentioned in the response to Question 3, the rescue results observed in the *piwil1* promoter-driven Stn1-Flag transgenic background *Tg(piwil1:stn1-Flag)* suggest that post-translational regulation of the germ cell-specifically expressed Stn1 likely plays a more critical role in its function during germ cell development. Furthermore, we analyzed the expression subpopulations of multiple Frizzled (Fzd) receptors. As shown in Figures 3–4b, the expression subpopulations of *fzd* overlap with that of *stn1*, although the number of germ cells expressing certain Fzd members is low. Thus, the overlapping expression subpopulations of *stn1*, *fzd*, and Wnt germ cell-specific targets do exist. Taken together, these results suggest that Stn1 likely influences Wnt activity and the expression of germ cell-specific genes.

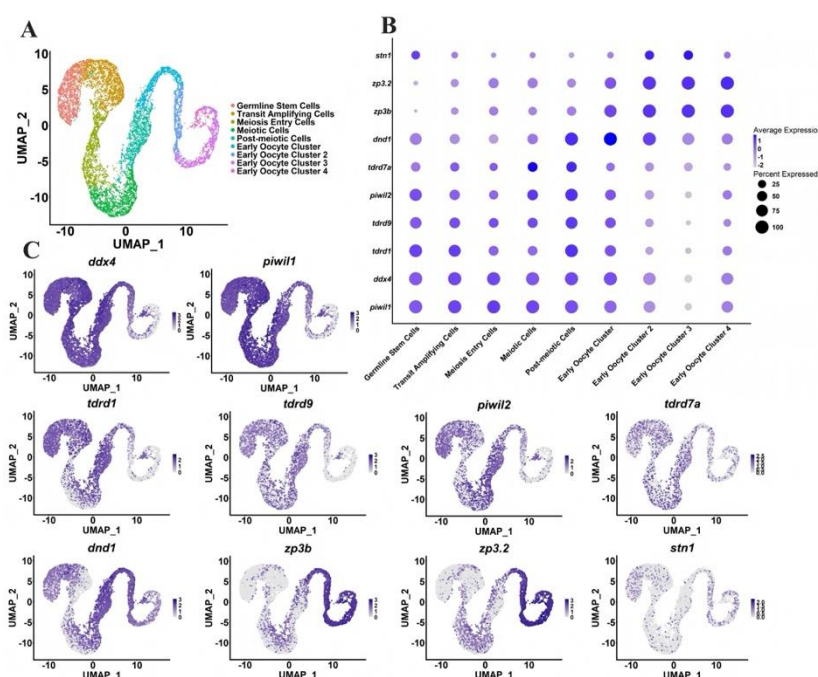


Figure 3-4a Gene expression UMAP plots of the Wnt germ cell-specific targets gene family. (A) UMAP plot of germ cell subclusters, with cells colored according to computationally inferred subtypes. (B) UMAP plots showing gene expression patterns for the indicated genes. (C) UMAP plots showing gene expression patterns for the indicated genes.

