Distinct roles of bendless in regulating FSC niche competition and daughter cell differentiation

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

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

We are aware that you may be experiencing disruption to the normal running of your lab that make experimental revisions challenging. If it would be helpful, we encourage you to contact us to discuss your revision in greater detail. Please send us a point-by-point response indicating where you are able to address concerns raised (either experimentally or by changes to the text) and where you will not be able to do so within the normal timeframe of a revision. We will then provide further guidance. Please also note that we are happy to extend revision timeframes as necessary.

Please attend to all of the reviewers' comments and ensure that you clearly highlight all changes made in the revised manuscript. Please avoid using 'Tracked changes' in Word files as these are lost in PDF conversion. I should be grateful if you would also provide a point-by-point response detailing how you have dealt with the points raised by the reviewers in the 'Response to Reviewers' box. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

This manuscript looks at the role of bendless (ben), an E2 ubiquitin ligase and member of the JNK signaling pathway, in the follicle stem cell lineage of the Drosophila ovary. ben mutant cells were previously shown to have a competitive advantage in the FSC niche, but the cause of this phenotype and the role(s) of ben in the early FSC lineage were not known. Here, the authors show that ben and other JNK pathway genes are essential for follicle cell differentiation and for upregulating Notch signaling, which was previously shown to be required for differentiation in this lineage. Unexpectedly, ben also has JNK-independent role in regulating Hedgehog signaling, proliferation, and niche competition in the early FSC lineage.

The Drosophila ovary is an excellent model for studying the regulation of stem cell maintenance and differentiation into multiple mature cell types. The data presented here are novel and high quality and contribute to our understanding of the complex combinations of signaling pathways that regulate the follicle stem cell lineage.

Comments for the author

The experiments are solid but the manuscript is at times difficult to follow the experiments are not always explained in enough detail to allow the reader to evaluate the conclusions, and it's not always clear what conclusions are to be made from the data, or which data support some of conclusions. The manuscript needs a careful and thoughtful re-writing.

Points to be addressed (more or less in the order in which they appear in the manuscript):
In the sentence "... benA mutant clones had severe follicle formation defects ... (Fig. 1C and 1F)," panel 1C depicts clones but 1F depicts ben RNAi. The text and/or figure references should be re-worked so that they match. Ideally to make it easier for the reader to follow along, figure panels should be arranged in the order in which they are mentioned in the text.

The "tube-like" and "expanded stalk" phenotypes shown in the figures are striking, but was this always the case? Was there a range of phenotype severity, and if so, what criteria were used to score a given germarium as normal or mutant?
For experiments described as "n = # replicates, n = # ovarioles," please clarify if the latter means total ovarioles from all replicates, or number of ovarioles per replicate. Also, please clarify what a different replicate means (flies heat-shocked on a different day?) and when the flies were examined (how many days after heat shock).

In Fig 1, in germaria with follicle cell clones, what is the status of the germ cells? In some panels, the germ cells appear to be GFP-. Are they also clones?
Does the status of the germ cells affect the phenotype of surrounding follicle cells? Also, does the extent of the follicle cell clone influence the phenotype? Is there a difference in phenotype severity in germaria when some vs. all follicle cells are mutant? Mosaic vs fully clonal ovarioles are indicated in later figures but not here.

In panel 1D, there appears to be some diffuse GFP staining within the expanded stalk region. Is this "background" staining or are "normal" GFP+ follicle cells sometimes found in these regions? Indicating the extent of the clones and / or adjusting the gain on the images might help to clarify this point.
In panel 1H, there is an extra parenthesis mark. Also, "CyO" will not make sense to anyone outside of the fly community. Explain or change to something more self-explanatory.
In panel 1K, are "multi-row stalks" the same as "expanded stalks"? If so please use one term for consistency.

In this sentence, "we found that cells in the expanded stalk regions of ovarioles [with?] ben[RNAi] driven by 109-30ts, remained cas+, Eya+ in 70.85% (± 10.8%, N=30) of the cases (Fig. 1J-M)," please indicate how many replicates (or explain how the confidence interval was determined, if there were not multiple replicates) and define "cases" (germaria / ovarioles?).

More importantly, the logic of this section, which shows that cells with loss of ben remain cas+, eya+, is confusing: "This suggests that the follicle formation defects caused by knockdown of ben are due to a failure of pFCs to fully differentiate into stalk cells." If pFCs are cas+, Eya+, and mature follicle cell types express one or the other marker, the fact that ben mutant cells continue to express both markers suggests that they remain in a pFC-like state and fail to fully differentiate into any type of mature follicle cell. It's not clear why differentiation into stalk cells is singled out. The header for this section ("bendless is required for pFC differentiation...") makes more sense.

Please explain or re-word the conclusions within the paragraph.
For the experiments shown in Fig S2, were the drivers combined with tub-Gal80[ts] and driven only in adult flies, or was knockdown happening throughout development? Also, please cite references for where these drivers are expressed in the germarium, or provide images of them driving GFP or another marker, to illustrate the expression patterns. These drivers seem crucial to the conclusion that ben is required not just for pFC differentiation but also for maintaining different follicle cell fates, but that point doesn't come through very clearly here. Minor point: in panel B, there appears to be some nuclear staining in a third (green) channel. Please remove or explain it.

Missing word: "To confirm these RNAi phenotypes, we generated follicle cell clones that are homozygous for hepG0107, which is a recessive [mutation?] (Baril et al., 2009; Guichard et al., 2006)."
As discussed above, the logic here is confusing: "... cells within the expanded stalks of hep[RNAi] mutants were cas+, eya+, indicating that they failed to fully differentiate into a stalk cell identity (Fig. 2D-H, 2J-K and S3)."

Again, please explain why continuing to express both markers indicates a defect specifically in stalk cell formation, rather than in pFC differentiation.
Otherwise, please re-word the conclusions in the paragraph and the header of this section ("JNK signaling is required for stalk specification and formation"). I also have trouble with the title of Fig 2. Aside from what the markers suggest, stalks are indeed forming (albeit abnormal ones), and there appear to be strong phenotypes outside the stalk region. Minor point: please use consistent capitalization (or not) for eya.

In the sentence "Therefore, we next investigated whether JNK signaling impairs JAK-STAT or Notch signaling," do you mean "loss of JNK signaling"?
It's clear from the data presented in Fig. 3 that NRE-GFP is often absent from polar cells upon loss of ben or hep, but the significance of this result is not clear. The stalks next to NRE-GFP- polar cells look normal, at least in this figure. Is this always the case? Do polar cells form normally despite the lack of Notch signaling, or do they form normally and then lose Notch signaling?
Does the loss of Notch signaling affect the polar cell fate in any way? Are there defects in later egg chambers associated with the loss of Notch signaling?
The conclusion that Notch signaling is impaired by loss of JNK is reasonable but what this means for the tissue needs to be clarified.

