The exocyst complex regulates *C. elegans* germline stem cell proliferation by controlling membrane Notch levels

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I have now received reviews of your manuscript from 3 experts. The reviewers' comments are appended below, or you can access them online: please go to BenchPress and click on the 'Manuscripts with Decisions' queue in the Author Area.

As you will all three reviewers' express interest in the study with some concerns. They each have numerous excellent suggestions to address and improve the study and the manuscript. In general, all three reviewers ask that the conclusions be softened to line up with the data that is presented and not overstated, which I agree with entirely. In addition, several statements in the text need to be better defined, for example as pointed by reviewer 3 the rachis of the gonad is not a lumen, and thus needs to be defined as a rachis. All three reviewers also request additional experiments to better support the model presented. Reviewer 1 requests a demonstration that modulating the surface levels of GLP-1 do in fact regulate Notch signaling levels, as a read out of Notch function. Reviewer 2 requests for clarity in experimental findings and details, and suggests an analysis of the GLP-1 RNA level to rule out other effects through which Notch signaling could be affected. Importantly, as stated by the reviewer the conclusion of the mammalian cultured cells to demonstrate that exocyst complex regulates Notch signaling at the plasma membrane needs to be supported by data or softened in the text. Both reviewer 2 and 3 request for further experimentation to support the germline autonomy. As stated by reviewer 3, rrf-1 is not a good reagent for this since it performs somatic RNAi. Thus, conduction of these experiments in the rde-1 strains indicated by the reviewer will be required.

I invite you to consider the reviewers' suggestions and submit a revised manuscript that addresses their concerns. Your revised manuscript would be re-reviewed, and acceptance would depend on your satisfactorily addressing the reviewers" concerns. Please note that Development normally

permits only one round of 'major revision' and that because of the pandemic we are happy to extend revision time frames as necessary.

In your revised manuscript, please clearly highlight all changes made in the revised version. You should avoid using 'Tracked Changes' in Word files as these are lost in PDF conversion. I also request a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of the reviewers' criticisms or suggestions, please explain why.
se to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this manuscript Pushpa et al. describe their work on the exocyst complex, its interaction with the Notch signaling pathway, and its role in regulating stem cell proliferation. They demonstrate that RNAi against exocyst complex components SEC-6 and -8 results in reduced progenitor zone sizes, and that RNAi in either somatic or germ cells results in this reduction. They demonstrate that this reduction of proliferative zone cells is also found in animals containing either partial loss-of-function or gain-of-function alleles of glp-1. They suggest that the effect that the exocyst complex has on Notch signaling and stem cell proliferation is linked to the amount of GLP-1 that is found on basal surface of the germ cells.
They provide data demonstrating that when exocyst function is reduced, GLP-1 on the basal surface is reduced. Furthermore, they demonstrate that the role of the exocyst complex in maintaining Notch on the cell surface is conserved in mammalian cells.

Overall, this is a logically presented model which increases our understanding of how an additional mechanism Notch signaling levels may be maintained in the C. elegans germ line and in other systems. Generally, the experiments appear to be well controlled and the figures properly present the results. However, I do have some concerns about the interpretation of some of the data, and how conclusively it supports their model.

Comments for the author

First, they demonstrate that reducing exocyst complex function results in germ line defects, including a reduction in the size of the proliferative zone, and that the exocyst complex may interact with Notch signaling (see below). They then demonstrate that GLP-1 basal surface levels are reduced when exocyst function is reduced. From this they conclude that the mechanism by which the exocyst is affecting progenitor zone size is through affecting GLP-1 surface levels.

However, there is not data linking these two observations. It is entirely possible that GLP-1 levels on the surface are in excess, and the reducing surface levels to this extent does not negatively impact Notch signaling levels (indeed INTRA levels are much lower than Notch surface levels). It seems that they would have to demonstrate that lowering GLP-1 surface levels to this extent does impact Notch signaling levels, or at least be much more measured in their conclusions.

Since they demonstrate that a reduction in exocyst function in the soma also reduces progenitor zone size, there must be more going on than just GLP-1 surface levels.

Second, the genetic interaction with bn18 and ar202 appears only to be additive and therefore does not conclusively demonstrate an interaction with the exocyst complex with Notch signaling. A reduction in Notch signalling is not the only cause of a smaller progenitor zone size. For example, a problem with the mitotic cell cycle could also cause a reduction. Therefore, if you have a smaller progenitor zone size due to bn18, and combine it with a mutant that causes a problem with the mitotic cell cycle, you would expect the phenotype of the double mutant to be a combination of the two phenotypes (smaller than both single mutants), even though each single mutant has a different cause for a smaller progenitor zone. The results would be more convincing if the double

mutant phenotype was more than additive (e.g. completely Glp phenotype). Therefore, the authors need to be more measured in their conclusions with this experiment.
However, lst-1 and sygl-1 data is much more convincing.

More minor points
1- Throughout the paper the authors state that a gene function is 'depleted' through the use of RNAi. This suggests the gene function is completely eliminated; however, RNAi only partially reduces gene function, emphasized by the fact that the mutant alleles have a stronger phenotype. Perhaps the authors could say that the gene function is 'reduced' when using RNAi.

2- The authors claim in the abstract and elsewhere that '…the exocyst complex regulates C. elegans GSC proliferation by modulating Notch signaling cell autonomously'. I do not believe they have demonstrated that cell autonomy. An active exocyst complex in a neighboring cell, or in the rachis, could affect GLP-1 accumalation levels.

3- The authors do not provide a rationale for why the first started looking at SEC-6 and -8. Providing a rationale would help the reader understand the logic of these first experiments.

4- I am not sure why the authors used sec-6(tm4536)/mIn1 heterozygotes for RNAi by sec-6. An explanation would help here.

5- Are tm4536 and ok2187 null alleles?

6- Why would sec-5 mutant not show a mutant phenotype, but sec-5 RNAi does?
Is it possible sec-5(RNAi) is targeting additional genes? For all genes, what is the evidence that RNAi was targeting the correct gene?

7- The authors state that they obtained a '…GLP-1::GFP::3xFLAG expressing strain…'. Where was this obtained and how has it been verified? Does this strain show any aberrant phenotypes? Reference?

8- Throughout paper exocyst complex is dropped to simply exocyst should be consistent and keep exocyst complex

9- The figure legends could be more informative, stating what stage animals were used

10- Page 3. remove 'in' - Exocyst complex members are required for recycling of the Delta-ligand in during the asymmetric division

11- Page 4. Don't need strain name just allele - compared to control (Figs. 1H, I, S1). We also examined the germline in the sec-8 genetic mutant VC2648

12- Page 4. "RNAi phenotype, sec-8(-/-) worm gonads showed significantly a smaller PZ as compared to sec-8(-/+)". Should be changed to "…showed a significantly smaller PZ…"

13- Page 5. s3a doesn't refer to SEC-6 expression – "cytoplasm, including the oocytes and sperm (Figs. 2D, S3A)."

14- Page 6. should just be figure 3 b since 3a is just ar202 at varying temps – "…(ar202) mutant at the restrictive temperature (Figs. 3A, B)."

15- Page 7. remove 'of' – "We next examined the role of the exocyst complex in regulating of GLP-1 localization to the membrane in…"

16- Page 7. Clean up reference – "…the exocyst complex has also been reported in literature (Boehm et al., 2017, Harsh Kumar, 2019 #269;"

17- Page 8. The authors claim that the did not observe any substantial changes in the apical levels of GLP-1::GFP with rab-5 or rab-11 RNAi. However the GFP looks much more cytoplasmic in the RNAi animals that the control in figure 5F

18- Page 9. "…depletion of human Sec5, Sec6 and Sec8 (Fig. 7C) revealed perceptible mis-localization…". Figure 7C does not show this.

19- Page 9. "…protein GM130 was absent from our plasma membrane fractions confirming a clean plasma membrane…". It appears as though there is a faint band in siGFP lane for GM130

20- space needed – "…germline stem cell (GSC) proliferation by maintaining optimal Notch receptor levels on the GSCsurface."

21- Page 13. Remove space – "…Notch2 levels on mammalian cell surface: Notch 2…"

22- Page 13. Add space – "… of five and upto ten regions of interest (ROI) per cell…"

23- Page 14. Add space – "…cropped to only display the progenitor zone going upto the first layer of the transition zone"

24- Add proper reference – "PMID: 25122090. For protein purification 1ml-streptactin…"

25- Figure 1. Legend – Italicize - depleted of exocyst complex subunits sec-6 and sec-8"

26- Figure 2 Legend- Gene names need to be italicisized.

27- Figure 3F- should mentioned that it is corrected for background

28- Figure 4- Legend does not mention purpose of arrows in 4B

29- Figure 5e- missing rab10 RNAi on graph

30- Figure 6b- missing par-3 on graph

31- Figure 7a- siSec6/8/5 labeled but everywhere else says siExocyst. Should be consistent

Reviewer 2

Advance summary and potential significance to field

In manuscript DEVELOP-2020-196345v1 by Pushpa et al, the authors have examined the role of the exocyst complex in the regulation of germline stem cell (GSC) proliferation in C. elegans. They first use RNAi and mutant analysis for sec6 and sec8 to show that exocyst is required for germ cell proliferation in this model. Using cell type-specific RNAi, they show that exocyst functions in both germ cells and somatic cells to regulate germline development. They then perform genetic interaction studies and staining to conclude that the exocyst complex positively regulates Notch signaling for germline stem cell proliferation. Using transgenic strains of C. elegans expressing tagged version of several proteins including GLP-1/Notch, the authors conclude the involvement of exocyst in intracellular trafficking in the germline and in the maintenance of membrane localization of GLP-1/Notch in germline. Similar observations are made for Rab5 and Rab11, suggesting a role for the exocyst in recycling of GLP-1 to the membrane. Using yeast two-hybrid and IP-mass spec experiments in C. elegans and a human osteosarcoma cell line (U2OS), the authors identify Par-5 as an interactor of sec-6. Combined with GLP-1 localization defects in the germline of Par-6 and Par-3 depleted worms, these data suggest that the anterior Par complex cooperates with exocyst to localize GLP-1 properly. Finally, using imaging and biotinylation assays in U2OS cells, the authors conclude that exocyst is also involved in surface localization of human NOTCH2.