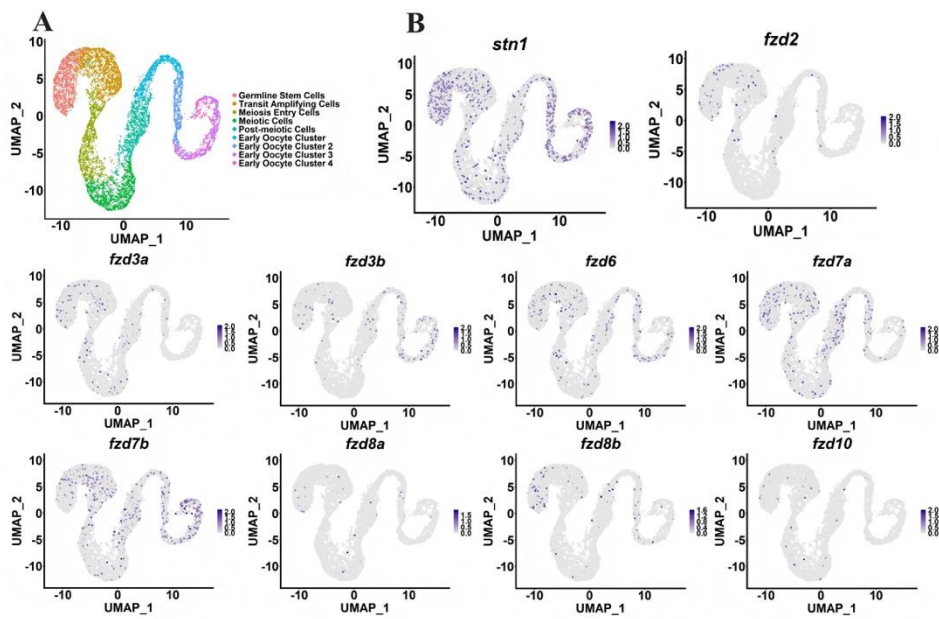


Figure 3-4b Gene expression UMAP plots of the *frizzled* gene family. (A) UMAP plot of germ cell subclusters, with cells colored according to computationally inferred subtypes. (B) UMAP plots showing gene expression patterns for the indicated Frizzled receptors.

- 5) Related to *stn1* expression, it is unclear how the cells/stages labeled by the Wnt reporter relate to the dynamic expression pattern of *stn1*.

Thank you for your comments. To address this question, we performed additional fluorescence *in situ* hybridization experiments on ovarian cryosections to investigate the expression of *stn1* and Wnt-activated *gfp* in ovaries across different developmental stages. The new data has been included in the revised Appendix Figure S1E and Figure EV1, respectively. The expression pattern of *stn1* closely resembles that of *gfp* in germ cells of varying sizes. These results suggest that Stn1 likely functions in Wnt-activated germ cells. The mRNA level of *stn1* may not accurately reflect its protein abundance in germ cells, as our biochemical analysis showed that Stn1 stability is regulated by Wnt/ β -catenin signaling activity at the post-translational level (Figure 3I).

- 6) "Conversely, in the *stn1* mutants, the oocytes gradually diminished, ultimately leaving only fibrous tissue in the gonads." What was the evidence that the tissue is fibrous tissue as opposed to somatic gonad tissue?

Thank you for your comments. There is no evidence to support the identification of this tissue as fibrous tissue. Therefore, this sentence has been revised as follows: "Conversely, in the *stn1* mutants, the oocytes gradually diminished from the gonads."

- 7) In several places, the germ cell genes are described as downregulated. However, it is

likely that the reduced expression is due to the reduced number and less advanced stages of germ cells in the mutant gonads. To distinguish between downregulation and less expression because there are fewer germ cells one would need to look at single cells, either with quantitative HCR (normalized to a "housekeeping" gene or single cell analysis).

Thank you for your comments and valuable suggestions. We totally agree with the opinion of the reviewer that describing germ cell genes as downregulated is inappropriate, given the reduced number of germ cells in the mutants. Following this suggestion, we performed additional fluorescence *in situ* hybridization on ovarian cryosections to examine the mRNA levels of each gene for comparison between siblings and mutants. The qRT-PCR results have been removed, and new data have been included as Appendix Figure S4F in the revised manuscript. Indeed, the expression of germ cell-specific genes is relatively lower in the residual germ cells of the mutants. Thank you again for your valuable suggestion.

- 8) "Wildtype larval zebrafish were treated from 19 to 25 dpf with XAV939 or PNU74654, which are Wnt/ β -catenin signaling specific inhibitors that activate Axin or inhibit the β -catenin/TCF complex, respectively (Huang et al, 2009; Trosset et al, 2006). Subsequently, the transcript levels of *ddx4*, *dnd1*, *piwil1*, *piwil2*, *tldr9*, *tldr1*, and *tldr7a* were evaluated. All transcripts were decreased following XAV939 or PNU74654 treatment, as indicated by qRT-PCR analysis (Fig. S4A, B)." Did these treatments lead to loss of germ cells or phenocopy *stn1* mutants? Were there any other phenotypes observed in treated fish? It is important to distinguish whether reduced expression was due to regulation, loss of germ cells, or other developmental delay phenotypes, and to determine the extent to which the phenotypes resemble *stn1* mutants. Examination of transcripts by PCR is not sufficient to distinguish between these possibilities.