In Fig. 3 and following, please define "fully labeled." Based on the diagram in Fig. 7, I think this means that the follicle lineage in this ovariole is fully clonal, but a different term ("fully clonal"?) might be clearer. "Fully labeled" in the context of negatively marked clones, which lack the GFP label and "unlabeled clone," referring to an ovariole that doesn't have a clone, are both confusing. In Fig 7, maybe "no clone," "mosaic," and "full clone" would be better? Also, for panels 3A-C, it would be helpful to show the RFP channel alone. In panel A especially, it's difficult to see that the 2A/2B boundary cells are RFP-.
In Fig S4, panel C, the yellow and white arrows appear to be reversed or mis-aligned with the image.

In Fig. 4, it would be helpful to add arrows or some other mark to point out where region 2b begins / where pERK staining should be off. The extent of pERK staining in Panel B (egr RNAi), especially, does not look that different from the control.
"... although loss of JNK signaling causes retention of pERK in pFCs, it does not fully recapitulate the effects of constitutive EGFR signaling": What are the effects of constitutive EGFR signaling? Please explain (and cite a reference).

It's clear that cell polarity does not seem to be affected, but then what is the significance of pERK retention? Can any of the phenotypes that result from loss of ben or JNK components be explained by an increase in EGFR signaling?

The experiments showing that loss of ben increases proliferation and niche competition are clear, convincing, and nicely presented, and the suggestion that ben regulates these events through Hh in a JNK-independent manner is entirely reasonable. It would be nice, but not essential, to show that a reduction in Hh signaling could suppress the increase in proliferation and niche competition.
Which data support the conclusion (in the abstract) that the "JNK-independent role of ben is important for proper specification of the main body follicle cell lineage"? Please make this clear in the text.
Please add antibody sources to the Methods section.

Reviewer 2

Advance summary and potential significance to field

How stem cells control their differentiation is critical to understanding tissue regeneration and homeostasis.
Here, the authors have used an exceptionally well-studied model system of follicle stem cells in Drosophila to understand the role of a E2 ubiquitin-conjugating enzyme and JNK pathway member, Bendless (Ben), in stem cell differentiation. Using FSCs, they have found that:

1. Ben and the JNK pathway are required for proper differentiation by promoting specification and maintenance of the polar and stalk cell fates.
2. Ben and JNK pathway are required for upregulation of Notch signaling and to limit EGFR signaling; with Notch playing an important role in polar cells.
3. Ben but not JNK pathway is required to limit hh signaling, which contributes to hypercompetion phenotype.

This study uncovers a role of JNK signaling in FSC differentiation and a role JNK independent role for Ben in limiting hh signaling.

Comments for the author

Major comments:
1. It is not clear to me how the overexpression of hh signaling upon depletion of ben contributes to the phenotype observed in ben mutants. It seems to me that the ben phenotype is completely be recapitulated by depletion of other JNK pathway members. If this is separate from the observed phenotype then this could be clearly stated in the text in the results section. For this reviewer, it was confusing to read.

2. The authors argue that the hyper-competition phenotype that they observe in ben mutants is due to hh over expression. They suppress the phenotype by slowing the cell cycle using dap. Can they do this under conditions where hh signaling is also limiting? This would be a direct test of what they are proposing.

Minor comments:
Including line numbers in the text will make feedback easier—I have cut and pasted the relevant sentences.
1. Adding a cartoon of JNK pathway in supplementary figure 2 will help readers.
2. Change wording of “we expressed hep, egr, grnd, or bsk RNAi” to “we depleted … using RNAi” for clarity?
3. Can the authors separate two paragraphs about EGFR and Hh using reporters that is under one subheading into two paragraphs with different headings? This is an important point.
4. In Figure 8, can the authors include what those reporters on the y axis are for? It is mentioned in the legend, but it would be easier for readers if it said (a reporter for Notch signaling) in the Y axis.
Congrats to the all the authors on finishing this manuscript under these difficult circumstances.

Reviewer 3

Advance summary and potential significance to field

Distinct roles of bendless in regulating niche competition and daughter cell differentiation by Tatapudy Peralta and Nystul

Follicle stem cells (FSC) and the differentiation of their progeny into different cell types in Drosophila ovaries serve as a model for investigating the mechanisms of stem-cell based production of various cell types in an organ. Multiple signaling pathways have been found to regulate number and behavior of FSCs and their differentiation. Here, the authors identify for the first time the JNK pathway as an important regulator of these processes. They had previously identified a mutation affecting Bendless (Ben), an E2 ubiquitin ligase and established component of the JNK pathway, as having an effect on FSC behavior. They show here that the JNK pathway as such plays an important role in the differentiation of FSC progeny. In addition to this canonical function of Ben, the authors provide some evidence that Ben might also have an JNK independent function in the regulation of FSCs.

The research data overall appear solid, figures are of good quality, and most experimental data have been quantified. The title of the manuscript is appropriate, and the interpretation in the result section and discussion is largely supported by the provided data.

The importance of this work lies in the identification of the JNK pathway as an additional signaling pathway that contributes to the differentiation of FSC progeny. This is a novel finding. Exactly what the function of Ben is remains unknown, however. The two main and largely contradictory effects on the interfollicular stalks remain unexplained.

Comments for the author

Main points:
1. The authors find that the ben mutation, but not the reduction of other JNK components causes increased Hh signaling and ‘super-competitiveness’ of FSCs. Increased Hh signaling is known to cause over-proliferation of FSC progeny and formation of extended stalks, which fits ben mutant phenotype.
However, it is puzzling then why JNK mutants would show the same phenotype? If Ben has functionally important JNK-dependent and independent functions, should one not expect differences in the phenotypes or in phenotypic strength? To test whether the effect on Hh signaling is functionally important one could try to reduce Hh signaling and see whether this provides a rescuing effect of the ben phenotype.

2. What causes the apparent opposite effects on the stalks by the same genotype, namely fused follicles without stalks (‘tube-like’) versus expanded multi-layered stalks (‘expanded stalks’)?

3. In general, how the effects on various factors/signaling pathways are related to the observed mutant phenotypes remains largely unexplored.

Additional points:

- The abstract does not fit the manuscript and needs to be thoroughly revised. The abstract speaks of ‘specification’ of cell types, which is not clearly supported by the data and runs contraire to the repeated message in the remainder of the manuscript that talks of ‘differentiation’ defects. Also, the information about Hh signaling is contradictory.

