JCB

# Gilgamesh (Gish)/CK1r regulates tissue homeostasis and aging in adult Drosophila midgut

Shuangxi Li, Aiguo Tian, Shuang Li, Yuhong Han, Bing Wang, and Jin Jiang

*Corresponding Author(s): Jin Jiang, Department of Molecular Biology*

## Review Timeline:

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|---|---|
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*Monitoring Editor: Mark Peifer*

*Scientific Editor: Marie Anne O'Donnell*

## Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

**DOI: https://doi.org/10.1083/jcb.201909103**

October 15, 2019

Re: JCB manuscript #201909103

Dr. Jin Jiang
Department of Molecular Biology
University of Texas Southwestern Medical Center at Dallas
6000 Harry hines Blvd.
Dallas, TX 5390

Dear Dr. Jiang,

Thank you for submitting your manuscript entitled "A CK1-Rho1-JNK signaling axis regulates tissue homeostasis and intestinal stem cell activity in adult Drosophila midgut". The manuscript was assessed by expert reviewers, whose comments are appended to this letter. We invite you to submit a revision if you can address the reviewers' key concerns, as outlined here.

As you will see, all three reviewers found the work of potential interest to the community, but all had significant suggestions for strengthening the work and some of the suggested connections. Reviewer #1 offers two concrete ways to strengthen the story. Reviewer #2 has some concrete suggestions for strengthening the conclusions. Reviewer #3 points #1-#4 and #8 also offer some reasonable ways to strengthen the existing story. Some of the other suggestions (e.g exploring further the role of Rho and examining why Gish levels decline during aging) are more open-ended and in our view perhaps more appropriate to be addressed by modifying the text.

While you are revising your manuscript, please also attend to the following editorial points to help expedite the publication of your manuscript. Please direct any editorial questions to the journal office.

GENERAL GUIDELINES:

Text limits: Character count for an Article is < 40,000, not including spaces. Count includes title page, abstract, introduction, results, discussion, acknowledgments, and figure legends. Count does not include materials and methods, references, tables, or supplemental legends.

Figures: Articles may have up to 10 main text figures. Figures must be prepared according to the policies outlined in our Instructions to Authors, under Data Presentation, http://jcb.rupress.org/site/misc/ifora.xhtml. All figures in accepted manuscripts will be screened prior to publication.

***IMPORTANT: It is JCB policy that if requested, original data images must be made available. Failure to provide original images upon request will result in unavoidable delays in publication. Please ensure that you have access to all original microscopy and blot data images before submitting your revision.***

Supplemental information: There are strict limits on the allowable amount of supplemental data. Articles may have up to 5 supplemental figures. Up to 10 supplemental videos or flash animations

are allowed. A summary of all supplemental material should appear at the end of the Materials and methods section.

The typical timeframe for revisions is three months; if submitted within this timeframe, novelty will not be reassessed at the final decision. Please note that papers are generally considered through only one revision cycle, so any revised manuscript will likely be either accepted or rejected.

When submitting the revision, please include a cover letter addressing the reviewers' comments point by point. Please also highlight all changes in the text of the manuscript.

We hope that the comments below will prove constructive as your work progresses. We would be happy to discuss them further once you've had a chance to consider the points raised in this letter.

Thank you for this interesting contribution to the Journal of Cell Biology. You can contact us at the journal office with any questions, cellbio@rockefeller.edu or call (212) 327-8588.

Sincerely,

Mark Peifer, PhD
Monitoring Editor, Journal of Cell Biology

Melina Casadio, PhD
Senior Scientific Editor, Journal of Cell Biology

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Reviewer #1 (Comments to the Authors (Required)):

In the current manuscript, the authors provide thorough and convincing data that Gish kinase regulates ISC proliferation in the fly midgut. They also show that this role involves multiple cell signaling pathways. Mechanistically, they provide genetic and biochemical data indicating the Gish's central role in regulating the JNK pathway occurs through direct phosphorylation of Rho1, an upstream JNK regulator. Lastly, they present intriguing data describing Gish's apparent role in ISC proliferation in aging guts. The main points are novel and well-supported by the data, including quantification of the major findings.
I have only 2 major suggestions that should help strengthen the interpretation of some results and provide more insight into the mechanisms involved. The first is to explore the potential relationship between loss of Gish, JNK activation, and cell death in the gut. The second is to more conclusively demonstrate that Gish actively represses JNK activity. Addressing these should involve only a few additional experiments. More detail is provided below.
The experiments are all very well done and given the importance of the subject matter (e.g., regulation of stem cell activity, identification of a novel JNK regulator, tissue homeostasis with aging implications, etc.), this study would be of interest to a wide range of scientists. I would therefore support the publication of the manuscript in the Journal of Cell Biology, if they can provide some additional insights into the major and minor points raised below.