Thank you for your comments. We totally agree with the reviewer's opinion that transcript examination by qRT-PCR is insufficient, and we should exclude the possibility you mentioned above. To address these questions, we performed additional experiments. After treatment with XAV939 or PNU74654 from 19 to 33 dpf, juvenile zebrafish were collected and subjected to body length measurement. Their ovaries were dissected for cryosection preparation to assess germ cell loss and to perform fluorescence *in situ* hybridization, which was used to examine the mRNA levels of each gene and quantify expression levels in oocytes of different sizes for comparison between the inhibitor-treated and DMSO control groups. Treatment with XAV939 or PNU74654 did not reduce the body length of zebrafish. In addition, morphological difference was not observed between the inhibitor-treated and DMSO control groups. Furthermore, slight or minimal germ cell loss was observed following exposure to either inhibitor. This observation may be attributed to the suboptimal concentration of the inhibitors administered, which was maintained at a lower level to minimize potential toxicity in juvenile zebrafish. Given that the *gfp* mRNA level in the *Tg(7×TCF-Xla.Siam:GFP)* transgenic line sharply increases in oocytes with diameters ranging from 10 to 35 μ m and then rapidly declines, we performed FISH to assess the

transcript levels of Wnt-induced *gfp* and the germ cell-specific target genes in oocytes measuring 10 to 30 μm in diameter. The mRNA levels of the examined genes were significantly reduced. Thus, the reduction in mRNA levels of the examined genes was not attributable to developmental delay, a finding that resembles the phenotype observed in *stn1* mutants. The new data have been included as Appendix Figure S6 in the revised manuscript. This issue has been discussed in the revised manuscript, page 9-10, lines 385-415. Thank you again for your valuable suggestion.

9) "Stn1 depletion weakened the association between β -catenin and the promoter regions of germ cell-specific genes at 25 dpf (Fig. 6B)." In a later sentence "strengthens" is used. The problem with these statements is that the stages of activation appear not to be achieved in the mutant and there are fewer germ cells, thus it is difficult to claim weaker association is directly due to loss of *stn1* rather than due to loss of the germ cells or stage differences between mutant and control gonads. This should be qualified.

Thank you for your comments. We totally agree with the reviewer's opinion that "it is difficult to claim a weaker association is directly due to loss of Stn1 rather than due to loss of the germ cells or stage differences between mutant and control gonads". To address this question, we performed new ChIP experiments. The ovaries of sibling zebrafish were dissected, and germ cells were dissociated from the gonad using a modified protocol based on Blokhina et al., 2019 (PLOS Genetics 15:e1007730). Given that the oocyte diameter of most germ cells in the mutants was less than 20 μm , the dissociated cells were filtered through a sieve to isolate germ cells with a diameter less than 20 μm . To ensure the reliability of the results, equal numbers of cells from siblings and mutants were then subjected to ChIP-qPCR analysis. These results indicate that Stn1 depletion resulted in a reduced association between β -catenin and the promoter regions of germ cell-specific genes at 25 dpf. The new data were included as revised Figure 5B in the revised manuscript.

10) "which acts as a novel binding partner of Tcf/Lef and cooperates with the β -catenin/Tcf complex to amplify Wnt/ β -catenin signaling in oocytes." The mutant data provided are not sufficient to support the statement that there is cooperative activity in oocytes.

Thank you for pointing out this issue. We have revised the sentence as follows: "which functions as a novel binding partner of Tcf/Lef and facilitates the activity of β -catenin/Tcf complex, thereby amplifying Wnt/ β -catenin signaling in oocytes" in the revised manuscript.

11) In Figure 4A, it appears that the mutant germ cells do not reach the stage when robust activation of the reporter is activated. Failure to reach the stage makes it challenging to establish a regulatory relationship. Initial expression/reporter expression in early GCs of *Ddx4* seems independent of *stn1*. Plus, as mentioned above, expression data indicates Stn1 is on in GSCs, then off then on in oocytes? How does reporter activation align with Stn1 expression? Stn1 expression should be validated.