- What type of allele is benA? Provide a description. Also, describe the hep mutation. Verification of RNAi tools is missing, although they seem to be supported by the phenotypes.
- Polar cells are mentioned in the abstract, but I could not find an analysis of the polar cells in the results.
What happens to polar cells in ben and hep mutants?
- Fig 1D’: It is puzzling that the multi-layered stalk seems to consist mainly of wild-type cells. Is this a cell-

non-autonomous effect?
- Fig 2 legend title: change to ….required for normal stalk formation
- Fig. 7A schematic drawing: It is confusing that the GFP is indicated in a ‘salt and pepper’ pattern. Follicle cell clones form normally continuous patches.
- Suppl Fig 1 D. In contrast to ben RNA which is clearly seen in follicle cells and germline, hep RNA seems to be largely confined to the germline. Are the authors convinced that there is signal in follicle cells?
- The scientific language, especially in the figure legends needs to be improved. For example, in several figure legends, it says “GFP+ cells are non-clonal, whereas GFP- cells are clonal”. This description makes little sense as all cells, whether GFP+ or - are part of a cell clones. Change to ‘mutant cell clones’ and ‘control cells’. Also, it is not self-explanatory what is meant by ‘fully labeled’ versus ‘mosaic’. This needs to be explained. Also, the label ‘GFP (clonal marker) is confusing in case of negative labeling of mutant cells.
- The whole manuscript needs careful editing, especially spelling and description of proteins and genotypes.
- Description of antibodies is missing from M&M.
- Statistics: replicas and sample size: does n represent the overall sample size from all replicas together or does it represent the sample size per replica. Do the means represent the mean of the replicas or samples? Please, clarify. Do graphs show SD or SEM?

First revision

Author response to reviewers' comments

Response to Reviewer Comments

We thank all three reviewers for their thorough review of our work and their excellent suggestions. We have addressed all the reviewer comments with revisions to the text, revisions to the figures, and new data. These changes have significantly strengthened the manuscript. Below, we provide a summary of our response to each of the reviewer comments.

Reviewer 1

This manuscript looks at the role of bendless (ben), an E2 ubiquitin ligase and member of the JNK signaling pathway, in the follicle stem cell lineage of the Drosophila ovary. ben mutant cells were previously shown to have a competitive advantage in the FSC niche, but the cause of this phenotype and the role(s) of ben in the early FSC lineage were not known. Here, the authors show that ben and other JNK pathway genes are essential for follicle cell differentiation and for upregulating Notch signaling, which was previously shown to be required for differentiation in this lineage. Unexpectedly, ben also has JNK-independent role in regulating Hedgehog signaling, proliferation, and niche competition in the early FSC lineage. The Drosophila ovary is an excellent model for studying the regulation of stem cell maintenance and differentiation into multiple mature cell types. The data presented here are novel and high quality and contribute to our understanding of the complex combinations of signaling pathways that regulate the follicle stem cell lineage.

We thank the reviewer for this summary and the positive assessment of our work.

The experiments are solid but the manuscript is at times difficult to follow, the experiments are not always explained in enough detail to allow the reader to evaluate the conclusions, and it's not always clear what conclusions are to be made from the data, or which data support some of conclusions. The manuscript needs a careful and thoughtful re-writing.

We have now thoroughly edited the manuscript for clarity and to emphasize the conclusions that we are making from the data in each figure.

In the sentence "... benA mutant clones had severe follicle formation defects ... (Fig. 1C and 1F),"

panel 1C depicts clones but 1F depicts ben RNAi. The text and/or figure references should be re-worked so that they match. Ideally, to make it easier for the reader to follow along, figure panels should be arranged in the order in which they are mentioned in the text.

We have corrected errors throughout so that the figure references in the text match the figures. We have also rearranged the figures so that, whenever possible, the panels are in the order that they are referred to in the text.

The "tube-like" and "expanded stalk" phenotypes shown in the figures are striking, but was this always the case? Was there a range of phenotype severity, and if so, what criteria were used to score a given germarium as normal or mutant?

The tube-like and expanded stalk phenotypes were highly penetrant. We provide quantifications of these phenotypes in Fig. 1K, 1L, 2F, 2K, and 5F. Indeed, as the reviewer suggests, we observed a range of tube-like and expanded stalk phenotypes. We now provide additional examples of these phenotypes in ovarioles with *ben*^{A} clones in Fig. S2A-D. We also describe this range of phenotypes as follows (page 3, line 30- page 4, line 2):

> We found that ovarioles in which the follicle cell population in the germarium was mosaic or fully marked with *ben*^{A} mutant clones had a range of follicle formation defects, including gaps in the follicle epithelium, “tube-like” phenotypes characterized by defective or absent stalks between the germarium and the downstream cysts, and “expanded stalk” phenotypes characterized by the presence of extra cells in the stalk region that form multiple rows (Fig. 1B-D, K and Fig. S2A-D).

For experiments described as "n = # replicates, n = # ovarioles," please clarify if the latter means total ovarioles from all replicates, or number of ovarioles per replicate. Also, please clarify what a different replicate means (flies heat-shocked on a different day?) and when the flies were examined (how many days after heat shock)

N indicates the total number of flies examined, and n indicates the total number of ovarioles examined. We now state this in the figure legends. For experiments with flies that have *tub-Gal80*^{ts}, the crosses were performed at 18°C and adults were shifted to 29°C for 14 days, unless specified otherwise. For experiments with clones, flies were dissected at 12 days post clone induction, unless specified otherwise. We now describe this in the Methods section (page 11, lines 3-6, page 14, lines 18-19)

In Fig 1, in germaria with follicle cell clones, what is the status of the germ cells? In some panels, the germ cells appear to be GFP-. Are they also clones? Does the status of the germ cells affect the phenotype of surrounding follicle cells?

With this clonal marking system, the GFP signal is much weaker in germ cells than in follicle cells so, when images are acquired and processed to facilitate the distinction between GFP^{-} and GFP^{+} follicle cells, germ cells can appear GFP^{-}. In response to these questions, we quantified the follicle formation phenotypes in ovarioles with and without GSC clones and found no significant differences (Fig. S2E). We now describe these results on page 4, lines 2-6 as follows:

> We also noticed a small but significant increase in the frequency of ovarioles with follicle formation defects in *ben*^{A}/+ heterozygous flies compared to wildtype controls (Fig. S2E), but the frequencies of follicle formation phenotypes were not significantly different between *ben*^{A}/+ ovarioles with or without *ben*^{A} homozygous germ cell clones (Fig. S2E). This suggests that *ben* is not required in germ cells for follicle formation.
> *Also, does the extent of the follicle cell clone influence the phenotype? Is there a difference in phenotype severity in germaria when some vs. all follicle cells are mutant? Mosaic vs fully clonal ovarioles are indicated in later figures but not here.*

All assays for follicle formation defects were performed at 12 dphs, when the large majority of

clones had progressed well beyond the germarium, so we cannot assess whether the extent of the follicle clone influences the phenotype. However, we strongly suspect that the clone would need to progress beyond the boundary of the germarium to produce a follicle formation defect. We observed morphological phenotypes in ovarioles that were either mosaic or fully marked with *ben*^{A} clones. In both cases, there were a similar range of phenotypes, so we considered the two categories together for our quantifications. We now state this in the figure legend for Figures 1 and 2 (page 20, lines 28-30 and page 21, lines 16-18).

In panel 1D, there appears to be some diffuse GFP staining within the expanded stalk region. Is this "background" staining or are "normal" GFP+ follicle cells sometimes found in these regions? Indicating the extent of the clones and / or adjusting the gain on the images might help to clarify this point.