Despite the importance of the exocyst complex in various physiological contexts, a cell-autonomous role for exocyst in stem cells was not reported previously. Moreover, a role for exocyt components

in the regulation of Notch signaling has only been reported in a couple of contexts in Drosophila, none of which was thought to be mediated by regulating the Notch receptor trafficking. Therefore, the manuscript focuses on a novel mechanism for the regulation of Notch signaling by exocyst, in a cell type not previously known to be regulated by the exocyt. However, unfortunately, a number of issues exist with the data presentation and quantification. Moreover, some of the conclusions of the paper do not seem to be supported by the data. These issues significantly reduce my enthusiasm for this manuscript.

Comments for the author

Major points

1. It is rather difficult to interpret the genetic interaction studies shown in Figure 3. First, why does the ar202 mutation exhibit higher Notch signaling at the restrictive temperature? Increased stability? Prolonged surface residence? Enhanced cleavage? Is this allele ligand-dependent? Knowing the nature of this allele is essential to interpret the implications of the partial rescue observed upon sec-6 and sec-8 RNAi. For example, if this allele behaves like NICD overexpression, then the data suggest that sec-6 and sec-8 function downstream of Notch signaling in this context. Second, the bn18 data are not convincing at all. At what temperature were these animals kept? What is the length of the mitotic zone in a wild-type animals kept at the same temperature? Presumably, the animals were kept at the permissive temperature (otherwise we would expect a complete loss of GSCs). If that is the case, then the worsening of the phenotype by sec-6 and sec-8 RNAi could mean that exocyst and Notch signaling operate in parallel during GSC development. By the way, the length of the mitotic zone for sec-6 and sec-8 RNAi should be shown in a control background (without GLP-1 mutation)

2. On a related note, to argue that exocyst positively regulates Notch signaling “in GSCs”, it would have been better to use the rrf-1 and ppw-1 systems in the genetic interaction studies. Otherwise, the effects could also be due to exocyst knock-down in the niche. Overall, despite the correlation between altered GLP-1 distribution in GSCs and the observed phenotypes, the manuscript does not present sufficient evidence to conclude that exocyst regulates Notch signaling in the GSCs to control GSC proliferation.

3. In section 1.4 (Figure 4B-E), the authors conclude that exocyst is required for intracellular trafficking in the germline. However, all of the markers used by the authors in this figure seem to show increased expression levels. Therefore, to conclude that the observed defects are due to impaired trafficking and not increased expression, qRT-PCR should be performed for these genes.

4. Page 6, “RME-2, which normally localizes on the plasma membrane, displayed significant accumulation on the membrane and cytosol upon exocyst depletion”. Do the authors mean that in exocyst RNAi conditions, RME-2 accumulates both at the plasma membrane and inside the cell? First, the RME-2 pattern in sec-6 and sec-8 RNAi are quite different from each other (not much at the membrane in sec-6 RNAi). Second, the green puncta are more likely to be in exocytic or endocytic vesicles as opposed to cytosolic.

5. Figure 4B, central panel, and 4D: The images shown in Figure 4B seem to have a significant increase in SYN-4::GFP at the membrane upon sec6/sec8 RNAi, However, the quantification does not show any difference. Are these images representative? Moreover, using nuclear and cell membrane markers would have made these data more convincing.

6. In the Discussion, the authors state “We found that the exocyst complex is required for regulating GSC proliferation by modulating Notch signaling”. The data presented in the manuscript is not sufficient to make this argument. To reach this conclusion, the authors would need to provide additional evidcen (for example, showing that by increasing the level of GLP-1 in the GSCs they can rescue the sec-6/sec-8 phenotype.)

7. The authors have used a strategy described in Figure S4 to measure the plasma membrane levels of Notch in C. elegans and mammalian cells. Has this methodology been shown to provide accurate surface expression levels for other proteins? This seems to be an unusual strategy for determining protein surface level, especially because the authors choose only some regions of the membrane for quantification, instead of using the whole perimeter of the cell.

8. On a related note, the conclusion that “exocyst is required for trafficking Notch to the plasma membrane in mammalian cells” is not supported by the data. First, the authors have used an antibody raised against the intracellular domain of NOTCH2 for these studies. To detect surface level of NOTCH2, an antibody against the extracellular domain should be used on non-permeabilized cells. Second, in the biotinylation assays, there is a GM130 band in the membrane-bound fraction of the control cells but not in siExocyst and siRab5 samples. Therefore, the quantification is not reliable. In addition, to convincingly show a parallel between their results in C. elegans and the mammalian system, the authors can consider performing a NOTCH2 signaling assay in U2OS cells upon exocyst RNAi.

9. Does RNAi for par-6, par-3, rab-11 and rab-5 recapitulate the germline phenotype of GLP-1 and exocyst components?

Other points

1. How did the authors determine the progenitor zone versus the transition zone in the figures? Although, WAPL-1 was used in one of the experiments as a PZ marker, it doesn’t seem to be restricted to PZ based on the green signals beyond the PZ in the stained gonad. Since a reduction in the size of PZ is a critical phenotype in exocyst mutations, it’s important to describe how the PZ was distinguished from the transition zone.

2. Was there any specific reason for choosing sec-6 and sec-8 for these studies, as opposed to other exocyst complex components?

3. The authors mention on page 5 that “the germline of sec-5 mutant did not show any discernible phenotypes”. Therefore, it is possible that the observed phenotypes only apply to a subset of exocyst components, not the whole complex. The authors should acknowledge this issue in the manuscript.

4. Results section, Heading 1.2. The title of this section can be misleading (“The exocyst functions in germ cells”). The conclusion of this section was that “the exocyst complex functions in both the germ cells and somatic cells for regulating germline development in C. elegans.” The title should reflect this conclusion.

5. What do the asterisks, arrows and arrowheads point to in Figure 3?

6. In the Discussion, the authors state “To our knowledge, this is the first report implicating exocyst function in any stem cell system”. This is not accurate. Mao et al. 2019 (which is cited by the authors) has shown a role for exocyte in niche cells to promote germline stem cell differentiation in Drosophila. Although, this paper did not show a cell autonomous role in stem cells, it is still a role for exocyst in a stem cell system. Therefore, it’s better to rephrase this sentence.

7. On page 8, the authors refer to Figure S5 for IP-mass spec in worms to identify sec-6 binding partners. However, Figure S5 does not seem to show any data related to IP-mass spec from worms. It contains yeast two-hybrid data from worms and IP-mass spec from U2OS cells.

8. The inconsistent PH::mCherry signal in the PZ in Figure 5 is concerning, as it suggests non-uniform fixation of the tissue.

9. Please label the genotypes in Figure S3C.

Reviewer 3

Advance summary and potential significance to field

The manuscript by Pushpa et al analyzes the role of the exocyst vesicle tethering complex, and the anterior Par complex, in germ cell proliferation in the C. elegans germline, focusing on the effects on GLP-1/Notch trafficking. Importantly this work identifies physical interactions between exocyst

components and PAR-5 and exocyst and GLP-1/Notch, and provides evidence that the anterior Par complex is important for recruitment of exocyst to the germ cell membrane. Overall I found the work to be of high quality. This work is likely to be of wide interest to the germ cell development and Notch signaling communities. There are some significant issues that need to be addressed however, including a tendency to overstate or over-interpret results in a few places.

Comments for the author

(1) The data supporting the assertion of germline autonomy for exocyst derives from analysis in putative germline-restricted RNAi using the rrf-1 mutant. rrf-1 mutants are known to retain some somatic RNAi capability, reducing the ability to interpret these results (Kumsta and Hansen 2012, PMID 22574120). The authors should explain the caveats to their interpretations with respect to the degree to which the germ cells themselves contribute to the exocyst and Par requirements. The authors achieved similar results with a soma-specific RNAi mutant ppw-1, further calling into question whether the effect with rrf-1 was due to residual somatic RNAi. Was the emo phenotype present in exocyst RNAi in the ppw-1 mutant in addition to the smaller proliferative zone? This section should be improved by using a different method to show a germline requirement. For instance they could use the CGC strain DCL569 rde-1(mkc36); sun-1p::rde-1 to do a more rigorous analysis. This is essential since the authors claim autonomy throughout the text, and are especially bold about this assertion in the abstract.

(2) With respect to Fig 4, sec-6 depletion effects on yolk uptake and RME-2::GFP localization were first reported in Balklava et al 2007 as high throughput data, supplemental tables 1 and 2 (PMID 17704769). This paper should be cited. More importantly, this was also the first paper to show endocytic recycling defects associated with loss of anterior Par complex components, and so should be cited for this reason as well. The authors should be aware of this paper and its results as they were already informed about this oversight when this work was reviewed at another journal.

(3) The finding that GLP-1 is differentially polarized with more GLP-1 on rachis associated membranes than outer membranes facing the DTC/pseudocoelom and the source of ligand is quite interesting. Could it be related to the radial polarity described for the anterior Par proteins in the embryo (PMID: 18583611)? I have a serious problem with the apical vs basolateral terminology used however. The rachis is not similar to a lumen as suggested in the text. It is a central shared cytoplasm and so is completely different than a lumen which is completely outside of the cytoplasm. The authors need to come up with a different terminology than apical and basal, and use in all of the text and figures. I would suggest inner and outer plasma membranes as names, but it’s just important not to misuse apical and basolateral, since those terms are misleading in this case.

(4) Which Rab5 was depleted from U2OS cells? Human cells express three separate Rab5 genes, A, B, and C. The antibody described in the methods section to detect Rab5 was directed at Rab5C. I don't see any information on the siRNA and which gene it targeted.

(5) In figure 4B it appears that the total levels of GFP::RAB-11 in oocytes are increased after sec-6 and sec-8 RNAi. Is this true? That is not a prediction of a failure to tether RAB-11 vesicles as might be expected after loss of exocyst. Please explain.

(6) In Fig 6E GFP::RAB-11 accumulates in the cortex of distal germ cells after sec-6 or par-6 RNAi. It’s an interesting correlation that par-6 and sec-6 produce similar effects, but the text describes it as GSC basal membrane localization. A fusion defect might be expected to accumulate recycling vesicles below the PM but the way it’s described makes it sound like RAB-11 is on the PM, which I don’t understand.

(7) In Fig 8, three exocyst components were co-depleted. Were the siRNAs used for this described anywhere in the paper?

(8) Fig S5B should have control data (PH::GFP as described in text) for comparison.