Major comments
1. There a number of important observations in the current study and previous studies that suggest it is important to investigate the possible role of cell death in mediating some of the major phenotypes presented in this manuscript. For example, Gish KD leads to increased ISC proliferation

in a cell non-autonomous manner, as well as increased JNK activity. It is well known that high levels of JNK activity can cause cell death, and in wing discs for example, increased cell death can trigger increased proliferation of neighboring cells (compensatory proliferation) due to release of growth factors, including the same growth factors identified in the current study of Gish loss. Indeed, tissue damage (apoptosis) in the midgut can trigger ISC proliferation (Jiang et al., Cell. 2009). This raises the question, does loss of Gish lead to JNK-dependent cell death and subsequent growth factor release that then promotes ISC proliferation?
There are also at least a few observations from the current study that indicate apoptosis may be an important part of this story. First, the authors conclude that loss of ISCs due to apoptosis is likely occurring when cells are depleted of both Gish and Puc, two negative regulators of JNK signaling. Second, the authors show that Gish KD in wing discs leads to some increased cell death, which is exacerbated when JNK activity is further increased by reducing Puc levels. Third, the current study suggests Gish acts on JNK signaling through Rho1, which was first identified as a JNK regulator through its role in apoptosis (Neisch et al., JCB. 2010). Thus, perhaps the non-autonomous proliferation in the midgut is a response to apoptotic damage? This should be quite simple to address by staining for cleaved Caspase 3 in the Gish KD guts. If time permits, it could also be examined more functionally by inhibiting apoptosis in Gish KD cells and measuring any effects on ISC proliferation rates. If inhibition of apoptosis reduces the level of ISC proliferation typically associated with Gish KD, it would help explain the mechanisms underlying the cellular and tissue level responses.

2. My second major suggestion addresses the interpretation of their data that Gish acts as a direct repressor of JNK signaling. While the current data are consistent with this interpretation, better support would seem to be demonstrating that Gish OE can repress the consequences of increased JNK activity. Specifically, can Gish OE prevent the increased proliferation caused by puc RNAi (as seen in Fig 4)? This would suggest that Gish is sufficient to repress JNK signaling.

Minor comments
1. It appears that JNK activation is cell-autonomous (Fig 3B). Is this correct? If so, it should be stated and discussed in the paper as it does provide some insight into which events may be directly related to Gish loss and which are secondary.
2. pg.10. "Therefore, under the stress condition caused by excessive JNK pathway activation, Gish is required for ISC maintenance by suppressing cell death." This interpretation of the data seem to suggest that Gish actively suppresses cell death. The data presented do not appear sufficient for that interpretation. One might simply infer that the excessive amount of JNK activation caused by reduction in both Gish and Puc leads to significantly increased cell death instead of proliferation.
3. Is it known if the relevant mitogenic signaling pathways (EGFR and JAK-STAT) are downstream of JNK signaling in tissue damage models in the midgut? An alternative hypothesis would be that these three pathways are each required but work in parallel to drive ISC proliferation. If this is not known, this could be tested by blocking JNK signaling to see if it prevents upregulation of growth factor production. This question is perhaps beyond the scope of the current manuscript, so only a suggestion for future work if the relationships are not known.
4. Gish was previously identified in a genetic screen as a modifier of Rho signaling (Gregory et al., Fly. 2007. PMID 18690061). Probably worth mentioning this as it seems to support one of the major findings of the current study.
5. Materials and Methods does not appear to include a description of the generation and expression of the FLAG:Rho1 constructs in the S2 cell experiments. Please include.
6. To demonstrate the importance of Rho1 in ISC proliferation, the authors misexpress a constitutively active form of Rho1 (RhoV14). However, the earlier study identifying Rho1 as a regulator of JNK signaling also demonstrated that this role is independent of its GTP-bound state