Yes, there are normal GFP^{+} cells in the stalk region of this ovariole. We now provide the following description in the figure legend (page 20, lines 21-22):

> In all cases, the clones extend well beyond the germarium, and the ovarioles are mosaic or fully marked by the lack of GFP.

In panel 1H, there is an extra parenthesis mark.

We thank the reviewer for catching this typo, and have now corrected it.

Also, "CyO" will not make sense to anyone outside of the fly community. Explain or change to something more self-explanatory.

We think it is important to emphasize the genotype here, so we have modified the figure legend to indicate that CyO is a balancer and that this line is a control, as follows (page 20, lines 22-23):

> (E-G) Ovarioles with *ben[RNAi]* over a CyO balancer (Control) (E) or with *109-30*^{ts} driving expression of *ben[RNAi]* (F-G) stained with Fas3 (magenta) and DAPI (blue).

In panel 1K, are "multi-row stalks" the same as "expanded stalks"? If so, please use one term for consistency.

Yes, these terms do refer to the same phenotype. We thank the reviewer for catching this inconsistency. The different terms reflect the evolution of our own terminology for these phenotypes, and we should have been more careful to describe them consistently in the paper. We now refer to this phenotype as “expanded stalk” throughout the paper.

In this sentence, "we found that cells in the expanded stalk regions of ovarioles [with?] ben[RNAi] driven by 109-30ts, remained cas+, Eya+ in 70.85% (± 10.8%, N=30) of the cases (Fig. 1J-M)," please indicate how many replicates (or explain how the confidence interval was determined, if there were not multiple replicates) and define "cases" (germaria / ovarioles?).

We examined 3 flies, and scored a total of 79 ovarioles. We calculated the penetrance of phenotypes within each fly, and performed a t-test to determine statistical significance. In the legend, we now report a total number of n’s and clarify that the replicates refer to the number of flies analyzed. Additionally, we also provide a supplemental file with all the compiled raw data.

More importantly, the logic of this section, which shows that cells with loss of ben remain cas+, eya+, is confusing: "This suggests that the follicle formation defects caused by knockdown of ben are due to a failure of pFCs to fully differentiate into stalk cells." If pFCs are cas+, Eya+, and mature follicle cell types express one or the other marker, the fact that ben mutant cells continue to express both markers suggests that they remain in a pFC-like state and fail to fully differentiate into any type of mature follicle cell. It's not clear why differentiation into stalk cells is singled out. The header for this section ("bendless is required for pFC differentiation...") makes more sense. Please explain or re-word the conclusions within the paragraph.

We thank the reviewer for this comment. Our original basis for the assertion that knockdown of ben (and hep) impairs stalk cell differentiation was that *cas*$^{+}$, *eya*$^{+}$ cells could be found in the stalk region and that stalks formed improperly in these mutant ovarioles. The reviewer's question prompted us to take a closer look at the expression of *cas* and *eya* in different cell types, and we noticed that main body follicle cells in *ben* or *hep* mutant ovarioles had the normal *cas*$^{-}$, *eya*$^{+}$ expression pattern, suggesting proper differentiation with respect to these two markers. Moreover, we noticed that mature polar cells, identified as clusters of cells with strong Fas3 staining, were frequently present in *ben* and *hep* mutant clones. These observations reinforced our conclusion that the follicle formation phenotypes we observed are due to impaired stalk cell differentiation. We describe these results as follows (page 4, lines 15-21):

> We found that a majority (70.9% ± 10.9%, N=30) of ovarioles with *ben[RNAi]* driven by *109-30*^{ts} contained *cas*$^{+}$, *eya*$^{+}$ cells in the regions between follicles, where stalk cells typically reside (Fig. 1I- J, M). However, the main body follicle cells surrounding recently budded follicles in these mutant ovarioles were *cas*$^{-}$, *eya*$^{+}$ (Fig. 1I-J), indicating that, unlike the cells in the stalk region, they were able to exit the *cas*$^{+}$, *eya*$^{+}$ state associated with pFC identity. Taken together, these results suggest that the follicle formation defects caused by knockdown of *ben* are due, at least in part, to a failure of mutant pFCs to differentiate into stalk cells.

We then describe the *cas* and *eya* expression patterns in ovarioles with reduced *hep* or overexpression of *ERK*^{SEM} at subsequent points in the paper, and discuss these results in the third paragraph of the discussion (page 9, lines 20-27).

For the experiments shown in Fig S2, were the drivers combined with tub-Gal80[ts] and driven only in adult flies, or was knockdown happening throughout development? Also, please cite references for where these drivers are expressed in the germarium, or provide images of them driving GFP or another marker, to illustrate the expression patterns. These drivers seem crucial to the conclusion that ben is required not just for pFC differentiation but also for maintaining different follicle cell fates, but that point doesn't come through very clearly here. Minor point: in panel B, there appears to be some nuclear staining in a third (green) channel.
Please remove or explain it.

Yes, in the experiments with *tub-Gal80*^{ts}, the transgenes were only driven in adult flies. In the Methods section (page 11, lines 3-5), we now state:

> For experiments with flies that have *tub-Gal80*^{ts}, the crosses were performed at 18°C and adults were shifted to 29°C for 14 days, unless specified otherwise.

The expression patterns of upd-Gal4, NRE-Gal4, and CG46339-Gal4 have all been reported elsewhere. However, in the revised manuscript, we have removed the descriptions of the phenotypes caused by driving *ben[RNAi]* in mature stalk or polar cells (previously Fig. S2) because the penetrance of these phenotypes is low. Thus, references to other papers showing where these drivers are expressed and modification of panel B are no longer needed.

Missing word: "To confirm these RNAi phenotypes, we generated follicle cell clones that are homozygous for hepG0107, which is a recessive [mutation?] (Baril et al., 2009; Guichard et al., 2006)."

We thank the reviewer for catching this typo and have made the correction. It now reads "...which is a recessive lethal mutation" (page 5, line 3).

As discussed above, the logic here is confusing: "... cells within the expanded stalks of hep[RNAi] mutants were cas+, eya+, indicating that they failed to fully differentiate into a stalk cell identity (Fig. 2D-H, 2J-K and S3)." Again, please explain why continuing to express both markers indicates a defect specifically in stalk cell formation, rather than in pFC differentiation. Otherwise, please

re-word the conclusions in the paragraph and the header of this section ("JNK signaling is required for stalk specification and formation").

As discussed above, our reanalysis of the expression patterns of *cas* and *eya* provide new support for the conclusion that the follicle formation phenotypes we observed are due to impaired stalk cell differentiation. We now describe this phenotype on page 5, lines 4-7 as follows:

> Moreover, as with knockdown of *ben*, we observed cells within the expanded stalks of *hep[RNAi]* mutants that were still cas^{+}, eya^{+}, whereas the cells in the main body cell region had matured into the cas^{-}, eya^{+} state (Fig. 2I-J, L). This suggests that RNAi knockdown of *hep* impairs differentiation of pFCs into stalk cells.