(9) Overstatements or incorrect statements in the text that should be toned down or fixed:

Abstract: “exocyst complex regulates C. elegans GSC proliferation by modulating Notch signaling cell autonomously.” Your results are correlative, and consistent with this model, but you did not do any experiments that show that Notch signaling is the sole contributor to the exocyst associated proliferation defect. Please tone this down.

“The anterior polarity protein Par6 is required for GSC Notch trafficking by regulating exocyst recruitment to the niche-signaling interface.” This also overstated. Should be toned down to reflect what was actually shown. or pose it as a model derived from the data.

Pg 3 “thus explaining the reduced Notch-Delta signaling.” “explaining” is an overstatement. Could say potentially explaining, or consistent with, or something along those lines.

Pg 4 “and that PAR-6 locally recruits SEC-6 to the basal GSC membrane.” Also overstated beyond what was actually shown.

Pg 6 “Exocyst complex function has been demonstrated in both endocytosis and secretion in C. elegans in other tissues (Armenti et al., 2014; Chen et al., 2014b; Jiu et al., 2014; Jiu et al., 2012; Zou et al., 2015), but not in the germline.” See comment 2 above.

Pg 7 “The rachis-facing GSC cell surface can be considered equivalent to the apical membrane of polarized cells…” This is incorrect. See comment 2 above.

First revision

Author response to reviewers' comments

Editor’s summary:

Reviewer 1 requests a demonstration that modulating the surface levels of GLP-1 do in fact regulate Notch signaling levels, as a read out of Notch function.

Response: With regard to this important query, we have now tried a few approaches to address his question:

Modulating the surface levels of GLP-1 to examine the change in Notch signaling is technically challenging, since no such *ts* mutants are available. Moreover, such transgenic lines are challenging to generate as they would not have been viable. Therefore, we have relied on overruling the other possibilities that could cause reduction in functional targets of Notch signaling, as summarized below.

1. The reviewer pointed out that the lowering of Notch signaling upon exocyst subunit depletion and the reduction in membrane Notch levels may not be connected. We agree that we have not provided direct evidence for this. However, we do show a significant reduction in the levels of membrane Notch receptor (about 40%) (Figs. 5D, E), and a similar reduction in the direct functional targets lst-1 and sygl-1 (Figs. 3H, J, K, M). Although there might be some percentage of receptor on the membrane available in excess, each event of Notch signaling results in the degradation of the receptor, and thus a significant reduction like this is bound to have an effect on signaling.

2. We show an autonomous role of exocyst components in germ cells, as follows: a) They are expressed in germ cells; b) Exocyst RNAi in sun-1p: rde-1 background results in a reduction of the proliferation zone (PZ) (Fig. 2G); and c) Germ cell-specific degradation of exocyst component sec6, using an auxin-degron system, also results in a smaller PZ (Fig. 2J). This assures us that the reduction in Notch signaling observed upon Exocyst knockdown is due to its role in germ cells. However, it does function in other somatic cell types to regulate GSC divisions.

3. Moreover, we show data that the exocyst is not directly playing a role in the cell cycle in GSC divisions. Our new data shows that exocyst RNAi does not affect other types of germline tumors that are not dependent on Notch signaling (Fig. 3E), thus ruling out a direct effect on GSC divisions through the cell cycle machinery. The data supports the argument that the exocyst complex regulates GSC divisions only in a Notch-dependent manner.

4. A large body of literature has demonstrated that the exocyst complex plays crucial roles in the intracellular trafficking of plasma membrane associated proteins across eukaryotes, especially in recycling and secretion.

5. We did try our best to observe changes in NICD levels in exocyst RNAi backgrounds by immunostaining, using the recently published CRISPR strains from the Kimble lab (Sorensen et al., 2020), but were unsuccessful in our attempts. It is worth noting that visualizing NICD levels by immunostaining is challenging in the adult worm (Gutnik et al., 2018; Sorensen et al., 2020)

Therefore, our most favored hypothesis is that the exocyst complex affects Notch signaling in GSCs by affecting the trafficking of membrane-bound Notch. However, due to technical limitations, we were unable to demonstrate a direct effect of reduction in membrane-bound receptors to a reduction in Notch signaling, and our data at best only points towards a positive correlation between these two phenotypes. We have edited the text acknowledging this gap in our model.

Reviewer 2 requests for clarity in experimental findings and details, and suggests an analysis of the GLP-1 RNA level to rule out other effects through which Notch signaling could be affected. Importantly, as stated by the reviewer the conclusion of the mammalian cultured cells to demonstrate that exocyst complex regulates Notch signaling at the plasma membrane needs to be supported by data or softened in the text.

Response: We have now provided all the experimental details requested by the reviewer in the pointwise responses. In summary, upon exocyst RNAi, total Notch protein levels remain similar to controls (checked by Westerns) in mammalian cells (Fig. 7C). However, we find a significant reduction in the expression of functional targets of Notch signaling, *lst-1/sygl-1* (Fig. 3H-M) in *C. elegans* and Herp2 and Hey1 (Fig. 7F) in U2OS cells.

Both reviewer 2 and 3 request for further experimentation to support the germline autonomy. As stated by reviewer 3, rrf-1 is not a good reagent for this since it performs somatic RNAi. Thus, conduction of these experiments in the rde-1 strains indicated by the reviewer will be required.

Response: As per the reviewer's suggestion, we have used a *sun-1p::rde-1* strain for the exocyst component RNAi to support the germline autonomy and have found that the exocyst is required in the germline for its proliferation (Fig. 2G). In addition, we generated a new transgenic strain expressing sec-6 tagged with a degron sequence at its endogenous locus and combined it with sun-1p::TIR-1 transgenic strain to enable auxin-regulated germ cell specific degradation of SEC-6. Degradation of SEC-6 in a germ cell-specific manner also resulted in a smaller PZ (Fig. 2I, J), indicative of an autonomous role of the exocyst complex in the germline.

Reviewer 1 Advance Summary and Potential Significance to Field:

In this manuscript Pushpa et al. describe their work on the exocyst complex, its interaction with the Notch signaling pathway, and its role in regulating stem cell proliferation. They demonstrate that RNAi against exocyst complex components SEC-6 and -8 results in reduced progenitor zone sizes, and that RNAi in either somatic or germ cells results in this reduction. They demonstrate that this reduction of proliferative zone cells is also found in animals containing either partial loss-of-function or gain-of-function alleles of glp-1. They suggest that the effect that the exocyst complex has on Notch signaling and stem cell proliferation is linked to the amount of GLP-1 that is found on basal surface of the germ cells.They provide data demonstrating that when exocyst function is reduced, GLP-1 on the basal surface is reduced. Furthermore, they demonstrate that the role of the exocyst complex in maintaining Notch on the cell surface is conserved in mammalian cells.

Overall, this is a logically presented model which increases our understanding of how an additional mechanism Notch signaling levels may be maintained in the C.elegans germ line and in other systems. Generally, the experiments appear to be well controlled and the figures properly present the results. However, I do have some concerns about the interpretation of some of the data, and how conclusively it supports their model.

Reviewer 1 Comments for the Author:

First, they demonstrate that reducing exocyst complex function results in germ line defects, including a reduction in the size of the proliferative zone, and that the exocyst complex may interact with Notch signaling (see below). They then demonstrate that GLP-1 basal surface levels are reduced when exocyst function is reduced. **From this they conclude that the mechanism by which the exocyst is affecting progenitor zone size is through affecting GLP-1 surface levels.**

Q1: However, there is not data linking these two observations. It is entirely possible that GLP-1 levels on the surface are in excess, and the reducing surface levels to this extent does not negatively impact Notch signaling levels (indeed, INTRA levels are much lower than Notch surface levels). It seems that they would have to demonstrate that lowering GLP-1 surface levels to this extent does impact Notch signaling levels, or at least be much more measured in their conclusions. Since they demonstrate that a reduction in exocyst function in the soma also reduces progenitor zone size, there must be more going on than just GLP-1 surface levels.

Response: We thank the reviewer for this insightful comment and agree that we have not shown direct evidence connecting the reduction of the membrane-bound receptors with a reduction in functional targets of Notch signaling. We would have loved to have been able to modulate the Notch surface levels to see if it would rescue the small PZ and reduced Notch signaling defect observed upon exocyst RNAi, but it is technically challenging. To the best of our knowledge, there are no genetic mutants/transgenic lines that would modulate the levels of glp-1 receptor on the membrane. Even the chemical agents that would block the deposition or endocytosis of membrane bound glp-1 would be accompanied by secondary effects, clouding the conclusions obtained from them. Notch receptors undergo proteolytic-cleavages upon ligand attachment while relaying the signal, resulting in degradation of the receptor with each successful signaling event. Therefore, in our opinion, a significant reduction in membrane-bound receptor levels is likely to have a negative effect on the signaling. However, our data shows only a positive correlation between surface Notch levels and Notch function in the GSCs, for which we offer our hypothesis as the most favored model rather than a firm conclusion. We have now edited the text to reflect this (lines 237-240). We have also added additional experiments supporting the function of the exocyst complex in the germline (fig 2G,H) in GSC proliferation, most likely in a Notch-dependent manner (Fig. 3G).

Q2: Second, the genetic interaction with bn18 and ar202 appears only to be additive, and therefore does not conclusively demonstrate an interaction with the exocyst complex with Notch signaling. A reduction in Notch signalling is not the only cause of a smaller progenitor zone size. For example, a problem with the mitotic cell cycle could also cause a reduction. Therefore, if you have a smaller progenitor zone size due to bn18, and combine it with a mutant that causes a problem with the mitotic cell cycle, you would expect the phenotype of the double mutant to be a combination of the two phenotypes (smaller than both single mutants), even though each single mutant has a different cause for a smaller progenitor zone. The results would be more convincing if the double mutant phenotype was more than additive (e.g. completely Glp phenotype). Therefore, the authors need to be more measured in their conclusions with this experiment. However, lst-1 and sygl-1 data is much more convincing.

Response: We thank the reviewer for this comment. The reviewer is correct in pointing out that we see an additive phenotype upon exocyst RNAi on the *bn18* and *ar202* backgrounds. We have now modified the text to reflect the same (lines 154 - 157). However, as the reviewer noted, we report a significant reduction in functional targets of Notch signaling, lst-1/sygl-1 in *C. elegans* (Fig. 3H-M). We have now also included functional data for Herp2 and Hey1, which are primary targets of Notch signaling in osteosarcoma (U2OS) cells (lines 314 - 317) (Fig. 7F).