Thank you for your comments and helpful suggestions. We totally agree with the reviewer's

opinion that establishing a regulatory relationship between Stn1 and Wnt activity is challenging. As the reviewer noted, mutant germ cells do not reach the developmental stage at which robust activation of the reporter occurs. Our results showed that Stn1 associates with and facilitates the activity of the β -catenin/Tcf complex, thereby amplifying Wnt/ β -catenin signaling. Thus, Stn1 does not function as a master regulator of these germ cell-specific genes, including *ddx4*, but rather as an amplifier to enhance Wnt/ β -catenin signaling levels. Hence, the observation that initial expression or reporter expression in early germ cells of *Ddx4* is independent of *stn1* is understandable. To address the question raised by the reviewer, we performed additional fluorescence *in situ* hybridization experiments on ovarian cryosections to examine the expression of *stn1* across different developmental stages. The new data has been included as Appendix Figure S1E. The expression of *stn1* in germ cells of varying sizes suggests that Stn1 may play a functional role in germ cells exhibiting Wnt activation. The mRNA level of *stn1* does not accurately reflect its protein abundance in germ cells, as our biochemical analysis showed that Stn1 stability is regulated by Wnt/ β -catenin signaling activity at the post-translational level.

12) Figure 4G, in the IP, all of the proteins also appear to come down with just GST.

Thank you for your comments. We acknowledge that the washing steps for the glutathione sepharose beads following incubation with cell lysate containing potentially interacting proteins were insufficient, resulting in incomplete removal of unbound proteins. Therefore, we repeated the GST pull-down assays with increased washing steps to ensure complete removal of unbound proteins. The new result has been included as Figure 3G in the manuscript. This result demonstrated that Stn1 binds to the Tcf712 fragment spanning residues 63–327.

13) For the luciferase assays, empty vector may not be the right control given how low the activity is for the germ cell genes compared to TOPFlash.

Thank you for your comments. We totally agree with the reviewer's opinion. To address this question, we performed new experiments using the FopFlash plasmid (a mutant version of TopFlash containing mutated TCF/LEF binding sites upstream of a luciferase reporter, serving as a control for Wnt reporter activity) as a negative control, and the promoter of the Wnt target gene *lef1* driven luciferase expression plasmid as a positive control. The relative Wnt reporter activity driven by the indicated gene promoters is presented as fold change relative to the FopFlash control. The new result has been included as the revised figure 4B. The expression of these reporters was induced when co-transfected with VP16-Tcf711 Δ N into HEK293T cells. Hence, the promoter regions of these germ cell-specific genes contain Wnt/ β -catenin-responsive elements.

14) Fig. 6F, it seems challenging to make these comparisons since there are different numbers and stages of germ cells and there is high variation from gonad to gonad, even in the controls. Histological evaluation is likely to provide more meaningful comparisons as the stages can be directly compared.

Thank you for your comments. We totally agree with the reviewer's opinion. To address this question, we performed additional experiments. Following heat shock treatment from 19 to 33 dpf, juvenile zebrafish were collected and subjected to body length measurement and genotyping analysis. Their ovaries were dissected for cryosection preparation to perform fluorescence *in situ* hybridization, which was used to examine the mRNA levels of the target genes and quantify expression levels in oocytes of different sizes for comparison between mutant groups with and without heat shock. The qRT-PCR results have been moved and the new data have been included as Figure EV4 in the revised manuscript. These results showed that temporal induction of Wnt/ β -catenin signaling activity alleviates the reduced mRNA levels of germ cell-specific genes caused by Stn1 loss.

Minor:

1) In Fig. 4H, there appears to be an extra plus sign in the line for Stn1-Flag.

The symbol "++" was used to indicate a higher dose compared to "+".

2) In the abstract the following sentence needs context. It reads like this is already known as written. "The dynamically intrinsic activity. "However, the dynamically intrinsic activity of Wnt/ β -catenin signaling in the progression of oocytes is poorly understood." Maybe "the dynamics of Wnt signaling in oocyte progression has not been reported".

Changed.

3) Stn1 should be spelled out and introduced the first time it is used.

Added.

4) In the abstract, "is enrichingly expressed in germ cells" should instead be "expression is enriched in..."

Changed.

5) Also in the abstract, "the" is needed before transcription factor.

Added.

6) Abstract: "stn1 leads to loss of massive oocytes prior to or during their development to the IB stage," "should instead be leads to massive loss of oocytes."

Changed.