As requested we have reworded the header of this section. It is now "JNK signaling is required for pFC differentiation"

I also have trouble with the title of Fig 2. Aside from what the markers suggest, stalks are indeed forming (albeit abnormal ones), and there appear to be strong phenotypes outside the stalk region.

In response to this comment, we have changed the title of Fig. 2 to "JNK signaling is required for pFC differentiation and follicle formation."

Minor point: please use consistent capitalization (or not) for eya.
We now use a lowercase e for eya throughout the paper, consistent with the way it is listed on FlyBase

In the sentence "Therefore, we next investigated whether JNK signaling impairs JAK-STAT or Notch signaling," do you mean "loss of JNK signaling"?

Yes, thank you for catching this typo. We have corrected this.

It's clear from the data presented in Fig. 3 that NRE-GFP is often absent from polar cells upon loss of ben or hep, but the significance of this result is not clear. The stalks next to NRE-GFP- polar cells look normal, at least in this figure. Is this always the case? Do polar cells form normally despite the lack of Notch signaling, or do they form normally and then lose Notch signaling? Does the loss of Notch signaling affect the polar cell fate in any way? Are there defects in later egg chambers associated with the loss of Notch signaling? The conclusion that Notch signaling is impaired by loss of JNK is reasonable, but what this means for the tissue needs to be clarified.

This is a very interesting question and prompted us to go back to search for phenotypes that might indicate a functional impairment of Notch signaling. While we occasionally found a follicle in *ben* mutant ovarioles that did not have a detectable anterior polar cell cluster, it was much more common to find polar cell clusters in *ben* and *hep* mutant clones. Thus, we have softened our conclusions in this section and now describe these observations on page 5, lines 27-32 as follows:

> In addition, we found that the *NRE-GFP* signal was also frequently absent from mature polar cells in ben^{A} or hep^{G0107} mutant clones compared to controls, which can be identified as small clusters of cells with high levels of Fas3 staining, in these ovarioles (Fig. 3D-F, 3H). However, since Notch signaling in Region 2b is required for the differentiation of pFCs into polar cells and we frequently observed polar cell clusters in ben^{A} and hep^{G0107} clones (Fig. 3D-F), the functional consequence of the loss of *NRE-GFP* signal in pFCs is unclear.

As shown in Fig. 1K and 2K, some ovarioles with *ben* and *hep* mutant clones have a normal stalk morphology. We observed *NRE-GFP*$^{-}$ polar cell clusters in both ovarioles with and without morphological phenotypes in the stalk cell region, but in ovarioles with morphological defects, it is difficult to determine the stage of the follicle and whether the polar cell cluster is an anterior or posterior cluster. Therefore, we chose to show ovarioles with normal stalk morphology in this figure to emphasize the polar cell phenotypes.

In Fig. 3 and following, please define "fully labeled." Based on the diagram in Fig. 7, I think this means that the follicle lineage in this ovariole is fully clonal, but a different term ("fully clonal"?) might be clearer. "Fully labeled" in the context of negatively marked clones, which lack the GFP label, and "unlabeled clone," referring to an ovariole that doesn't have a clone, are both confusing. In Fig 7, maybe "no clone," "mosaic," and "full clone" would be better?

Yes, our intention was to indicate that the follicle lineage in the ovariole is fully clonal. In response to this comment, we now describe the clonal marking system more thoroughly and use the terms "unmarked," "mosaic," and "fully marked" throughout the text. We also indicate that we are referring to the status of the follicle cell population. For example, in the figure legend for Fig. 3, we state (page 21, lines 23-24):

> (A-F) Ovarioles in which the follicle cell populations in the germarium and first two follicles are fully marked by the lack of RFP stained for GFP (green), RFP clonal label (magenta) and Fas3 (white).

Also, for panels 3A-C, it would be helpful to show the RFP channel alone. In panel A especially, it's difficult to see that the 2A/2B boundary cells are RFP-.

We now provide the RFP channel alone for panels in Fig. 3A-C as panels A'-C'. Note, we have also chosen a different image for panel A that more clearly shows the RFP^- cells at the Region 2a/2b boundary.

In Fig S4, panel C, the yellow and white arrows appear to be reversed or mis-aligned with the image.

We have moved the yellow arrow over to the right to better align it with the RFP^+ cells.

In Fig. 4, it would be helpful to add arrows or some other mark to point out where region 2b begins / where pERK staining should be off. The extent of pERK staining in Panel B (egr RNAi), especially, does not look that different from the control.

In Fig. 4, we indicate the location of the pFCs that should be $pERK^-$ with white dashed lines. In response to this comment, we have more carefully positioned these lines to highlight the regions where the pERK staining should be off and made the lines thicker, so they are easier to see.

"... although loss of JNK signaling causes retention of pERK in pFCs, it does not fully recapitulate the effects of constitutive EGFR signaling": What are the effects of constitutive EGFR signaling? Please explain (and cite a reference).

We were referring to the impaired maturation of apical cell polarity that is caused by constitutive activation of EGFR signaling in the FSC lineage. We now state this more clearly and provide the reference, as requested (page 6, lines 12-17):

> Constitutive activation of EGFR signaling interferes with the maturation of apical cell polarity in the early FSC lineage (Castanieto et al., 2014). However, we observed no defects in the localization of apical or lateral markers in ben^A pFCs (Fig. S6), indicating that, although loss of JNK signaling causes retention of pERK in pFCs, it does not phenocopy the impaired maturation of cell polarity that has been observed with constitutive EGFR signaling (Castanieto et al., 2014).

It's clear that cell polarity does not seem to be affected, but then what is the significance of pERK retention? Can any of the phenotypes that result from loss of ben or JNK components be explained by an increase in EGFR signaling?

This is a very good question, and we provide new data in the revised manuscript to address this point. Specifically, we found that overexpression of a constitutively active allele of *ERK* (ERK^{SEM})

in follicle cells with *109-30^{ts}* phenocopied the follicle formation phenotypes that we observed in *ben* and *hep* mutants. We also noticed that overexpression of *ERK^{SEM}* had the same effect on the pattern of *cas* and *eya* in stalk cells and main body follicle cells as we observed in *ben* and *hep* mutants. These observations suggest that the activation of *ERK* contributes to the pFC differentiation defects and follicle formation defects we observed in JNK pathway mutants. The new data are in Fig. 5 and Fig. S7 and described in the second paragraph of the section titled "Impaired JNK signaling causes retention of phosphorylated ERK in pFCs, which blocks pFC differentiation" (page 6, lines 18-26).

The experiments showing that loss of ben increases proliferation and niche competition are clear, convincing, and nicely presented, and the suggestion that ben regulates these events through Hh in a JNK-independent manner is entirely reasonable. It would be nice, but not essential, to show that a reduction in Hh signaling could suppress the increase in proliferation and niche competition.