Additionally our newly included data shows that exocyst subunit RNAi does not affect other types

of germline tumors that are not dependent on Notch signaling (Fig. 3G). Thus, it is unlikely that the exocyst complex acts on GSC divisions by virtue of a direct role in the cell cycle. Overall, our results support a role for the exocyst complex in regulating Notch signaling in GSCs, thus affecting the GSC divisions in a Notch-dependent manner.

More minor points

Comment 1-Throughout the paper the authors state that a gene function is 'depleted' through the use of RNAi. This suggests the gene function is completely eliminated; however, RNAi only partially reduces gene function, emphasized by the fact that the mutant alleles have a stronger phenotype. Perhaps the authors could say that the gene function is 'reduced' when using RNAi.

Response: We have now suitably replaced the word depletion at various places in the text.

Comment 2-The authors claim in the abstract and elsewhere that '…the exocyst complex regulates C. elegans GSC proliferation by modulating Notch signaling cell autonomously'. I do not believe they have demonstrated that cell autonomy. An active exocyst complex in a neighboring cell, or in the rachis, could affect GLP-1 accumalation levels.

Response: We thank the reviewer for this comment. By cell autonomous, we meant to convey a germline autonomous role of the exocyst complex in GSC proliferation, as opposed to a role in somatic cells such as the DTC, wherein the exocyst complex is also known to function. Our data with *rrf-1*, *sun-1p::rde-1* and auxin-degron mediated germline specific degradation of *sec-6* (Fig. 2F, G, I, J) indicates a germline-specific role of the exocyst complex. We have now included the new *sun-1p::rde-1* and auxin-degron data and also corrected the manuscript at the relevant places to reflect germline autonomy, as opposed to GSC autonomy (lines 126-142).

Comment 3: The authors do not provide a rationale for why the first started looking at SEC-6 and -8. Providing a rationale would help the reader understand the logic of these first experiments.

Response: We thank the reviewer for this comment. Our lab has been working on SEC-6 (Mylavarapu *et al* 2005, Mylavarapu *et al* 2006, Kumar et al., 2019), of which SEC-8 is a well documented interaction partner in the exocyst complex. As stated in the introduction, when we started this study, not much was known about the role of these two proteins in *C. elegans*, although in other systems, the conserved *sec-6* has been shown to play a vital role in localization of the exocyst complex to sites of secretion. We therefore started working with *sec-6* and *sec-8*.

Comment 4: I am not sure why the authors used sec-6(tm4536)/mIn1 heterozygotes for RNAi by sec-6. An explanation would help here.

Response: Our RNAi did not result in 100% sterility and the sec-6 mutants were not viable, dying at the L1 stage. Therefore, in an attempt to achieve a near-complete removal of gene function and to obtain a more severe phenotype, we performed the RNAi on the heterozygous mutant. However, our analysis showed that *sec6 (RNAi)* on the wild type and on the mutant was not significantly different.

Comment 5: Are tm4536 and ok2187 null alleles?

Response:

1. Tm4536 (sec-6): This mutant deletes 272 bp in the beginning of the protein, resulting in a frameshift mutation right after the deletion, which produces a truncated protein of only 91 residues, encoded by only the first two exons. The crystal structure of sec-6 as well as functional data from other systems suggest that this truncated protein is very likely to be non- functional because it will miss part of the "CoREx" domain essential for assembly of the holo complex. This region is also required for sec-6's interaction with sec9, sec1 and snc2 and for its recruitment to the vesicles (Mei et al.,2018, Meio and Guo, 2018.). These mutants die as early L1 larvae after hatching. Thus, this allele is likely to be a null mutant.

2. Ok2187 (sec-8): This mutant deletes 1128 bp in the middle of the Sec-8 protein, producing a

truncated polypeptide of 247 residues, which represents approximately only one third of the full length protein. This mutant will have an intact CorEx domain for initial complex assembly, but the complex will not be very stable, as the C-terminus of the protein is required to mediate the interaction with the other Sec10-Sec15-Exo-84 subcomplex (Mei et al.,2018). This mutant grows to adulthood, but is sterile and exhibits a short lifespan, indicating a strong loss-of-function, but may not be a null mutant.

Comment 6: Why would sec-5 mutant not show a mutant phenotype, but sec-5 RNAi does? Is it possible sec-5(RNAi) is targeting additional genes? For all genes, what is the evidence that RNAi was targeting the correct gene?

Response: Deletion in pk2357 leaves the N-terminal overhang, CorEX domain, CAT-A and CAT-B (partially) intact. CorEX is the most important domain for the pairwise interaction with sec3 and the rest of the complex. In addition the N-terminal overhang and the CAT-B domain further help to form the sec3-sec5-sec6-sec8 sub-complex. However, the complex will be less stable because the C-terminus is required for the interactions with the Sec10-Sec15-Exo-84 subcomplex, albeit this is a minor role to Sec-8 (Mei et al.,2018). As explained above, the *sec-5* mutant is not a null mutant, which might explain the lack of a strong germline phenotype in the mutant. However, the mutant is virtually sterile, bearing very few embryos (that are also not viable) and exhibit a short lifespan (Frische EW, et al. 2007). However, performing RNAi on the mutant background, which would reduce levels of the truncated protein, could disrupt the exocyst complex more efficiently than the mutant itself.

Using qRT-PCRs, we have confirmed robust knockdown of *sec-6*, p*ar-6* and *par-2* (Fig. S4). Our qRT-PCRs did not work well for *sec-5* and *sec-8* due to technical reasons; however, specific depletion of these two genes using agarose gel band intensities from semi-quantitative RT-PCR is evident (Fig. S4B for sec-5 and Fig. S1C in Kumar et al., 2019 for sec-8). The targeted mRNA of these genes is reduced significantly in their respective RNAi.

Comment 7: The authors state that they obtained a '…GLP-1::GFP::3xFLAG expressing strain…'. Where was this obtained and how has it been verified? Does this strain show any aberrant phenotypes? Reference?

Response: We had obtained this strain from the CGC (originated from the Christian Eckmann laboratory) and out-crossed it thrice. The strain did not show any aberrant phenotypes in the germline at any developmental stages. The length of PZ and brood count were similar to wild type animals. The expression pattern of the transgene was very similar to the expression pattern observed by immunostaining for the V5-tag in the GLP-1::V5 strain (V5 tag present at the C-terminus of GLP-1 in the endogenous locus; Somerson et al., 2020). We have also indicated these details in the manuscript in the Materials and Methods section (lines 419-420).

Comment 8: Throughout paper exocyst complex is dropped to simply exocyst should be consistent and keep exocyst complex

Response: We apologize for this error and have maintained the nomenclature as the exocyst complex.

Comment 9: The figure legends could be more informative, stating what stage animals were used

Response: Due to constraints of word limits, we were unable to include the details in the figure legends. However, we have included this information in the Materials and Methods and/or Results sections.

Comment 10: Page 3. remove 'in' - Exocyst complex members are required for recycling of the Delta-ligand in during the asymmetric division

Response: We have now corrected this error.

Comment 11: Page 4. Don't need strain name just allele - compared to control (Figs. 1H, I, S1). We also examined the germline in the *sec-8* genetic mutant VC2648

Response: We have now corrected this error as suggested (lines 100-103).

Comment 12: Page 4. “RNAi phenotype, sec-8(-/-) worm gonads showed significantly a smaller PZ as compared to sec-8(-/+)”. Should be changed to “…showed a significantly smaller PZ…”

Response: We have now modified the text as suggested.

Comment 13: Page 5. s3a doesn’t refer to SEC-6 expression – “cytoplasm, including the oocytes and sperm (Figs. 2D, S3A).”

Response: We have now corrected this error.

Comment: 14-Page 6. should just be figure 3 b since 3a is just ar202 at varying temps– “…(ar202) mutant at the restrictive temperature (Figs. 3A, B).”

Response: We appreciate the reviewer’s suggestion. We wished to show panel A at its present location so as to make it easy for the reader to appreciate the rest of the figure. Therefore, we would request, if possible, for this panel to be retained. However, we would be happy to move it to the supplementary information if so advised.

Comment 15: Page 7. remove ‘of’ – “We next examined the role of the exocyst complex in regulating of GLP-1 localization to the membrane in…”

Response: We have now corrected this error.

Comment 16: Page 7. Clean up reference – “…the exocyst complex has also been reported in literature (Boehm et al., 2017, Harsh Kumar, 2019 #269;”

Response: We have now corrected this error.

Comment 17: Page 8. The authors claim that the did not observe any substantial changes in the apical levels of GLP-1::GFP with rab-5 or rab-11 RNAi. However, the GFP looks much more cytoplasmic in the RNAi animals that the control in figure 5F

Response: Our quantitation on the apical (rachis-facing, now named “inner”) membrane presented in Fig 5G shows that there is no substantial change in membrane Notch receptor intensity. Therefore, it is likely that the cytoplasmic accumulation observed (for sec-6, Rab-5, Rab-11) is dominated by GLP-1-positive vesicles that have formed but are unable to fuse at the basal (niche-facing, now named “outer”) membrane (Fig. 5D). This phenotype is consistent with the classic exocyst phenotype, wherein vesicles form but are unable to fuse with the plasma membrane (Novick and Schekman, 1979).

Comment: 18-Page 9. “…depletion of human Sec5, Sec6 and Sec8 (Fig. 7C) revealed perceptible mis-localization…”. Figure 7C does not show this.

Response: 7C refers to the blots showing the knockdown efficiency for the exocyst complex, which led to mislocalization of the exocyst complex as depicted in Fig. 7A, B).

Comment 19: Page 9. “…protein GM130 was absent from our plasma membrane fractions, confirming a clean plasma membrane…”. It appears as though there is a faint band in siGFP lane for GM130

Response: We now repeated the experiment and replaced it with a better blot, which does not show GM130 in the plasma membrane fraction.

Comment 20: space needed – “…germline stem cell (GSC) proliferation by maintaining optimal Notch receptor levels on the GSCsurface.”

Response: We have now corrected this error.

Comment 21-Page 13. Remove space – "…Notch2 levels on mammalian cell surface: Notch 2…"

Response: We have now corrected this error.

Comment 22-Page 13. Add space – "… of five and upto ten regions of interest (ROI) per cell…"

Response: We have now corrected this error.