(Neisch et al., JCB. 2010). Indeed there appears to be no difference between misexpression of Rho1[V14] (~15 pH3+ cells/gut; Fig 5R) compared to wildtype Rho1 (~15 pH3+ cells/gut; Fig 6K). It seems worth mentioning that these findings agree with the previous study on Rho1 in terms of the GTP-bound state not being important.
7. The repression of proliferation in aging guts by GishOE is impressive. Based on the model proposed, the ectopic Gish is phosphorylating and destabilizing the increased Rho1 levels associated with older guts. This begs the question, does GishOE repress the increased Rho1-GFP levels seen in aging guts? Can these changes in Rho1-GFP levels be shown by Western, where appropriate loading controls can be included? Time constraints may preclude the ability to perform this experiment, however, if the authors continue to explore this process in aging guts, perhaps they can test this in future work.
8. Some abbreviations should be written out the first time they are used (e.g. pg 3 - BMP and N).
9. Pg 4. Line 11. Should be "tissue damage" not "damaging".
10. JAK-STAT pathway is written as "JAK-Stat" at different times (pg 7 for example), please capitalize throughout.
11. Figure 4N. Colors in legend do not match graph (green and gray appear switched).
12. Pg.10. "However, when RNAi was conducted for 5 days, Gish RNAi appeared to reverse the effect on ISC proliferation caused by Puc RNAi because Gish and Puc double RNAi resulted in less pH3 positive cells compared with Puc RNAi alone (Fig. 4E-H, 4N)." No statistical significance indicated to support this conclusion.
13. Pg. 11 "promoted" should be "prompted".

Reviewer #2 (Comments to the Authors (Required)):

The manuscript by Li et al is an overall technically sound study that goes further into depth on understanding of cell signaling that promotes self renewal of the Drosophila midgut epithelium. In the first part of the paper, the authors show that loss of the CK1 family member Gish leads to heightened proliferation in the midgut, which can be suppressed by manipulating the activity of numerous known midgut proliferation signaling pathways. They then decide to focus on the JNK pathway, performing more in depth analyses which lead again to the conclusion that Gish suppresses midgut proliferation, and that manipulation of JNK can counter the hyperproliferation caused by Gish loss. Taking a cue from mammalian literature, the authors then identify that genetic manipulation of the small GTPase Rho1 can mimic the effects of Gish loss, suggesting a possible conserved interaction between Rho1 and Gish. The authors then move to the S2 cell system to further probe the interaction between Gish and the small GTPase Rho1. They are able to identify specific amino acid residues in Rho1 that are important for Gish phosphorylation of Rho1, shown with an in vitro kinase assay. Finally, they move back into the fly and identify physiological aging as a mechanism that tips the balance between Gish/Rho1 regulation, causing high intestinal proliferation rates. Overall, the authors do a good job of mechanistically characterizing the Gish/Rho1 interaction in the midgut epithelium. This study adds one more node to the already large complexity of regulation of cell signaling in the proliferating midgut. While there is little question that the work is of good quality and mechanistic rigor, I am not totally clear of whether this study convincingly provides an important conceptual advance for the field beyond adding to the already large complexity of midgut proliferation regulation. Also, while likely beyond the scope of the study, I am left with wondering mechanistically what Rho1 (which has well-characterized roles in actin regulation in numerous contexts) is doing in the midgut that ultimately lead it to regulate numbers of PH3+ cells.

Specific comments:

Please specify the precise posterior midgut region that was analyzed in these studies, as proliferation rates can vary dramatically by region.

Numbers of PH3+ cells support the conclusions in all cases, but it is very hard to see in the images. Red on black PH3 staining has little contrast. I suggest changing these images to black and white in these panels and possibly enhancing the contrast.

Fig 2Q- the authors point out that it is notable that Dome RNAi did not completely suppress the PH3 phenotype- this could be explained by incomplete knockdown, so the authors should be careful about their conclusion here.

Typo p. 6- "USA-Gish"

Puc lacZ is introduced in the context of being a negative regulator of JNK, which it is, but it is also a target of JNK activity. The way the authors discuss this to the readers as it pertains to Fig3A is confusing- the unfamiliar reader may wonder why the authors are claiming that upregulation of a negative regulator suggests pathway activation (as opposed to the opposite). The authors should cite prior work on the dual nature (target/repressor) of puckered activity, so the non-JNK expert understands the nuances involved. This will also help later with discussing the puc genetic manipulation results.

Figure 4N- there is an issue with bar graph coloring relative to the legend- PucRNAi is green in the legend but to me looks grey in the figure (if I am wrong then I have mis-interpreted the data- also a problem). Also, the dark blue (control) vs the light blue (Diap1 experiment) is very hard to discern.

Figure 4, Diap experiment- the authors conclude too strongly that Diap1 expression restores PH3+ numbers- looks like a partial (though significant) restoration. Please clarify in the text.

p.11 typo- "promoted" I think should be "prompted"

p.11 typo- "that express" I think should be "that expresses"

Figure 6A- I could not find any information about how fluorescence levels were normalized between conditions. Without this information, I cannot assess the validity of this experiment. Figure 7A-C and E-F: same comment.