In response to this comment and similar comments by the other two reviewers, we tested whether RNAi knockdown of the Hh pathway effector, *smoothened*, could suppress the hypercompetition phenotype in *ben^{A}* clones. Indeed, we found that it does. The new data are in Fig 8J, and are discussed in the last paragraph of the Results section, on page 8, lines 22-33.
Which data support the conclusion (in the abstract) that the "JNK-independent role of ben is important for proper specification of the main body follicle cell lineage"? Please make this clear in the text.

This conclusion is based on our observation that *zfh1* is aberrantly expressed in the main body follicle cells of *ben* mutant ovarioles but not in *hep* mutant ovarioles. However, since our prior submission, we have noticed that *ben* mutant main body follicle cells do acquire the proper pattern of *cas* and *eya* expression. Thus, we have removed this conclusion from the abstract and softened our interpretation of these data in the main text. In the Discussion, we now state (page 9, lines 20-27):

> Likewise, we found that RNAi knockdown of *ben* caused the main body follicle cells to retain *zfh- 1* expression, a downstream target of Hh signaling, throughout the germarium and even into Stage 2 in some cases, where it is completely absent in wildtype tissue. However, we also found that *ben* mutant main body follicle cells were able to mature into a *cas^{+}*, *eya^{-}* state, indicating proper differentiation with respect to these two markers. Thus, while loss of *ben* does not fully impair main body follicle cell differentiation, it may influence the process through its role as an upstream regulator of *zfh-1* expression. Further study will be required to understand the functional significance of decreased *cas* and *zfh-1* expression during main body follicle cell differentiation.

Please add antibody sources to the Methods section.

We thank the reviewer for noticing this omission. We have now added this to the Methods section (page 11, line 28-page 12, line 4)

Reviewer 2

How stem cells control their differentiation is critical to understanding tissue regeneration and homeostasis. Here, the authors have used an exceptionally well-studied model system of follicle stem cells in Drosophila to understand the role of a E2 ubiquitin-conjugating enzyme and JNK pathway member, Bendless (Ben), in stem cell differentiation. Using FSCs, they have found that: 1. Ben and the JNK pathway are required for proper differentiation by promoting specification and maintenance of the polar and stalk cell fates. 2. Ben and JNK pathway are required for upregulation of Notch signaling and to limit EGFR signaling; with Notch playing an important role in polar cells. 3. Ben but not JNK pathway is required to limit hh signaling, which contributes to hypercompetion phenotype. This study uncovers a role of JNK signaling in FSC differentiation and a role JNK independent role for Ben in limiting hh signaling.

We thank the reviewer for this summary of our manuscript, which highlights the main insights

provided by our study.

It is not clear to me how the overexpression of hh signaling upon depletion of ben contributes to the phenotype observed in ben mutants. It seems to me that the ben phenotype is completely be recapitulated by depletion of other JNK pathway members. If this is separate from the observed phenotype then this could be clearly stated in the text in the results section. For this reviewer, it was confusing to read.

We apologize for the confusion. The main differences between *ben* mutants and other JNK pathway mutants that we described in the original submission are that *ben* mutants but not other JNK pathway mutants caused
(1) overactivation of Hh signaling and expansion of *zfh-1* expression in the germarium and Stage 2 follicles; (2) increased proliferation; and (3) FSC niche competition. We now summarize these differences in the Discussion (page 10, lines 19-27) as follows:

> Taken together, our findings indicate that ben has JNK-dependent and independent roles in regulating pFC cell fate decisions as they differentiate into polar, stalk and main body follicle cells. Specifically, our findings support a model (Fig. 9) in which JNK signaling promotes the differentiation of pFCs during normal homeostasis by helping to downregulate EGFR signaling in early pFCs.…Separately, *ben* inhibits Hh signaling in a JNK-independent manner, thereby controlling pFC proliferation, regulating FSC niche competition, and possibly contributing to main body follicle cell differentiation.

The authors argue that the hyper-competition phenotype that they observe in ben mutants is due to hh over expression. They suppress the phenotype by slowing the cell cycle using dap. Can they do this under conditions where hh signaling is also limiting? This would be a direct test of what they are proposing.

Yes, we thank the reviewer for this suggestion. In response to this comment and similar comments from the other two reviewers, we have conducted this experiment and found that RNAi knockdown of *smo* suppresses the hypercompetition phenotype of ben^{A} mutants, as described in our response to Reviewer 1 above.

Including line numbers in the text will make feedback easier

We apologize for this oversight. We have added line numbers to the revised draft.

Adding a cartoon of JNK pathway in supplementary figure 2 will help readers.

We now provide this in Fig. S3A

Change wording of "we expressed hep, egr, grnd, or bsk RNAi" to "we depleted … using RNAi" for clarity?

We have made this change to the sentence on page 4, line 35.

Can the authors separate two paragraphs about EGFR and Hh using reporters that is under one subheading into two paragraphs with different headings? This is an important point.

We thank the reviewer for this suggestion and now put these two paragraphs under different subheadings.

In Figure 8, can the authors include what those reporters on the y axis are for? It is mentioned in the legend, but it would be easier for readers if it said (a reporter for Notch signaling) in the Y axis.

Yes, we have made this change, as requested (Fig. 9 in the revised draft).

Congrats to the all the authors on finishing this manuscript under these difficult circumstances.

Thank you! This is a very kind sentiment to include in a manuscript review and it is much appreciated!

Reviewer 3

Distinct roles of bendless in regulating niche competition and daughter cell differentiation by Tatapudy, Peralta and Nystul. Follicle stem cells (FSC) and the differentiation of their progeny into different cell types in Drosophila ovaries serve as a model for investigating the mechanisms of stem-cell based production of various cell types in an organ. Multiple signaling pathways have been found to regulate number and behavior of FSCs and their differentiation. Here, the authors identify for the first time the JNK pathway as an important regulator of these processes. They had previously identified a mutation affecting Bendless (Ben), an E2 ubiquitin ligase and established component of the JNK pathway, as having an effect on FSC behavior. They show here that the JNK pathway as such plays an important role in the differentiation of FSC progeny. In addition to this canonical function of Ben, the authors provide some evidence that Ben might also have an JNK independent function in the regulation of FSCs. The research data overall appear solid, figures are of good quality, and most experimental data have been quantified. The title of the manuscript is appropriate, and the interpretation in the result section and discussion is largely supported by the provided data. The importance of this work lies in the identification of the JNK pathway as an additional signaling pathway that contributes to the differentiation of FSC progeny. This is a novel finding. Exactly what the function of Ben is remains unknown, however. The two main and largely contradictory effects on the interfollicular stalks remain unexplained.

We thank the reviewer for this thorough and complementary summary of our manuscript. We appreciate the Reviewer's recognition of the importance and novelty of our finding that the JNK pathway is an additional signaling pathway that contributes to the differentiation of FSC progeny, and agree that further study will be needed to fully understand the functions of *ben* in the FSC lineage and its role in stalk formation.