Comment 23-Page 14. Add space – "…cropped to only display the progenitor zone going upto the first layer of the transition zone"

Response: We have now corrected this error. -

Comment 24: Add proper reference – "PMID: 25122090. For protein purification 1ml-streptactin…"

Response: We have now corrected the endnote entry

Comment 25-Figure 1. Legend – Italicize - depleted of exocyst complex subunits sec-6 and sec-8"

Response: We have now corrected this error.

Comment 26-Figure 2 Legend- Gene names need to be italicisized.

Response: We have now corrected this error.

Comment 27-Figure 3F- should mentioned that it is corrected for background

Response: We have now corrected this error.

Comment 28-Figure 4- Legend does not mention purpose of arrows in 4B

Response: We have now corrected this error.

Comment 29-Figure 5e- missing rab10 RNAi on graph

Response: We have now included this data in the manuscript (Figs. 5E).

Comment 30-Figure 6b- missing par-3 on graph

Response: We have now included this data in the manuscript (Figs. 6B).

Comment 31-Figure 7a- siSec6/8/5 labeled but everywhere else says siExocyst. Should be consistent

Response: We have now corrected this error in the manuscript.

Reviewer 2 Advance Summary and Potential Significance to Field:

In manuscript DEVELOP-2020-196345v1 by Pushpa et al, the authors have examined the role of the exocyst complex in the regulation of germline stem cell (GSC) proliferation in C. elegans.

They first use RNAi and mutant analysis for sec6 and sec8 to show that exocyst is required for germ cell proliferation in this model. Using cell type-specific RNAi, they show that exocyst functions in both germ cells and somatic cells to regulate germline development. They then perform genetic interaction studies and staining to conclude that the exocyst complex positively regulates Notch signaling for germline stem cell proliferation. Using transgenic strains of C.

elegans expressing tagged version of several proteins including GLP-1/Notch, the authors conclude the involvement of exocyst in intracellular trafficking in the germline and in the maintenance of membrane localization of GLP-1/Notch in germline. Similar observations are made for Rab5 and Rab11, suggesting a role for the exocyst in recycling of GLP-1 to the membrane. Using yeast two-hybrid and IP-mass spec experiments in C. elegans and a human osteosarcoma cell line (U2OS), the authors identify Par-5 as an interactor of sec-6. Combined with GLP-1 localization defects in the germline of Par-6 and Par-3 depleted worms, these data suggest that the anterior Par complex cooperates with exocyst to localize GLP-1 properly. Finally, using imaging and biotinylation assays in U2OS cells, the authors conclude that exocyst is also involved in surface localization of human NOTCH2.

Despite the importance of the exocyst complex in various physiological contexts, a cell-autonomous role for exocyst in stem cells was not reported previously. Moreover, a role for exocyt components in the regulation of Notch signaling has only been reported in a couple of contexts in Drosophila, none of which was thought to be mediated by regulating the Notch receptor trafficking. Therefore, the manuscript focuses on a novel mechanism for the regulation of Notch signaling by exocyst, in a cell type not previously known to be regulated by the exocyt. However, unfortunately, a number of issues exist with the data presentation and quantification. Moreover, some of the conclusions of the paper do not seem to be supported by the data. These issues significantly reduce my enthusiasm for this manuscript.

Reviewer 2 Comments for the Author:

Major points

Comment 1. It is rather difficult to interpret the genetic interaction studies shown in Figure 3. First, why does the ar202 mutation exhibit higher Notch signaling at the restrictive temperature? Increased stability? Prolonged surface residence? Enhanced cleavage? Is this allele ligand-dependent? Knowing the nature of this allele is essential to interpret the implications of the partial rescue observed upon sec-6 and sec-8 RNAi. For example, if this allele behaves like NICD overexpression, then the data suggest that sec-6 and sec-8 function downstream of Notch signaling in this context.

Response: ar202 mutation is a well-characterized temperature sensitive gain-of-function mutation and most widely used for glp-1 genetic analysis (Hubbard and Schedl, Genetics, 2019). The molecular lesion causes a single base mutation in the negatively regulated region (NRR) that causes excessive Notch signaling. This region is known to mask the second cleavage site from Adam-metalloproteases and thus keeps the receptor from self activation.

When the ligand interacts with the receptor it causes stretching of the receptor, unmasking the cleavage-site. The molecular lesion in *ar202* might make the GLP-1 receptor at the membrane more amenable to cleavage at a higher temperature, causing an increase in signaling in a temperature-dependent manner.

The mutation displays a degree of ligand-independent cleavage, but is also responsive to the ligand (Pepper et al., 2003; McGgovern et al., 2009; Hubbard and Schedl 2019); however, it is unlike NICD over expression, which is truly ligand-independent. In the context of our work, the reduction of the *ar202* tumor is significant because we observe a role of the Exocyst complex in maintaining optimal levels of Notch receptors on the membrane for the right amount of signaling. The GLP-1(ar202) receptor would also need to be trafficked to the membrane before cleavage (by adam-proteases) could happen, and therefore we propose that reduction in exocyst function would lead to reduction in ar202-led tumorigenesis.

Comment 2: Second, the bn18 data are not convincing at all. At what temperature were these animals kept? What is the length of the mitotic zone in a wild-type animals kept at the same temperature? Presumably, the animals were kept at the permissive temperature (otherwise we would expect a complete loss of GSCs). If that is the case, then the worsening of the phenotype by sec-6 and sec-8 RNAi could mean that exocyst and Notch signaling operate in parallel during GSC development. By the way, the length of the mitotic zone for sec-6 and sec-8 RNAi should be shown in a control background (without GLP-1 mutation).

Response: We apologize for not explaining these experiments in detail due to word limitations. This has now been corrected in the manuscript (lines 151-154) and summarized below.

Glp-1(bn18) is a temperature-sensitive, loss-of-function mutation in the NICD domain. The number of germ cells in bn18 is less even at the permissive temperature of 15-20 deg C as compared to wild type animals (numbering about 120-130 and 210-230, respectively) (Fox and Shedl, 2015, Genetics).

Although the worsening of the phenotype by combining *bn18* and exocyst depletion can be interpreted as working in parallel pathways. However, our data show a Notch-dependent function of the Exocyst complex in GSC proliferation (Fig. 3E) in a germline-autonomous manner (Fig. 2F, G, J). We also observe a reduction in direct Notch transcriptional targets (lst-1/ sygl-1) upon exocyst complex depletion (Fig. H-M). Our study therefore indicates that the already compromised signaling due to the *bn18* mutation (at the permissive temperature) is further worsened due to exocyst complex depletion, since even the available (compromised) receptors on the membrane are further reduced in number.

We would like to draw attention to the fact that we had already included the data showing the length of the PZ for both *sec-6* and *sec-8* RNAi (Figs. 1F, 1G). We have now used the data from 1F in Fig. 3D also to show everything together for ease of understanding.

Comment 2: On a related note, to argue that exocyst positively regulates Notch signaling "in GSCs", it would have been better to use the rrf-1 and ppw-1 systems in the genetic interaction studies. Otherwise, the effects could also be due to exocyst knock-down in the niche. Overall, despite the correlation between altered GLP-1 distribution in GSCs and the observed phenotypes, the manuscript does not present sufficient evidence to conclude that exocyst regulates Notch signaling in the GSCs to control GSC proliferation.

Response: We agree with the reviewer's comment that performing the epistasis on the *rrf-1(-)* background would have been good, and we did try to generate the *glp-1(bn18);; rrf-1* strain, but both our attempts failed. The resulting mutants were not healthy and we could not maintain them for further experiments. In lieu of this, we now show through multiple experiments that the exocyst complex functions in the germline (Fig and affects GSC proliferation in a Notch signaling-dependent manner.

1. Exocyst functions in the germline:

a. We have now used a *sun-1p::rde-1* strain that specifically knocks down the exocyst components only in the germline.

b. We have used rrf-1, which allows processing of RNAi in the germline and a few other somatic cells (Kumsta and Hansen, 2012), but not in the somatic niche cell (DTC).

c. In addition, we have also reduced the protein levels of the exocyst component sec-6 in the germline, using an auxin-degron system.

All of the above results indicate that the exocyst complex is functional in the germline for GSC proliferation.

2. Reduction of GSCs upon exocyst depletion is Notch-dependent:

We used two other germline tumors that are formed due to uncontrolled proliferation of GSCs, but are independent of Notch signaling, namely *gld-2(q497);gld-1(q361)* and *gld-3(q730);nos- 3(q650)*. In both these genetic backgrounds, GSCs fail to undergo differentiation and proliferate in an uncontrolled manner and remain unaffected by reduction of Notch signaling (Fig. 3E). Upon exocyst subunit depletion, these tumors do not show any reduction, suggesting that exocyst is not likely to function in GSC divisions by influencing the differentiation pathways or by directly affecting the cell cycle; rather, it regulates GSC proliferation in a Notch-dependent manner.

We agree that we have not provided a conclusive, causative connection between the reduction in glp-1 receptor levels at the signaling-active, niche-facing membrane with the lowering of glp-1 signaling in GSCs (reduction of LST-1/SYGL-1 protein levels). However, as also articulated above in response to another comment of reviewer 1, this model represents the best hypothesis that explains our data about the role of the exocyst complex in GSCs.

Comment 3: In section 1.4 (Figure 4B-E), the authors conclude that exocyst is required for intracellular trafficking in the germline. However, all of the markers used by the authors in this figure seem to show increased expression levels. Therefore, to conclude that the observed defects are due to impaired trafficking and not increased expression, qRT-PCR should be performed for these genes.

Response: We agree that looking at these images, one can perceive a slight increase in the expression of rme-2 and syn-4 because of the mislocalized GFP throughout the cytoplasm, but our qRT-PCRs (as suggested by the reviewer) do not show any increase in the expression at the mRNA levels, suggesting mislocalization rather than upregulation. However, we do see an approximately 20% increase in the *rab-11* mRNA levels (Fig. S4A), which indicates a transcriptional upregulation upon exocyst depletion, perhaps through a compensatory feedback loop to try and maintain normal levels of recycling. This increase, however, is still lower than the ~2 fold increase in the mislocalization underneath the membrane (Fig. 4E). Our data suggest that there is both a modest transcriptional upregulation of *rab-11*, leading to higher protein levels, as well as mislocalization underneath the plasma membrane. We focused on the clearly evident redistribution in this study. The RT-PCR data has now been discussed in the text appropriately (lines 194-198).