Reviewer #3 (Comments to the Authors (Required)):

In this paper, Li et al. provide compelling evidence to show that Gish/Ck1 maintains Drosophila midgut homeostasis during the aging process by restricting JNK pathway activity. Although it has been reported that the JNK pathway plays a fundamental role during midgut aging (by Heinrich Jasper's lab), how the activity of JNK is up-regulated upon aging is still somewhat unclear. This paper illustrates a part of one possible mechanism. Some major questions are raise here, however, that are not answered. For instance, why does Gish expression decline with aging? How does Rho1 activated the JNKKK, Slipper? Although the paper would be more satisfying with answers to these questions, it is nevertheless a well-written paper, and the data are substantial, good quality and

presented clearly and logically. It should be a good candidate for JCB after a minor revision. Comments are listed below.

Major:

1. As the authors mention, they did a kinome screen in gut progenitors. It would be of great benefit for readers and the field if the authors could exhibit their screen results in a supplementary table.

2. Base on Figure 2, it seems that GishRNAi upregulates not only JNK but also EGFR, JAK-STAT, and Wnt signaling. However, it remains unclear whether GishRNAi actives these pathways in parallel, or if GishRNAi specifically actives JNK first, and then EGFR, JAK-STAT, and Wnt signaling are stimulated later as a result of JNK activity or the stress of hyperplastic ISC division. The authors should make this limitation to their analysis more transparent in their conclusions. Alternately, they may wish to address this issue experimentally. A simple way to do this is to artificially block the proliferation of ISC at the same time as Gish knockdown (esgts>StringRNAi+GishRNAi), then examine the upregulation of EGFR, JAK-STAT, and Wnt ligands as shown in Fig.2A. If those upregulations diminish, it means the activation of EGFR, JAK-STAT, and Wnt signaling is a secondary effect of GishRNAi-driven ISC proliferation.

3. In Figure 2B&2C, it is hard to see in which cell types upd3-lacZ is upregulated (progenitors? ECs? or both?). Please show a zoom-in picture and describe the expression pattern.

4. To confirm that the regulation of JNK by Gish is conserved from ISCs/EBs to ECs, it would be great to repeat the type of experiments shown in Figure 3E-M using Myo1Ats driver.

5. As noted above, some information on why Gish expression declines with aging would enhance the paper. We also request better data on this phenomenon.

6. As noted above, the paper would be better with some information about how Rho1 activates JNKKK. They cite (Neish 2010) but don't say anything about the mechanism. In fact that paper doesn't have much on mechanism besides a Rho1:Slpr physical interaction and genetic epistasis, and the work was done mostly in wing discs, so there is more to be done on this topic. The authors should at the very least add better discussion of what exactly is known about the Rho:Slpr interaction. Better would be to add new data on this topic, to advance from where Neish et al left off.

7. The paper has a number of typos/grammatical mistakes that need correction.

8. Regarding Figure3,S4 and results part "Loss of Gish leads to JNK pathway activation'.
It is unclear in which cells puc-lacZ is upregulated following esg>gishRNAi and Myo>gishRNAi. The authors claim that the effect on JNK activity is direct rather than a secondary effect of ISC over-proliferation, however in Fig3B,3D and S4B (3days) puc-lacZ seems to me to be upregulated both in the progenitors (ISC+EB) and the differentiated epithelial cells (enterocytes). Thus, the Gish RNAi would have both cell-autonomous and non-cell autonomous effects.
Given this, we think the authors should modify their conclusions, and also provide much clearer descriptions of which cell types upregulate puc-lacZ after the various treatments. This can be easily determined by co-staining with cell identity markers, a standard procedure in this field. If the authors wish to show that the effect is direct (cell autonomous) they should provide earlier time-point and quantification data or an an experimental treatment that seperates cell autonomous and

non-autonomous effects. This could be done using clonal expression, or by blocking cell divisions triggered by GISH, or by epistasis with other things that activate JNK.

Minor:

1. Based on Fig.1, the authors found that Gish acts as a general repressor for JNK activity in the whole ISC-EB-EC lineage. What about the function of Gish in EEs?

2. In Figure 4N, what does the grey column represent? (pucRNAi?)

3. In Figure 7I, it seems that overexpression of Gish in esg+ cells significantly repressed the levels of puc-lacZ in ECs, but not in ISCs/EBs. Please discuss the meaning of this result.

UT Southwestern Medical Center

**Dr. Jin Jiang**
Professor

Eugene McDermott Endowed Scholar in Biomedical Science

**Departmen**

Dear Mark and Melina:

Enclosed please find a copy of our revised manuscript entitled "**A CK1γ-Rho1-JNK signaling axis regulates tissue homeostasis and intestinal stem cell activity in adult *Drosophila* midgut**" for submission to JCB.