The authors find that the ben mutation, but not the reduction of other JNK components causes increased Hh signaling and 'super-competitiveness' of FSCs. Increased Hh signaling is known to cause over-proliferation of FSC progeny and formation of extended stalks, which fits ben mutant phenotype. However, it is puzzling then why JNK mutants would show the same phenotype? If Ben has functionally important JNK-dependent and independent functions, should one not expect differences in the phenotypes or in phenotypic strength? To test whether the effect on Hh signaling is functionally important, one could try to reduce Hh signaling and see whether this provides a rescuing effect of the ben phenotype.

It was, indeed, challenging to parse out which phenotypes in *ben* mutant ovarioles are also present in JNK mutants. As described above in response to the other two reviewers' comments, our data indicate that the JNK independent functions of ben are to regulate (1) the patterning of Hh signaling in the early FSC lineage;
(2) pFC proliferation; and (3) FSC niche competition. Since Hh signaling has also been shown to regulate proliferation and niche competition in the FSC lineage, it is likely that the ben regulates proliferation and niche competition via its effects on Hh signaling. The JNK-dependent roles of ben are to regulate (1) the patterning of pERK; and (2) pFC differentiation toward the stalk cell fate.

What causes the apparent opposite effects on the stalks by the same genotype, namely fused follicles without stalks ('tube-like') versus expanded multi-layered stalks ('expanded stalks')?

In response to this and a similar comment from Reviewer 1, we reanalyzed the *ben* mutant ovarioles and identified a range of phenotypes (Fig. S2A-D). We also provide additional evidence that impaired JNK signaling interferes with pFC differentiation into stalk cells, as described above in our response to Reviewer 1. These observations suggest that the seemingly contradictory effects on interfollicular stalks we observed in *ben* and *hep* mutant ovarioles can both be caused by a failure of pFCs to differentiate into mature stalk cells. We now discuss this possibility in the Discussion section as follows (page 9, lines 6-9):

> The range of tube-like and expanded stalk morphological phenotypes we observed could both be explained by an inability of cells in the stalk regions between follicles to facilitate the pinching off of follicles from the germarium or to intercalate into a single row after the pinching off process has been initiated.

In general, how the effects on various factors/signaling pathways are related to the observed mutant phenotypes remains largely unexplored.

In response to this comment and related comments by the other two reviewers, we now provide new data that explore how the effects on ERK and Hh signaling are related to the observed mutant phenotypes. As described above in response to Reviewer 1, we found that overexpression of a constitutively active allele of *ERK* phenocopied the follicle formation phenotypes and *cas* and *eya* expression patterns that we observed in *ben* and *hep* mutants. This suggests that the activation of ERK in JNK pathway mutants contributes to the pFC differentiation defects we observed. In addition, we now show that RNAi knockdown of *smo* suppresses the hypercompetition phenotype of ben^{A} mutant clones, which provides further support for our model that increased Hh signaling in *ben* mutant cells causes the hypercompetition phenotype.

The abstract does not fit the manuscript and needs to be thoroughly revised. The abstract speaks of 'specification' of cell types, which is not clearly supported by the data and runs contraire to the repeated message in the remainder of the manuscript that talks of 'differentiation' defects. Also, the information about Hh signaling is contradictory.

We use the word "specification" in conjunction with the phrase "differentiation defect" because the process of cell fate specification in this lineage involves the differentiation of pFCs into mature cell types. For example, a mutation could cause a differentiation defect that prevents the specification of the stalk cell fate. However, to avoid confusion, we have removed the word "specification" from the abstract. We also corrected an error in our description of the role of Hh signaling in FSC niche competition that we believe the reviewer is referring to: We incorrectly stated that Hh signaling suppresses FSC niche competition; we now state more generally "Hedgehog signaling is known to regulate follicle cell proliferation and FSC niche competition." and elaborate in the Discussion section as follows (page 10, lines 9-13):

> Previous studies established that increased proliferation causes hypercompetition (Wang et al., 2012), and that the proliferative response to Hh signaling is the key mediator of the FSC niche competition phenotypes in Hh pathway mutants (Huang and Kalderon, 2014). Therefore, the hypercompetition phenotype of *ben* mutant clones is likely caused in large part by the increased Hh signaling levels and proliferation rates in these mutants.

What type of allele is benA? Provide a description. Also, describe the hep mutation.

We now state that the ben^{A} allele, "contains a single nonsense mutation in the C-terminal domain of the protein and is homozygous lethal." (page 3, line 1-2), and that the hep^{G0107} allele "is a recessive lethal mutation" (page 5, line 2-3)

Verification of RNAi tools is missing, although they seem to be supported by the phenotypes.

We validated the two most commonly used RNAi lines in this study, *ben[RNAi]* and *hep[RNAi]* by confirming that they were effective in reducing the fluorescence in situ HCR signal in follicle cells in the germarium when driven with 109-30-Gal4 (Fig. S1B-E).

Polar cells are mentioned in the abstract, but I could not find an analysis of the polar cells in the results. What happens to polar cells in ben and hep mutants?

This is a very interesting question. In response to this question and a similar question from Reviewer 1, we investigated this and found that polar cells still frequently form in *ben* and *hep* mutant clones. Based on this, we have softened our interpretation of the effects of JNK pathway mutants on NRE-GFP expression, as described above in our response to Reviewer 1.

Fig 1D': It is puzzling that the multi-layered stalk seems to consist mainly of wild-type cells. Is this a cell-non- autonomous effect?

Indeed, the stalk region in this ovariole contains both GFP^+ cells and GFP^- cells. We found that the follicle formation phenotypes can occur even when the stalk regions are mosaic in this way. In response to this comment and a similar comment from Reviewer 1, we now state in the legend for Figure 1 (page 20, lines 21- 22):

> In all cases, the clones extend well beyond the germarium, and the ovarioles are mosaic or fully marked by the lack of GFP.

Fig 2 legend title: change to ….required for normal stalk formation

Reviewer 1 also objected to our previous title for Fig. 2. In response to this comment and the comment from Reviewer 1, we have changed it to: "JNK signaling is required for pFC differentiation and follicle formation." We hope this is satisfactory for both reviewers but are happy to consider other options.

Fig. 7A schematic drawing: It is confusing that the GFP is indicated in a 'salt and pepper' pattern. Follicle cell clones form normally continuous patches.

We thank the reviewer for pointing this out and have modified the schematic (now Fig. 8A)

Suppl Fig 1 D. In contrast to ben RNA which is clearly seen in follicle cells and germline, hep RNA seems to be largely confined to the germline. Are the authors convinced that there is signal in follicle cells?

In response to this comment, we developed a protocol for combining HCR with immunofluorescence. We now show that the HCR signal for *hep* is clearly detectable in $vasa^-$ follicle cells in the germarium (Fig. S1D).

The scientific language, especially in the figure legends needs to be improved. For example, in several figure legends, it says "GFP+ cells are non-clonal, whereas GFP- cells are clonal". This description makes little sense as all cells, whether GFP+ or - are part of a cell clones. Change to 'mutant cell clones' and 'control cells'. Also, it is not self-explanatory what is meant by 'fully labeled' versus 'mosaic'. This needs to be explained. Also, the label 'GFP (clonal marker) is confusing in case of negative labeling of mutant cells.