Comment 4: Page 6, "RME-2, which normally localizes on the plasma membrane, displayed significant accumulation on the membrane and cytosol upon exocyst depletion". Do the authors mean that in exocyst RNAi conditions, RME-2 accumulates both at the plasma membrane and inside the cell? First, the RME-2 pattern in sec-6 and sec-8 RNAi are quite different from each other (not much at the membrane in sec-6 RNAi). Second, the green puncta are more likely to be in exocytic or endocytic vesicles as opposed to cytosolic.

Response: We thank the reviewer for urging us to clarify our writing. We agree that the "cytosolic" accumulation observed very likely reflects accumulated RME-2 in plasma membrane-directed vesicles that have failed to fuse with the plasma membrane upon exocyst complex depletion (a classical exocyst complex depletion phenotype). While individual oocytes can show seemingly different cytosolic distribution patterns of RME-2-positive vesicles (Fig. S5), the vesicles consistently show accumulation underneath the membrane, or spill over to other regions of the cytosol. We have now clarified this discussion in the manuscript (lines 185–188).

Comment 5: Figure 4B, central panel, and 4D: The images shown in Figure 4B seem to have a significant increase in SYN-4::GFP at the membrane upon sec6/sec8 RNAi, However, the quantification does not show any difference. Are these images representative? Moreover, using nuclear and cell membrane markers would have made these data more convincing.

Response: We thank the reviewer for pointing this out regarding the image, and have now included more images that reflect the quantification more accurately (Fig. S5). Indeed, there appears to be a slight increase in overall SYN4::GFP levels underneath the plasma membrane, which is consistent with the accumulation of SYN4::GFP-vesicles accumulating near the plasma membrane due to compromised fusion, a hallmark of exocyst complex dysfunction. We agree that there is a potential for the higher plasma membrane accumulation observed to be either within or just under the membrane, or possibly a combination of both. Accumulation of post- Golgi secretory vesicles and recycling endosomes under the membrane is a classical phenotype of exocyst complex depletion in many systems (He and Guo, 2009; Heider and Munson, 2012; Wu and Guo, 2015; Zhu et al., 2017). Accumulation within the membrane would signify defects in endocytosis, a role also attributed to the exocyst complex in other systems (Boehm et al., 2017, Sommer et al., 2005, Murthy and Schwarz, 2004, Kumar et al., 2019). Teasing out these mechanisms would be a very interesting and worthwhile exercise that would require an independent and detailed study not under the scope of this manuscript. Nevertheless, our data in Figs. 4 and S5 supports a definitive role for the exocyst complex in regulating intracellular

trafficking in the *C. elegans* germline. We have now modified the relevant text (lines 201 - 205) to more accurately reflect this conclusion.

Comment 6: In the Discussion, the authors state "We found that the exocyst complex is required for regulating GSC proliferation by modulating Notch signaling". The data presented in the manuscript is not sufficient to make this argument. To reach this conclusion, the authors would need to provide additional evidence (for example, showing that by increasing the level of GLP-1 in the GSCs they can rescue the sec-6/sec-8 phenotype.)

Response: We agree with the reviewer on this and as written in the response to reviewer 1, evidence by modulating the levels of GLP-1 receptor on the membrane is technically challenging. As also detailed in the responses above, we have already presented results delineating the effect of exocyst depletion on direct Notch signaling targets (lst-1/sygl-1), supporting a role for the exocyst complex in regulating Notch signaling in GSCs. We have now added new evidence, also explained above in response to comment 2, that the exocyst complex regulates GSC proliferation in a Notch-dependent manner and does not affect the tumors which are not Notch-dependent.

Comment 7: The authors have used a strategy described in Figure S4 to measure the plasma membrane levels of Notch in C. elegans and mammalian cells. Has this methodology been shown to provide accurate surface expression levels for other proteins? This seems to be an unusual strategy for determining protein surface level, especially because the authors choose only some regions of the membrane for quantification, instead of using the whole perimeter of the cell.

Response: In mammalian cells it is routine to use a few linescans across the membrane per cell to quantitate the amount of protein on the surface (Wit et al., 2006, Rajan et al., 2012, Solis J et al., 2012, Leitch et al., 2014), which has been shown to give a reasonable representation of the whole membrane. The membrane marker WGA shows punctate staining all along the membrane - we chose only clearly WGA-positive regions to ensure that we measured true membrane levels of Notch (Fig. S6B, C). We have now verified this method by analyzing approximately double the number of linescans per cell for 20 cells in sec-6 siRNA treated cllsle cells and plotted it as a ratio of membrane vs. cytoplasm (Fig. S6D), which reproduces the trends of the results presented earlier (Fig. 7B). In *C. elegans*, we could use the total cell surface because the transgenic line showed more uniform expression all along the membrane.

Comment 8: On a related note, the conclusion that "exocyst is required for trafficking Notch to the plasma membrane in mammalian cells" is not supported by the data. First, the authors have used an antibody raised against the intracellular domain of NOTCH2 for these studies. To detect surface level of NOTCH2, an antibody against the extracellular domain should be used on non-permeabilized cells.

Response: We appreciate the reviewer's comment. We used multiple antibodies against Notch1, Notch2 and Notch4 to try to observe membrane-bound levels of Notch in mammalian cells. In our experience, the antibody we used works best in immunostaining to show membrane-bound Notch in both U2OS and Hela cells. The same antibody has been used in a previous study for measuring Notch membrane levels (Li et al., 2015). Unfortunately, antibodies targeting the N-terminal (extracellular) region of Notch2 work well for Western blots, but not for immunostaining (Li et al., 2015). Although we agree with the reviewer's concern that the antibody targets the C-terminus of the Notch protein, and so would show all possible cleavage stages of the protein, it should also show the full length receptor bound to the membrane. To ensure that we quantitate only the membrane-bound fraction, we used a plasma membrane marker for our analysis. For our *C. elegans* work, all of our transgenic fusion lines for Notch (GLP-1) are also C-terminally tagged with GFP/V5, which show clear membrane localization (Figs. 5, S3B). In addition, the biochemical purification of the membrane bound fraction from mammalian cells also supports our model that the exocyst complex affects Notch levels on the plasma membrane (Fig. 7D).

Comment 9: Second, in the biotinylation assays, there is a GM130 band in the membrane- bound fraction of the control cells but not in siExocyst and siRab5 samples. Therefore, the quantification is not reliable. In addition, to convincingly show a parallel between their results in C. elegans and the mammalian system, the authors can consider performing a NOTCH2 signaling assay in U2OS cells upon exocyst RNAi.

Response: We thank the reviewer for pointing this out. We have now repeated these experiments and show a new blot with robust GM130 signals only in the cytoplasmic fractions and not in the membrane-bound fractions (Fig. 7D). To gauge the functional effect of Notch mislocalization on signaling, we measured the mRNA levels of herp2 and hey1 (direct Notch targets) in osteosarcoma cells, and found them to be significantly downregulated upon exocyst knockdown, indicating a reduction in Notch signaling (Fig. 7G).

Comment 9: Does RNAi for par-6, par-3, rab-11 and rab-5 recapitulate the germline phenotype of GLP-1 and exocyst components?

Response: We thank the reviewer for this comment. We had indeed anecdotally observed a reduction in the size of the PZ upon depletion of each of these proteins. We have now quantified these phenotypes and reported fewer cells in the PZ as compared to control for each of these proteins, similar to exocyst subunit knockdown. We have now included these results as a new supplementary figure (**Fig. S6**) in the revised manuscript.

Other points

Comment 10: How did the authors determine the progenitor zone versus the transition zone in the figures? Although, WAPL-1 was used in one of the experiments as a PZ marker, it doesn't seem to be restricted to PZ based on the green signals beyond the PZ in the stained gonad. Since a reduction in the size of PZ is a critical phenotype in exocyst mutations, it's important to describe how the PZ was distinguished from the transition zone

Response: The different stages of germ cells can be easily recognized based on their different nuclear morphology (Pazdernik et al., 2013, Hubbard et al., 2007, Hirsh et al., 1976), as reflected in our DAPI-stained images (Fig. 1E, J). As suggested by the reviewer, we have now included magnified insets representing nuclei from each stage from a wild type gonad. The GSCs present in close contact with the niche cell (extreme left of the images) show floret- shaped nuclei (Fig. 1E). As the cells move away from the niche and start transitioning into the meiotic program, the nuclei become crescent-shaped (thought to be due to chromosome pairing). Further on, the nuclei in the pachytene zone (meiotic zone) have a distinct noodle- shaped morphology (Hubbard 2007). Additionally, the PZ can also be stained by using an antibody against the nuclear protein WAPL-1 (Fig. 1J), which negatively regulates cohesin loading on the chromosomes and is involved in the DNA repair machinery. WAPL-1 staining correlates very well with the DAPI staining of mitotic nuclei, and starts fading from the nuclei as cells enter meiosis in the transition zone (Fig. 1J). We have also included this description in the Materials and Methods section (lines 552 – 557).

Comment 11: Was there any specific reason for choosing sec-6 and sec-8 for these studies, as opposed to other exocyst complex components?

Response: As explained in response to a previous comment (comment no. 3 of reviewer 1), our lab has been working on these two components of the exocyst complex in mammalian cells for some time now (Kumar et al., 2019, Sivaram et al., 2006, Sivaram et al., 2005). When we began working on the role of the exocyst complex in *C. elegans*, nothing much was known about sec-6 in this system. However, sec-6 has been shown to be a crucial, highly conserved component of the complex required for targeting the entire complex to the plasma membrane in yeast (Songer and Munson, 2009), encouraging us to further delineate the role of these genes.

Comment 12: The authors mention on page 5 that "the germline of sec-5 mutant did not show any discernible phenotypes". Therefore, it is possible that the observed phenotypes only apply to a subset of exocyst components, not the whole complex. The authors should acknowledge this issue in the manuscript.

Response: The *sec-5* mutant did not show a perceptibly smaller PZ (Fig. S2A), which is why we did not quantify GSC numbers. In contrast, the *sec-6* RNAi and *sec-8* mutant/ RNAi showed clearly reduced PZs as documented in the paper (Fig. 1). This apparent discrepancy between exocyst subunits could perhaps be due to the fact that the sec-5 mutant is not a null mutant (as also explained above in response to comment 9 of reviewer 1). However, the *sec-5* mutants are almost

sterile with very low brood counts (our unpublished observations), consistent with earlier reports (Armenti et al., 2014). However, we do acknowledge the possibility that the phenotypes we report may be related only to some subunits of the complex, given the existence of subcomplexes of the exocyst complex in some contexts (Moskalenko et al., 2003, Singh et al., 2019, Katoh et al., 2015). We thank the reviewer for this comment and have now acknowledged this possibility in the manuscript (lines 112 - 114).