7) Abstract: By "male-like phenotype" I assume the authors mean "phenotypically male" or "sterile male".

Thank you for pointing out this issue. This sentence has been revised to “male-like phenotype associated with infertility”.

8) Abstract: The following sentence is unclear as written. "Temporally induced activation of the Wnt/ β -catenin signaling partially reverses Stn1 depletion induced loss of germ cells and eventually promotes development of oocytes to the stage IB."

Thank you for pointing out this issue. This sentence has been changed to “The loss of germ cells caused by Stn1 depletion can be partially restored by temporal activation of the Wnt/ β -catenin signaling pathway, which facilitates oocyte development to stage IB”.

9) Abstract: I believe this is a typo and that "facilitating" should be "facilitating".

Changed.

10) Introduction: The following sentence is unclear as written. "In ovaries, a key germ cell-intrinsic activity of signaling pathway, Wnt/ β -catenin signaling that govern their development is evolutionarily conserved across the animal kingdom (Le Rolle *et al*, 2021; Takase & Nusse, 2016)."

Thank you for pointing out this issue. This sentence has been changed to “In ovaries, the germ cell-intrinsic activity of the Wnt/ β -catenin signaling pathway, which regulates their development, is evolutionarily conserved across the animal kingdom (Le Rolle *et al*, 2021; Takase & Nusse, 2016)”.

11) Introduction: "is crucial for its pluripotent maintenance." "its" is not needed.

Deleted.

12) Introduction: "meiosis entry" is typically written as "meiotic entry"

Changed.

13) Introduction: "regulated in progression". Consider instead "regulated during the progression".

Changed.

14) The conclusion "These results imply that Stn1 may regulate Wnt/ β -catenin signaling pathway and play key roles in germ cell development." Isn't really supported by the data presented immediately before it is made.

Thank you for pointing out this issue. This sentence has been changed to “Collectively, these

results suggest that Stn1 may play a role in germ cell development”.

15) The following is a complex sentence. Consider splitting it into two sentences. Hence, an anti-active-caspase-3 antibody was employed to immunostain stn1 mutant embryos with a Tg(piwil1:EGFP-UTRnanos3) genetic background at 25 dpf. In agreement with phenomenon in vasa mutants (Hartung et al, 2014), the abundance of active-caspase-3-positive oocytes in mutants was not higher than in siblings despite oocyte sin stn1 mutants are apparent loss (Fig. 2F)."

Thank you for pointing out this issue. This sentence has been changed in the revised manuscript.

16) In the section titled "Loss of Stn1 reduces the expression abundance of germ cell-specific genes" hpf is used several times to refer to the stage, but I think these are typos and should be dpf. - also, there is a typo in this title "abundance".

Changed.

17) The following sentence is unclear as written. "(Fig. 3E). Taken together, these results suggest that loss of Stn1 leads to the germ cell numbers are greatly reduced in mutant gonads at 25 while not 19 hpf.

Thank you for pointing out this issue. This sentence has been changed to “Taken together, these results suggest that loss of Stn1 leads to a significant reduction in germ cell numbers in mutant gonads starting from 25 dpf”.

18) The first "the" is not needed in this sentence "The Stn1 bound with the Tcf7l2 fragment spanning residues 63-327 (Fig. 4F and G).

Deleted.

19) The basis for describing HEK cells as a "wnt off" line should be discussed briefly.

Thank you for pointing out this issue. The following sentence has been added: “Human HEK293T cells exhibit a functional Wnt signaling system with basal Wnt/ β -catenin activity and are commonly regarded as a Wnt-off cell line. This cell line is highly amenable to induction of Wnt/ β -catenin signaling activity through modulation of Wnt activators at multiple levels.”.

20) There is a typo in the following sentence. "Thus, Stn1 likely functions by binding with Tcf/Lef at target promoters and promoting the association of β -catenin/Tcf complexe with the target gene promoters.

Changed.

21) "And its genetic deletion in zebrafish leads to loss of oocytes and results in a male-like phenotype and infertility." It appears to lead to loss of the stem cells too.

Thank you for pointing out this issue. The sentence has been revised as “And its genetic deletion in zebrafish leads to germ cell loss and results in a male-like phenotype associated with infertility.”

22) "its actin in oogenesis." Should "actin" be "action".