We have carefully addressed the reviewers' comments by conducting requested/suggested experiments and modified the text accordingly. The major changes are as following:

- We have provided new data to show that blocking cell death partially suppressed the non-autonomous ISC proliferation induced Gish KD in ECs (revised Fig. S4).
- We have provided new data showing that Gish overexpression can suppress ISC proliferation caused by Puc RNAi (revised Fig. 3N-R)
- We have provided new data to show that blocking JNK signaling can suppress the upregulation of multiple growth factors caused by Gish RNAi (Fig. S4)
- We included a new experiment to show that blocking JNK signaling can partially block ISC overproliferation caused by Gish inactivation in ECs (revised Fig. S4A-E).
- We clarified the autonomous and non-autonomous upregulation of upd3-lacZ (Fig 2C) and puc-lacZ (Fig. 3B) caused by Gish RNAi.
- We provided new data to show that Gish overexpression could suppress the upregulation of Rho1-GFP in old guts (Fig. 7E-G).
- We provided internal controls for immunostaining in the aging experiments (Fig. 7A-G).
- We converted the single pH3 channel into black-and-white image for all the figures.
- To cut the supplementary figures down to 5, we combined the previous FigS1 and 2 into revised FigS1 and previous FigS3 and 4 into revised FigS3.

Please see our point-to-point responses to reviewers' comments for detailed changes in the text. I hope we have addressed the reviewers' comments satisfactorily. I thank all the reviewers for their constructive comments that have greatly improved our manuscript. I look forward to your favorable decision.

Sincerely

Jin Jiang, Ph.D.

Location: 6000 Harry Hines Blvd. ND5.136AE / Dallas, Texas / (214) 645-5914 Telefax (214) 648-1196
Mail: 5323 Harry Hines Blvd. / Dallas, Texas 75390-9148 / jin.jiang@utsouthwestern.edu

Reviewer #1 (Comments to the Authors (Required)):

*In the current manuscript, the authors provide thorough and convincing data that Gish kinase regulates ISC proliferation in the fly midgut. They also show that this role involves multiple cell signaling pathways. Mechanistically, they provide genetic and biochemical data indicating the Gish's central role in regulating the JNK pathway occurs through direct phosphorylation of Rho1, an upstream JNK regulator. Lastly, they present intriguing data describing Gish's apparent role in ISC proliferation in aging guts. The main points are novel and well-supported by the data, including quantification of the major findings.*
*I have only 2 major suggestions that should help strengthen the interpretation of some results and provide more insight into the mechanisms involved. The first is to explore the potential relationship between loss of Gish, JNK activation, and cell death in the gut. The second is to more conclusively demonstrate that Gish actively represses JNK activity. Addressing these should involve only a few additional experiments. More detail is provided below.*
*The experiments are all very well done and given the importance of the subject matter (e.g., regulation of stem cell activity, identification of a novel JNK regulator, tissue homeostasis with aging implications, etc.), this study would be of interest to a wide range of scientists. I would therefore support the publication of the manuscript in the Journal of Cell Biology, if they can provide some additional insights into the major and minor points raised below.*

We thank this reviewer for the positive comments.

*Major comments*
*1. There a number of important observations in the current study and previous studies that suggest it is important to investigate the possible role of cell death in mediating some of the major phenotypes presented in this manuscript. For example, Gish KD leads to increased ISC proliferation in a cell non-autonomous manner, as well as increased JNK activity. It is well known that high levels of JNK activity can cause cell death, and in wing discs for example, increased cell death can trigger increased proliferation of neighboring cells (compensatory proliferation) due to release of growth factors, including the same growth factors identified in the current study of Gish loss. Indeed, tissue damage (apoptosis) in the midgut can trigger ISC proliferation (Jiang et al., Cell. 2009). This raises the question, does loss of Gish lead to JNK-dependent cell death and subsequent growth factor release that then promotes ISC proliferation? There are also at least a few observations from the current study that indicate apoptosis may be an important part of this story. First, the authors conclude that loss of ISCs due to apoptosis is likely occurring when cells are depleted of both Gish and Puc, two negative regulators of JNK signaling. Second, the authors show that Gish KD in wing discs leads to some increased cell death, which is exacerbated when JNK activity is further increased by reducing Puc levels. Third, the current study suggests Gish acts on JNK signaling through Rho1, which was first identified as a JNK regulator through its role in apoptosis (Neisch et al., JCB. 2010). Thus, perhaps the non-autonomous proliferation in the midgut is a response to apoptotic damage? This should be quite simple to address by staining for cleaved Caspase 3 in the Gish KD guts. If time permits, it could also be examined more functionally by inhibiting apoptosis in Gish KD cells and measuring any effects on ISC proliferation rates. If inhibition of apoptosis reduces the level of ISC proliferation typically associated with Gish KD, it would help explain the mechanisms underlying the cellular and tissue level responses.*

Response:

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To address whether cell death could contribute to increased ISC proliferation caused by Gish inactivation, we inhibited apoptosis by overexpressing Diap1 in conjunction with Gish RNAi either in progenitor cells (*esg$^{ts}$*) or in ECs (*Myo1A$^{ts}$*). We found that blocking cell death did not affect ISC overproliferation induced by Gish RNAi in progenitor cells but partially suppressed ISC overproliferation caused by Gish RNAi in ECs, suggesting that cell death contributed to the non-autonomous proliferation induced by Gish RNAi. These results have been incorporated into Fig. S4 (Fig.S4H-L).