Comment 13: Results section, Heading 1.2. The title of this section can be misleading ("The exocyst functions in germ cells"). The conclusion of this section was that "the exocyst complex functions in both the germ cells and somatic cells for regulating germline development in C. elegans." The title should reflect this conclusion.

Response: We thank the reviewer for pointing this out and have now edited the title to more accurately reflect the results showing a function of the exocyst complex in GSC proliferation in both germline and somatic cells (line 115).

Comment 14: What do the asterisks, arrows and arrowheads point to in Figure 3?

Response: The asterisks mark the distal end of the gonad. In the case of germline tumors, it is difficult to distinguish the distal and the proximal end by just looking at the dissected gonad because all the cells are in the mitotic stage and no gametes are formed in the proximal end. Therefore, as general practice, the distal end is indicated by asterisks. We apologize for the oversight. The relevant details have now been added in the figure legend (lines 1013-1014 and 1020).

Comment 15: In the Discussion, the authors state "To our knowledge, this is the first report implicating exocyst function in any stem cell system". This is not accurate. Mao et al. 2019 (which is cited by the authors) has shown a role for exocyte in niche cells to promote germline stem cell differentiation in Drosophila. Although, this paper did not show a cell autonomous role in stem cells, it is still a role for exocyst in a stem cell system. Therefore, it's better to rephrase this sentence.

Response: We wish to point out that the Mao et al. 2019 paper described the role of the exocyst complex in the germarial sheath cells (somatic cells) in *Drosophila*, which remain in contact with and support the differentiating germ cells; inactivation of the exocyst complex causes defects in germ cell differentiation. Here, we report a role of the exocyst complex in regulating the proliferation of undifferentiated germline stem cells themselves by influencing Notch signaling in a germline autonomous manner. Therefore, in our understanding, this is the first report of a role for the exocyst complex in any type of stem cell. We have now clarified this point in the manuscript by removing the word "system" (lines 345– 348).

Comment 16: On page 8, the authors refer to Figure S5 for IP-mass spec in worms to identify sec-6 binding partners. However, Figure S5 does not seem to show any data related to IP-mass spec from worms. It contains yeast two-hybrid data from worms and IP-mass spec from U2OS cells.

Response: We apologize for this mistake. The IP followed by MS/MS data is from *C.elegans* sec-6 pulldowns. We have now corrected the legend for the table accordingly.

Comment 17: The inconsistent PH::mCherry signal in the PZ in Figure 5 is concerning, as it suggests non-uniform fixation of the tissue.

Response: The concerned images are not immunostained tissue, but are fluorescence micrographs of whole mount, anesthetized live animals without using any fixative, expressing the PH::mCherry and GLP-1::GFP in the germline. The difference in the intensity of red fluorescence between the left and right halves of the outer ("basal") PH::mCherry is likely due to the curvature at the distal (left) end of this gonad. The differential effect is less pronounced, or almost absent, in the corresponding inner ("apical") side, which is not as curved by virtue of being in the centre of the tubular gonad.

Comment 18: Please label the genotypes in Figure S3C.

Response: This has now been incorporated as suggested.

Reviewer 3 Advance Summary and Potential Significance to Field:

The manuscript by Pushpa et al analyzes the role of the exocyst vesicle tethering complex, and the anterior Par complex, in germ cell proliferation in the C. elegans germline, focusing on the effects on GLP-1/Notch trafficking. Importantly this work identifies physical interactions between exocyst components and PAR-5, and exocyst and GLP-1/Notch, and provides evidence that the anterior Par complex is important for recruitment of exocyst to the germ cell membrane.

Overall I found the work to be of high quality. This work is likely to be of wide interest to the germ cell development and Notch signaling communities. There are some significant issues that need to be addressed however, including a tendency to overstate or over-interpret results in a few places.

Reviewer 3: Comments for the Author:

Comment 1: The data supporting the assertion of germline autonomy for exocyst derives from analysis in putative germline-restricted RNAi using the rrf-1 mutant. rrf-1 mutants are known to retain some somatic RNAi capability, reducing the ability to interpret these results (Kumsta and Hansen 2012, PMID 22574120). The authors should explain the caveats to their interpretations with respect to the degree to which the germ cells themselves contribute to the exocyst and Par requirements. The authors achieved similar results with a soma-specific RNAi mutant ppw-1, further calling into question whether the effect with rrf-1 was due to residual somatic RNAi. Was the emo phenotype present in exocyst RNAi in the ppw-1 mutant in addition to the smaller proliferative zone? This section should be improved by using a different method to show a germline requirement. For instance they could use the CGC strain DCL569 rde-1(mkc36); sun-1p::rde-1 to do a more rigorous analysis. This is essential since the authors claim autonomy throughout the text, and are especially bold about this assertion in the abstract.

Response: We appreciate this important comment by the reviewer. As suggested, and also explained above in response to similar comments from the other reviewers, we have now used the *sun-1p::rde-1* strain (DCL569) to delineate the function of the exocyst complex in the germline vs soma. Knock down of the exocyst complex on this background also leads to a significant reduction in the number of GSCs as compared to controls, indicating a germline- specific role of the exocyst complex in GSC proliferation (Fig. 2G). In addition, we also found that about 40-50% of gonads display the emo phenotype.

We also similarly used the *lag-2p::rde-1* (JK4143) strain to observe the effect of the reduction of exocyst subunit levels in the niche cells (DTC) on GSC proliferation. However, these experiments were inconclusive, since the PZ is small and the GSC numbers very low in this strain compared to the wild type.

In addition, we have now added data from a newly generated transgenic strain wherein we tagged sec-6 at the endogenous locus with an auxin-responsive degron sequence using CRISPR-Cas9 editing. Our results show that degrading SEC-6 specifically in the germline using this system also results in a significantly smaller pool of GSCs (Fig. 2I, J), phenocopying the germline-specific RNAi data.

Comment 2: With respect to Fig 4, sec-6 depletion effects on yolk uptake and RME-2::GFP localization were first reported in Balklava et al 2007 as high throughput data, supplemental tables 1 and 2 (PMID 17704769). This paper should be cited. More importantly, this was also the first paper to show endocytic recycling defects associated with loss of anterior Par complex components, and so should be cited for this reason as well. The authors should be aware of this paper and its results as they were already informed about this oversight when this work was reviewed at another journal.

Response: We sincerely apologize for our inadvertent oversight and have now included the reference and also discussed our results in the context of this paper in the text (lines 179, 187-

188, 273-274).

Comment 3: The finding that GLP-1 is differentially polarized with more GLP-1 on rachis associated membranes than outer membranes facing the DTC/pseudocoelom and the source of ligand is quite interesting. Could it be related to the radial polarity described for the anterior Par proteins in the embryo (PMID: 18583611)? I have a serious problem with the apical vs basolateral terminology used however. The rachis is not similar to a lumen as suggested in the text. It is a central shared cytoplasm and so is completely different than a lumen which is completely outside of the cytoplasm. The authors need to come up with a different terminology than apical and basal, and use in all of the text and figures. I would suggest inner and outer plasma membranes as names, but it's just important not to misuse apical and basolateral, since those terms are misleading in this case.

Response: We thank the reviewer for these important comments and suggestions. The paper referred to by the reviewer (Anderson et al., 2008) describes a radial polarity for aPars in *C. elegans* embryos wherein PAR-6 is enriched on the contact-free surfaces of the cell. Later, another article from the same group (Armenti et al., 2014) reported a similar radial polarity for the exocyst complex in the embryo and also reported a requirement of aPars for exocyst complex localization on the contact-free membranes of the cell. It is therefore interesting to envision that PAR-6 shows a similar radial polarity in the GSCs, although this remains to be experimentally demonstrated. Unfortunately, our attempts to visualize PAR-6 and PAR-3 in GSCs were not successful with the existing transgenic lines due to the weak fluorescence intensity. Similar to Armenti *et al.*, 2014, we show a polarized localization of the exocyst complex on the GSC membrane. It is enriched on/near the niche-facing surface and its localization near this surface is likely to be dependent on the aPar PAR-6 (Figs. 2D, 6C).

We had used the "apical" and "basal" terminology to describe the two surfaces of the GSCs based on a recent publication (Agarwal et al., 2018). However, we now recognize that this could be misleading, and are thankful to the reviewer for pointing this out. As suggested, we have now renamed them as "inner" (rachis-facing, earlier "apical") and "outer" (niche-facing, earlier "basal") throughout the manuscript.

Comment 4: Which Rab5 was depleted from U2OS cells? Human cells express three separate Rab5 genes, A, B, and C. The antibody described in the methods section to detect Rab5 was directed at Rab5C. I don't see any information on the siRNA and which gene it targeted.

Response: We used siRNA against all three isoforms of Rab5. References for siRNAs used have now been described in the Materials and Methods (lines 656-657).

Comment 5: In figure 4B it appears that the total levels of GFP::RAB-11 in oocytes are increased after sec-6 and sec-8 RNAi. Is this true? That is not a prediction of a failure to tether RAB-11 vesicles as might be expected after loss of exocyst. Please explain.

Response: We thank the reviewer for this comment. As also suggested by reviewer 2, we have now performed whole-worm RT-PCRs for *rab-11* upon control and *sec-6* RNAi, and find that *rab-11* is slightly increased (about 20%) upon sec-6 RNAi, possibly as a compensatory mechanism. However, quantitation of RAB-11-GFP images from oocytes upon sec-6 knockdown reveals an approximately 40-50% increase in the accumulation of rab-11 underneath the membrane (Fig. 4B, E). The data therefore suggests that the increased fluorescence near the membrane upon sec-6 knockdown is a result of both increased expression and mislocalization. We have now included the RT-PCR data (Fig. S4) and discussed this point in the manuscript (lines 194-198).

Comment 6: In Fig 6E GFP::RAB-11 accumulates in the cortex of distal germ cells after sec-6 or par-6 RNAi. It's an interesting correlation that par-6 and sec-6 produce similar effects, but the text describes it as GSC basal membrane localization. A fusion defect might be expected to accumulate recycling vesicles below the PM, but the way it's described makes it sound like RAB-11 is on the PM, which I don't understand.