Changed. Thank you.

23) "In zebrafish, Ddx4 is required for germ cell differentiation and maintenance, while its disruption results in sterile adult male-like fish (Hartung et al., 2014). Similarly, Dnd1 is implicated in germ cell fate maintenance; its depletion in zebrafish leads to complete loss of germ cells (Chu et al, 2023; Weidinger et al, 2003)." The Ddx4 and Dnd1 phenotypes both lead to loss of the germline but are very different phenotypes from one another. In Dnd1 loss, the primordial germ cells are lost within the first few days of development and in ddx4 mutants they are lost much later in gonad development. Thus, they should not be described as "similar"

Thank you for pointing out this issue. The term "similar" has been removed to improve clarity and precision in the description.

24) For the genotypes +/- is used throughout the figures. Instead, label these, which I assume to be either heterozygotes or homozygous WT, as "wild type", and assuming this is the case, in the figure state that no differences were observed between heterozygotes and homozygous WT, thus both genotypes are referred to as wild type.

We appreciate the reviewer's helpful comment. Your assumption is correct, and in response, we have replaced "+/?" with "wild type" in all figures and text. Because wild type and heterozygous individuals exhibited no significant differences, both are described as "wild type." Thank you.

Dear Dr. Zhou

Thank you for the submission of your revised manuscript to EMBO reports. Please accept my sincere apologies regarding the delay in handling your manuscript. Unfortunately, referee #3, despite having raised a number of concerns, was not responsive and available to review your revised manuscript, but I acknowledge that you have performed extensive additional experimentation to address all concerns. Referee #1 and #2 have evaluated your revised manuscript and both are very positive about your study and recommend publication.

Before I can accept the manuscript, I need you to address some minor points below:

- Your manuscript will be published in our Reports section. In order to comply with this format, I kindly ask you to combine the Results and Discussion section.
- Regarding the Author Contributions, we now use CRediT to specify the contributions of each author in the journal submission system. CRediT replaces the author contribution section, which therefore needs to be removed from the manuscript text. You can use the free text box in our system if you wish to provide more detailed descriptions. See also guide to authors <https://www.embopress.org/page/journal/14693178/authorguide#authorshipguidelines>.
- References: please use et al after 10 author names.
- Author checklist: please select responses from the pull-down menu, in column D.
- In the Author checklist lines 81 and 82 you refer to statements about blinding or randomization in the methods section, which I could not locate. Please make sure these are present.
- Please add the fund ZR2025MS429 in the online manuscript handling system. The information in the system and in the text must be congruent. In the manuscript text all funding information needs to go under Acknowledgments; a separate section (heading) should not be used.
- Please add a callout for Fig. EV1
- Appendix: please remove the legend for Appendix Table S1 as the table is not part of this file but a separate dataset.
- The Appendix Table S1 file should be renamed to Dataset EV1. The Excel file needs to be uplidd without the zip folder; the legend should be provided in another tab of the file; the correct nomenclature should be Dataset EV1 in all places (source file name, legend in the Excel file, title in the system, callouts in the manuscript.)
- Appendix Figure legends: please specify whether 'n' refers to technical or biological/independent replicates.
- Appendix Figure S3H, K: please define the statistical test used.
- Materials and methods should be Methods
- Please add information related to n in the legends of figures 3H, I.
- Please make sure that it is clear in all legends what 'n' refers to, in the case of cell culture please specify whether it is technical or independent replicates and please make sure to apply statistical analysis only to independent experiments.
- Data availability section: this section should only refer to the deposited data. Please remove the statement on data sharing on request.
- The link to the Single Cell Portal in line 615 seems broken. I received an error message when clicking on it. Could you please check?
- You reanalysed the scRNA-seq dataset from Liue et al, 2022. You can add a data citation that refers to the dataset in addition to citing the paper it was published in.
In the text this would look like (Liu et al., 2022, data ref: Liu et al., 2022).
The data ref in the reference list should refer to the dataset using its URL instead of journal name etc. and have the tag [DATASET] at the end of the reference.
- Why are the numbers for WT male and WT female the exact same in Appendix Fig. S3K and the associated source data .xls file (fertilization rate and number of embryos)?
- For Figure 5B, source data file, it appears that the number showin in Column A, line 8 is the same as that in line 9. The same applies to column B, column E and column F lines 8 and 9. It appears that the numbers have been duplicated (line 8 = line 9). Please check and confirm whether this is intended or a mistake.