We have now thoroughly rewritten the figure legends. Also, in response to this comment and a similar comment by Reviewer 1, we now use the terms "unmarked," "mosaic," and "fully marked" to refer to the different clone patterns, and indicate that we are referring to the status of the follicle cell population.

The whole manuscript needs careful editing, especially spelling and description of proteins and genotypes.

We have carefully edited the manuscript and standardized the way we refer to proteins and genotypes.

Description of antibodies is missing from M&M.

We thank the reviewer for catching this omission and have now added an antibodies section.

Statistics: replicas and sample size: does n represent the overall sample size from all replicas together or does it represent the sample size per replica. Do the means represent the mean of the replicas or samples? Please, clarify. Do graphs show SD or SEM?

n represents the overall sample size from all replicates. The means referred to in the text refer to

the means of the replicas. The graphs show standard deviation. We now state this in the figure legends and the “Statistics and graphs” section of the Methods (page 14, lines 14-19)

Second decision letter

MS ID#: DEVELOP/2021/199630

MS TITLE: Distinct roles of bendless in regulating FSC niche competition and daughter cell differentiation

AUTHORS: Sumitra Tatapudy, Jobelle Peralta, and Todd Nystul
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. In terms of the comments of Reviewer 1, I won't have the MS sent out for review again, but I would ask you to please implement those suggestions that you agree would improve clarity, in your final files that you submit for publication. In particular I agree with the Reviewer that it is important to capitalize protein names.

Reviewer 1

Advance summary and potential significance to field

I have re-read the paper entitled "Distinct roles of bendless in regulating FSC niche competition and daughter cell differentiation" by Tatapudy et al.

Comments for the author

The authors have addressed all my concerns, and I believe the paper is now ready for publication.

Reviewer 2

Advance summary and potential significance to field

How stem cells control their differentiation is critical to understanding tissue regeneration and homeostasis.
Here, the authors have used an exceptionally well-studied model system of follicle stem cells in Drosophila to understand the role of a E2 ubiquitin-conjugating enzyme and JNK pathway member, Bendless (Ben), in stem cell differentiation. Using FSCs, they have found that: 1. Ben and the JNK pathway are required for proper differentiation by promoting specification and maintenance of the polar and stalk cell fates. 2. Ben and JNK pathway are required for upregulation of Notch signaling and to limit EGFR signaling; with Notch playing an important role in polar cells. 3. Ben but not JNK pathway is required to limit hh signaling, which contributes to hypercompetion phenotype. This study uncovers a role of JNK signaling in FSC differentiation and a role JNK independent role for Ben in limiting hh signaling.

Comments for the author

The authors have suitably addressed all my concerns. This manuscript is suitable of publication.

Reviewer 3

Advance summary and potential significance to field

please, see my previous review

Comments for the author

Distinct roles of bendless in regulating niche competition and daughter cell differentiation by Tatapudy Peralta and Nystul
The manuscript is clearly improved. The authors provide now clear evidence for JNK-dependent and independent functions of Ben. The important critique points of the reviewers appear addressed.
I like to raise one point for consideration and otherwise have a number of small suggestions:
Point 1 The authors found a reduction in the expression of the Notch activity reporter NRE-GFP in ben and hep mutant follicle cell clones, but did not find any phenotypic effects that would be expected from a loss of Notch activity. They conclude that the functional relevance of the abnormally low NRE-GFP expression remains unknown. I think that this needs to be reinvestigated with a second Notch reporter [E(spl)] to confirm that Notch activity is indeed reduced. Also, I am wondering whether the speckled staining in region 2b might be an artifact as this reporter seems to be mostly detected in cell nuclei based on images in this manuscript and other papers. However, I consider the effect on the N reporter a minor aspect of this work that does not contribute much to the overall conclusion and message, and suggest to either remove this chapter + Figure 3 from the manuscript or move the Figure to Supplementary.

Small points
- I like to ask the authors to adhere to the generally accepted convention of starting Drosophila protein names with a capital letter: Bendless (Ben), Hh, Eya, Cas, Zfh1 etc. It makes for an easier read and in figures it clearly signals that it is the protein that is being looked at.
- The description of the marking of the clones is still clumsy, incorrect or difficult to follow. For example p3L31: "follicle cell population .... was mosaic or fully marked with benA mutant clones". One could say e.g.: follicle cell population .... was mosaic (partially marked) or only consisted of benA mutant cells (fully marked)...

Similar changes need to be made to the labeling in figures - see below.
- wildtype -> wild-type (if used as adjective)
Abstract:
21: in the proper time -> at the proper time 22-29: The abstract talks about Proteins (e.g. pathway effector, kinase, ubiquitin ligase etc.). All mentioned factors should therefore be indicated in non-italics and starting with a capital first letter.
Intro:
p2, L6: downregulate niche signals -> reduce response to niche signals p2, L9 and p3,L32: follicle epithelium -> follicular epithelium p3,L12/13: see abstract 22-29
Results:
p4,L13-16 and p5,L5-6: cells are not negative or positive for the genes eye and cas, but for the proteins.
Change to: Cas and Eya (non-italics). Make according changes throughout the manuscript.
p4,L35: we deplete hep -> we depleted expression of hep p5,L2: To confirm these RNAi phenotypes -> To further confirm the requirement of Hep for stalk differentiation p5,L23: express NRE-GFP -> expresses NRE-GFP p6,L32-33: add: (Fig. 6B,D,E) after "throughout the germarium". (Fig 6B-F) -> (Fig. 6C, F)
p6,L33: To further explore this result -> To further explore the effect of Hh signaling p7, L4-10: replace "cyst(s)" with 'follicle(s)' or 'egg chambers'. (Cyst refers to the germline portion of a follicle) p7,L14: increased in Hh signaling -> increased Hh signaling p7,L23-24: this sentence needs to be revised p7,L28: with ben[RNAi] driven by -> with ben[RNAi] or hep[RNAI] driven by p7,L35: I don't understand why the authors would state that "ben suppresses competition" even though competition normally takes place between FSCs in a wild-type germarium. Please, clarify the title.
p8,L10-11: this sentence needs to be revised. The use of "fully marked" for two different things does not sound right.
p8,L27: to control -> to the control p9,L34: lateral epidermis -> dorsal epidermis
M&M:
p11, L11: indicate that NRE-pGR is called NRE-GFP in main text
Figure 1: Panel I: Labels says that Cas is in green and Eya in red, but the image shows Eya in green and Cas in red!!

Figure 3: Title statement is too strong. "is required for" -> affects Figure 4C, 5F, 7G and H, 8E and I: replace "unmarked, mosaic, fully marked clones" with 'unmarked mosaic, fully marked follicle cell lineage'
Figure 5: Title: causes stalk formation and differentiation defects -> causes defects in stalk formation and differentiation Figure 5: Panels D'", E'" are not mentioned in text Figure 6: patterning Hh signaling -> patterning of Hh signaling Figure 6 K and L: font is too small for some of the labels