Response: We thank the reviewer for correcting the terminology. As rightly pointed out, the sub-surface fluorescence is very likely due to the accumulation of rab-11 positive vesicles that have

failed to fuse, a classical phenotype observed upon depletion of the exocyst complex (He and Guo, 2009; Heider and Munson, 2012; Wu and Guo, 2015; Zhu et al., 2017). We have now corrected the text to reflect this accurately (lines 272- 273).

Comment 7: In Fig 8, three exocyst components were co-depleted. Were the siRNAs used for this described anywhere in the paper?

Response: We apologize for inadvertently omitting the description of these siRNAs. We have now included its reference in the Materials and Methods section (lines 656 - 657).

Comment 8: Fig S5B should have control data (PH::GFP as described in text) for comparison.

Response: Fig S5B displays the interactors that were found by both Y2H and IP followed by MS/MS. We have now included the complete list of interactors in our control pull down (PH::GFP) as well as the SEC-6::GFP as supplementary files.

Comment 9: Overstatements or incorrect statements in the text that should be toned down or fixed:

Abstract: "exocyst complex regulates C. elegans GSC proliferation by modulating Notch signaling cell autonomously." Your results are correlative, and consistent with this model, but you did not do any experiments that show that Notch signaling is the sole contributor to the exocyst associated proliferation defect. Please tone this down.

Response: We thank the reviewer for this comment. We agree that while our results are consistent with the model that the exocyst complex regulates GSC proliferation through Notch signaling, we had not earlier provided conclusive evidence for the same. As also pointed out by reviewer 1 to test whether the role of the exocyst complex in GSC proliferation is Notch- dependent, we performed sec-6 RNAi on two different Notch signalling-independent germline tumor backgrounds namely, *gld-1(-); gld-2(-)* and *gld-1(-); nos-3(-)* (Fig 3E). Both of these tumors are non-responsive to either excessive or reduced Notch signaling, because the GSCs in these mutants fail to undergo differentiation and continue to proliferate in an uncontrolled manner to produce germline tumors (Kadyk and Kimble, 1998, Hansen et al., 2004). In case the exocyst functions in another parallel pathway or in a Notch-independent manner to promote GSC proliferation, exocyst subunit depletion would have shown a likely reduction in these two tumors. However, we did not observe any reduction in the tumor in either of the two mutant backgrounds (Fig. 3G). These observations, and our other results in the *sun1::rde1* mutant background and the auxin-responsive degron data (Fig. 2G, J; see comment 1 above) indicate that the exocyst complex functions in GSC proliferation by affecting Notch-signaling, most likely in a germline autonomous manner.

Comment 10: "The anterior polarity protein Par6 is required for GSC Notch trafficking by regulating exocyst recruitment to the niche-signaling interface." This also overstated. Should be toned down to reflect what was actually shown. or pose it as a model derived from the data.

Response: We show that similar to embryos (as shown by Armenti et al., 2014), the aPar complex protein PAR-6 is required for the enrichment of SEC-6 near the niche-facing surface of the GSCs (Fig. 6C, D). In addition, we show that similar to *sec-6* knockdown, *par-6*/*par-3* RNAi reduces the level of Notch receptors on this surface of the GSCs (Fig. 6A, B) and also results in accumulation of rab-11-positive endosomes underneath the membrane (Fig. 6E, F). We also now show that RNAi of the aPars (*par-3, par-6*) significantly reduces the GSC numbers in the PZ similar to exocyst complex RNAi (Fig. S6). All of these data put together suggest a model wherein the aPars regulate GSC proliferation, perhaps by regulating exocyst complex localization near the niche-facing surface and modulating levels of notch receptors on this surface. Therefore, as suggested, we have now modified the abstract to offer this as a plausible model based on our data.

Comment 11: Pg 3 "thus explaining the reduced Notch-Delta signaling." "explaining" is an overstatement. Could say potentially explaining, or consistent with, or something along those lines.

Response: We have now corrected the statement as suggested.

Comment 12: Pg 4 “and that PAR-6 locally recruits SEC-6 to the basal GSC membrane.” Also overstated beyond what was actually shown.

Response: We have now corrected it to convey that depletion of Par-6 results in reduced levels of sec-6 and an accumulation of rab-11-GFP levels (likely on recycling endosomes) underneath the niche-facing outer GSC surface (lines 270-272).

Comment 13: Pg 6 “Exocyst complex function has been demonstrated in both endocytosis and secretion in C. elegans in other tissues (Armenti et al., 2014; Chen et al., 2014b; Jiu et al., 2014; Jiu et al., 2012; Zou et al., 2015), but not in the germline.” See comment 2 above.

Response: We apologize for this oversight and have now referred to this article and discussed our results in the light of it as explained in the response to comment 2 (lines 179, 187-188, 273- 274).

Comment 14: Pg 7 “The rachis-facing GSC cell surface can be considered equivalent to the apical membrane of polarized cells…” This is incorrect. See comment 2 above.

Response: We have now changed the terminology as suggested by the reviewer, and also explained in response to comment 3 above.

Second decision letter

MS ID#: DEVELOP/2020/196345

MS TITLE: The Exocyst Complex Regulates C. elegans Germline Stem Cell Proliferation by Controlling Membrane Notch Levels

AUTHORS: Kumari Pushpa, Sunayana Dagar, HARSH KUMAR, Diksha Pathak, and Sivaram Mylavarapu

I have received all the reviewer comments, and as you will see the overall evaluation is positive and we would like to publish a revised manuscript in Development. However, reviewer 1 highlights some textual edits, including edits to the title, which will greatly improve the clarity of the manuscript. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. I do not expect to send the study back to the reviewers, it is important that you make the suggested edits. Please highlight all the textual changes in the revised manuscript. If you do not agree with any of their criticisms or suggestions explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

As stated in my initial review of this manuscript, Pushpa et al. describe their work on the exocyst complex, its interaction with the Notch signaling pathway, and its role in regulating stem cell proliferation. They demonstrate that RNAi against exocyst complex components SEC-6 and -8 results in reduced progenitor zone sizes, and that RNAi in either somatic or germ cells results in this reduction.
They demonstrate that this reduction of proliferative zone cells is also found in animals containing either partial loss-of-function or gain-of-function alleles of glp-1. They suggest that the effect that the exocyst complex has on Notch signaling and stem cell proliferation is linked to the amount of GLP-1 that is found on basal surface of the germ cells. They provide data demonstrating that when exocyst function is reduced, GLP-1 on the basal surface is reduced.
Furthermore, they demonstrate that the role of the exocyst complex in maintaining Notch on the cell surface is conserved in mammalian cells. The original submission of ‘The Exocyst Complex

Regulates C. elegans Stem Cell Proliferation by Controlling Membrane Notch Levels' received three thorough reviews that had many of the same concerns regarding the manuscript. I believe that the authors have successfully addressed the concerns of the reviewers.

Comments for the author

I have only a few minor comments.

Line 69 'notch/glp-1' should be 'Notch/GLP-1'

Line 98 The authors should provide a rational for doing RNAi on heterozygous animals

Line 176 This heading is too strong as the complex may not be 'required' for all intracellular trafficking. They could say 'required for proper trafficking' or that the 'Exocyst complex is involved in proper intracellular…'

Line 295 Again, this heading is too strong. A suggested change is '…Notch and is required for its proper trafficking…'

Reviewer 3

Advance summary and potential significance to field

I am satisfied with the revised version of the manuscript

Comments for the author

I am satisfied with the revised version of the manuscript

Second revision

Author response to reviewers' comments

Reviewer 1 Advance Summary and Potential Significance to Field:
As stated in my initial review of this manuscript, Pushpa et al. describe their work on the exocyst complex, its interaction with the Notch signaling pathway, and its role in regulating stem cell proliferation. They demonstrate that RNAi against exocyst complex components SEC-6 and -8 results in reduced progenitor zone sizes, and that RNAi in either somatic or germ cells results in this reduction. They demonstrate that this reduction of proliferative zone cells is also found in animals containing either partial loss-of-function or gain-of-function alleles of glp-1. They suggest that the effect that the exocyst complex has on Notch signaling and stem cell proliferation is linked to the amount of GLP-1 that is found on basal surface of the germ cells. They provide data demonstrating that when exocyst function is reduced, GLP-1 on the basal surface is reduced. Furthermore, they demonstrate that the role of the exocyst complex in maintaining Notch on the cell surface is conserved in mammalian cells. The original submission of 'The Exocyst Complex Regulates C. elegans Stem Cell Proliferation by Controlling Membrane Notch Levels' received three thorough reviews that had many of the same concerns regarding the manuscript. I believe that the authors have successfully addressed the concerns of the reviewers.

Reviewer 1 Comments for the Author:

I have only a few minor comments.

Comment: Line 69 'notch/glp-1' should be 'Notch/GLP-1'

Response: We have now corrected this as suggested to Notch/GLP-1.

Comment: Line 98 The authors should provide a rational for doing RNAi on heterozygous animals

Response: We have now provided the rationale for doing RNAi on heterozygous animals. In lieu of a viable genetic mutant, we wanted to examine the phenotype of a near-complete removal of *sec-6* gene activity, and for this we performed *sec-6* RNAi on heterozygous mutants, anticipating a severe reduction in gene activity as animals carried only one wild type copy of the gene.

Comment: Line 176 This heading is too strong as the complex may not be 'required' for all intracellular trafficking. They could say 'required for proper trafficking' or that the 'Exocyst complex is involved in proper intracellular…'
Response: As suggested, we have now corrected this to "….required for proper trafficking".

Comment : Line 295 Again, this heading is too strong. A suggested change is '…Notch and is required for its proper trafficking…'

Response: We have now changed it as per the suggestion of the reviewer.

Reviewer 3 Advance Summary and Potential Significance to Field:
I am satisfied with the revised version of the manuscript

Response: We would like to thank the reviewer for all the comments that helped us to greatly improve the manuscript.

Reviewer 3 Comments for the Author:
I am satisfied with the revised version of the manuscript

Response: We thank the reviewer for their comments that helped us to greatly improve the manuscript.

Third decision letter

MS ID#: DEVELOP/2020/196345

MS TITLE: The Exocyst Complex Regulates C. elegans Germline Stem Cell Proliferation by Controlling Membrane Notch Levels

AUTHORS: Kumari Pushpa, Sunayana Dagar, HARSH KUMAR, Diksha Pathak, and Sivaram Mylavarapu

ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Development, pending our standard ethics checks.