*2. My second major suggestion addresses the interpretation of their data that Gish acts as a direct repressor of JNK signaling. While the current data are consistent with this interpretation, better support would seem to be demonstrating that Gish OE can repress the consequences of increased JNK activity. Specifically, can Gish OE prevent the increased proliferation caused by puc RNAi (as seen in Fig 4)? This would suggest that Gish is sufficient to repress JNK signaling.*

Response:

We thank reviewer for the suggested experiment. We found that overexpression of Gish could suppress ISC overproliferation caused by puc RNAi (revised Fig. 3N-R), suggesting that excessive Gish activity is sufficient to repress JNK signaling.

*Minor comments*
*1. It appears that JNK activation is cell-autonomous (Fig 3B). Is this correct? If so, it should be stated and discussed in the paper as it does provide some insight into which events may be directly related to Gish loss and which are secondary.*

Response:

*puc-lacZ* is upregulated both cell-autonomous and non-cell autonomously. We have now included an inset in Fig.3B' to indicate the non-cell autonomous upregulation of *puc-lacZ* caused by Gish KD in progenitor cells. We have rewritten this part as the following:

"Careful examination of *puc-lacZ* expression in *esg$^{ts}$>GFP+Gish$^{RNAi}$* guts revealed that JNK pathway activation also occurred in ECs that are GFP negative (arrows in Fig. 3B'), which could explain the non-cell-autonomous activation of *upd3-lacZ* in *esg$^{ts}$>GFP+Gish$^{RNAi}$* guts (Fig. 2B). However, Gish RNAi in progenitor cells for a shorter period of time (3 days at 29 $^{0}$C) did not significantly increased ISC proliferation and resulted in increased *puc-lacZ* expression primarily in GFP$^{+}$ progenitor cells (Fig. S3A-C), suggesting that cell-autonomous JNK activation could be a direct effect of Gish inactivation while the non-cell-autonomous JNK activation observed in prolonged Gish RNAi guts is likely due to epithelial stress caused by ISC overproliferation similar to what has described by a previous study (Patel et al., 2015). Consistent with Gish regulating JNK, Gish inactivation in wing imaginal discs also resulted in ectopic *puc-lacZ* expression (Fig. S3E-G')".

*2. pg.10. "Therefore, under the stress condition caused by excessive JNK pathway activation, Gish is required for ISC maintenance by suppressing cell death." This interpretation of the data seems to suggest that Gish actively suppresses cell death. The data presented do not appear sufficient for that interpretation. One might simply infer that the excessive amount of JNK activation caused by reduction in both Gish and Puc leads to significantly increased cell death instead of proliferation.*

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Response:

We agree with the reviewer and therefore we deleted “by suppressing cell death”.

*3. Is it known if the relevant mitogenic signaling pathways (EGFR and JAK-STAT) are downstream of JNK signaling in tissue damage models in the midgut? An alternative hypothesis would be that these three pathways are each required but work in parallel to drive ISC proliferation. If this is not known, this could be tested by blocking JNK signaling to see if it prevents upregulation of growth factor production. This question is perhaps beyond the scope of the current manuscript, so only a suggestion for future work if the relationships are not known.*

Response:

A previous study has demonstrated JNK signaling activates EGFR and JAK-STAT pathway ligands (Jiang et al., 2009). We included this information in the revised text. We also conducted experiments to show that blocking JNK signaling by a dominant-negative Bsk ($Bsk^{DN}$) inhibits the upregulation of cytokines and growth factor production caused by Gish RNAi (revised Fig. S4F-G).

*4. Gish was previously identified in a genetic screen as a modifier of Rho signaling (Gregory et al., Fly. 2007. PMID 18690061). Probably worth mentioning this as it seems to support one of the major findings of the current study.*

Response:

We have included this information in the revised text.

*5. Materials and Methods does not appear to include a description of the generation and expression of the FLAG:Rho1 constructs in the S2 cell experiments. Please include.*

Response:

We have included this information in the revised Method.