- As a standard procedure, we modify abstract and title to make them more accessible to our general readership. Please find my suggestions copied below my signature and please try to shorten the abstract further to 175 words.

We look forward to seeing a final version of your manuscript as soon as possible.

With kind regards,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Referee #1:

The authors addressed all of my previous critiques and suggestions. The new data provide much stronger support for the conclusions they draw.

Referee #2:

The authors should be commended for the thoughtful approach to addressing reviewer concerns. They performed additional experiments that added significant value to their manuscript in rigor and clarity.

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Stn1 promotes zebrafish oocyte development via amplifying Wnt/ β -catenin signaling

Wnt/ β -catenin signaling regulates oocyte development in vertebrates. However, the dynamics of Wnt signaling in oocyte progression has not been reported. Here, we analyse oocytes across different developmental stages in zebrafish and find that the activity of Wnt/ β -catenin signaling sharply increases when oocytes reach a size of 10 to 45 μ m (stage IA to middle stage IB) and subsequently declines rapidly. In addition, we show that expression of the CST complex subunit Stn1 is enriched in germ cells. Stn1 interacts with the transcription factor Tcf/Lef, facilitates its association with promoters of various germ cell-specific genes, thereby enhancing Wnt/ β -catenin signaling activity in oocytes. Genetic deletion of stn1 leads to massive loss of oocytes prior to or during their development to stage IB, resulting in a male-like phenotype associated with infertility. The loss of germ cells caused by Stn1 depletion can be partially restored by temporal activation of the Wnt/ β -catenin signaling pathway, which facilitates oocyte development to stage IB. Our findings highlight the importance of Wnt/ β -catenin signaling in promoting the expression of germ cell-specific genes and provide novel insights into the physiological functions of Stn1 in maintaining oocyte development in zebrafish.

The authors have addressed all minor editorial requests.

Dr. Jianfeng Zhou
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Dear Dr. Zhou,

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

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If you have any questions, please do not hesitate to contact the Editorial Office. Thank you for your contribution to EMBO Reports.

Yours sincerely,

Martina Rembold, PhD
Senior Editor
EMBO reports

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Corresponding Author Name: Jianfeng Zhou
Journal Submitted to: EMBO Reports
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Reporting Checklist for Life Science Articles (updated January

This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: [10.31222/osf.io/9sm4x](#)). Please follow the journal's guidelines in preparing your

Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for figures

1. Data

The data shown in figures should satisfy the following conditions:

- the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

2. Captions

Each figure caption should contain the following information, for each panel where they are relevant:

- a specification of the experimental system investigated (eg cell line, species name).
- the assay(s) and method(s) used to carry out the reported observations and measurements.
- an explicit mention of the biological and chemical entity(ies) that are being measured.
- an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.
- the exact sample size (n) for each experimental group/condition, given as a number, not a range;
- a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
- a statement of how many times the experiment shown was independently replicated in the laboratory.
- definitions of statistical methods and measures:
 - common tests, such as t-test (please specify whether paired vs. unpaired), simple χ^2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
 - are tests one-sided or two-sided?
 - are there adjustments for multiple comparisons?
 - exact statistical test results, e.g., P values = x but not P values < x;
 - definition of 'center values' as median or average;
 - definition of error bars as s.d. or s.e.m.

Please complete ALL of the questions below.

Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	Not Applicable
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For antibodies provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Reagents and Tools Table
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Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Reagents and Tools Table
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Report if the cell lines were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Materials and Methods
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Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, have they been described?	Yes	Materials and Methods
Include a statement about blinding even if no blinding was done.	Yes	Materials and Methods
Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Yes	Materials and Methods
If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.		
For every figure, are statistical tests justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Materials and Methods

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In the figure legends: state number of times the experiment was replicated in laboratory.	Yes	Figure Legends
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Studies involving experimental animals : State details of authority granting ethics approval (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Materials and Methods
Studies involving specimen and field samples : State if relevant permits obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	Not Applicable

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Are computational models that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	Not Applicable
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