“To generate *UAS-Flag-Gish*, *UAS-Flag-Rho1* and *UAS-Flag-Rho1*$^{SA}$ constructs, DNA fragments encoding Gish, Rho1 with wild type or mutated CK1 sites were amplified by PCR and inserted into the *Flag-pUAST* vector (Tong and Jiang, 2007)”.

“*UAS-Flag-Rho*/*UAS-Flag-Rho*$^{SA}$ was cotransfected with actin-Gal4 to express the Flag-Rho/$Rho^{SA}$ in S2 cells”.

*6. To demonstrate the importance of Rho1 in ISC proliferation, the authors misexpress a constitutively active form of Rho1 (RhoV14). However, the earlier study identifying Rho1 as a regulator of JNK signaling also demonstrated that this role is independent of its GTP-bound state (Neisch et al., JCB. 2010). Indeed there appears to be no difference between misexpression of Rho1[V14] (~15 pH3+ cells/gut; Fig 5R) compared to wildtype Rho1 (~15 pH3+ cells/gut; Fig 6K). It seems worth mentioning that these findings agree with the previous study on Rho1 in terms of the GTP-bound state not being important.*

Response:

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We did not compare Rho1V14 and Rho1 side by side. In addition, the expression levels of these transgenes are likely to be different, making it difficult to compare their relative activities. Nevertheless, we have added the following in the discussion. “How Rho1 inhibits JNK pathway remains an open question. A previous study revealed that Rho1 physically interacts with Slipper (Slpr)/JNK kinase kinase (JNKKK) regardless its GDP/GTP-binding state and that Rho1 promotes Slpr cortical localization (Neisch et al., 2010). Therefore, it is possible that plasma-membrane-associated Rho1 promotes JNK pathway activation by increasing the local concentration of Slpr/JNKKK at the plasma membrane. The precise biochemical mechanism by which Rho1 activates Slpr/JNKKK awaits further investigation”.

*7. The repression of proliferation in aging guts by GishOE is impressive. Based on the model proposed, the ectopic Gish is phosphorylating and destabilizing the increased Rho1 levels associated with older guts. This begs the question, does GishOE repress the increased Rho1-GFP levels seen in aging guts? Can these changes in Rho1-GFP levels be shown by Western, where appropriate loading controls can be included? Time constraints may preclude the ability to perform this experiment, however, if the authors continue to explore this process in aging guts, perhaps they can test this in future work.*

Response:

We have now provided data to show that GishOE can repress the increased Rho1-GFP levels seen in aging guts (revised Fig. 7). We also co-stained the guts with Arm or phalloidin as internal control for immunostaining.

*8. Some abbreviations should be written out the first time they are used (e.g. pg 3 - BMP and N).*

We provided the full names.

*9. Pg 4. Line 11. Should be "tissue damage" not "damaging".*

We corrected it.

*10. JAK-STAT pathway is written as "JAK-Stat" at different times (pg 7 for example), please capitalize throughout.*

We changed to “JAK-STAT” throughout.

*11. Figure 4N. Colors in legend do not match graph (green and gray appear switched).*

We corrected this, sorry for the confusion.

*12. Pg.10. "However, when RNAi was conducted for 5 days, Gish RNAi appeared to reverse the effect on ISC proliferation caused by Puc RNAi because Gish and Puc double RNAi resulted in less pH3 positive cells compared with Puc RNAi alone (Fig. 4E-H, 4N)." No statistical significance indicated to support this conclusion.*

We provided the statistics.

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*13. Pg. 11 "promoted" should be "prompted".*

We corrected it. Thanks.

Reviewer #2 (Comments to the Authors (Required)):

*The manuscript by Li et al is an overall technically sound study that goes further into depth on understanding of cell signaling that promotes self renewal of the Drosophila midgut epithelium. In the first part of the paper, the authors show that loss of the CK1 family member Gish leads to heightened proliferation in the midgut, which can be suppressed by manipulating the activity of numerous known midgut proliferation signaling pathways. They then decide to focus on the JNK pathway, performing more in depth analyses which lead again to the conclusion that Gish suppresses midgut proliferation, and that manipulation of JNK can counter the hyperproliferation caused by Gish loss. Taking a cue from mammalian literature, the authors then identify that genetic manipulation of the small GTPase Rho1 can mimic the effects of Gish loss, suggesting a possible conserved interaction between Rho1 and Gish. The authors then move to the S2 cell system to further probe the interaction between Gish and the small GTPase Rho1. They are able to identify specific amino acid residues in Rho1 that are important for Gish phosphorylation of Rho1, shown with an in vitro kinase assay. Finally, they move back into the fly and identify physiological aging as a mechanism that tips the balance between Gish/Rho1 regulation, causing high intestinal proliferation rates. Overall, the authors do a good job of mechanistically characterizing the Gish/Rho1 interaction in the midgut epithelium. This study adds one more node to the already large complexity of regulation of cell signaling in the proliferating midgut. While there is little question that the work is of good quality and mechanistic rigor, I am not totally clear of whether this study convincingly provides an important conceptual advance for the field beyond adding to the already large complexity of midgut proliferation regulation. Also, while likely beyond the scope of the study, I am left with wondering mechanistically what Rho1 (which has well-characterized roles in actin regulation in numerous contexts) is doing in the midgut that ultimately lead it to regulate numbers of PH3+ cells.*

Response:

Although many signaling pathways have been identified to regulate ISC proliferation, how these pathways are regulated in midgut homeostasis and regeneration are still poorly understood. Our study identifies Gish/CK1$\gamma$ as a novel regulator of Rho1 and gatekeeper of tissue homeostasis whose activity is compromised in aging guts. In addition, our study provides a novel insight into how JNK pathway activity is kept in check during adult tissue homeostasis and how JNK pathway activity might be deregulated in aging tissues. Because the CK1 phosphorylation sites on Rho1 are conserved in mammalian Rho family members including RhoA, RhoB and RhoC, CK1 may play a conserved role in the regulation of mammalian Rho activity. I believe that our work not only represents an important step forward in the field but also has a broader impact on cell signaling, stem cell biology, and cancer biology.

Specific comments:

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*Please specify the precise posterior midgut region that was analyzed in these studies, as proliferation rates can vary dramatically by region.*

Response:

We counted pH3 signals in the entire guts ($pH3^+$ cells/gut) for all the study. For examining the clone size, we only included ISC lineage clones in the R4 region of posterior midguts for quantification because of the regional difference in ISC proliferation rate (Buchon et al., 2013; Marianes and Spradling, 2013). We provided this information in the revised text.

*Numbers of PH3+ cells support the conclusions in all cases, but it is very hard to see in the images. Red on black PH3 staining has little contrast. I suggest changing these images to black and white in these panels and possibly enhancing the contrast.*

Response:

Thanks for the suggestion. We converted all pH3 panels into black-and-white images.

*Fig 2Q- the authors point out that it is notable that Dome RNAi did not completely suppress the PH3 phenotype- this could be explained by incomplete knockdown, so the authors should be careful about their conclusion here.*

Response:

We stated that the incomplete suppression by Dome RNAi is likely due to incomplete knockdown.

*Typo p. 6- "USA-Gish"*

We corrected this, thanks.

*Puc lacZ is introduced in the context of being a negative regulator of JNK, which it is, but it is also a target of JNK activity. The way the authors discuss this to the readers as it pertains to Fig3A is confusing- the unfamiliar reader may wonder why the authors are claiming that upregulation of a negative regulator suggests pathway activation (as opposed to the opposite). The authors should cite prior work on the dual nature (target/repressor) of puckered activity, so the non-JNK expert understands the nuances involved. This will also help later with discussing the puc genetic manipulation results.*

We rephrased the sentence: “we found that Gish RNAi in either progenitor cells or ECs resulted in upregulation of a JNK pathway reporter gene *puc-lacZ* ($puc^{E96}$) (Fig. 3A-D”), an enhancer trap line inserted in the *puckered* (*puc*) locus, which encodes a phosphatase that mediates negative feedback regulation of the JNK pathway (Martin-Blanco et al., 1998)”.

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*Figure 4N- there is an issue with bar graph coloring relative to the legend- PucRNAi is green in the legend but to me looks grey in the figure (if I am wrong then I have mis-interpreted the data- also a problem). Also, the dark blue (control) vs the light blue (Diap1 experiment) is very hard to discern.*

We corrected this, sorry for the confusion.

*Figure 4, Diap experiment- the authors conclude too strongly that Diap1 expression restores PH3+ numbers- looks like a partial (though significant) restoration. Please clarify in the text.*

We restated that "expression of the cell death inhibitor Diap1 in Gish and Puc double RNAi progenitor cells partially restored *esg>GFP* positive cells and pH3 positive cells (Fig. 4M-N)"

*p.11 typo- "promoted" I think should be "prompted"*

We corrected this, thanks.

*p.12 typo- "that express" I think should be "that expresses"*

We corrected this, thanks.

*Figure 6A- I could not find any information about how fluorescence levels were normalized between conditions. Without this information, I cannot assess the validity of this experiment.*

In $esg^{ts}$>$Gish^{RNAi}$ guts, Rho-GFP was only upregulated in progenitor cells but not in ECs. Therefore, EC signals in control and RNAi guts were used to normalize Rho-GFP expression. Similarly, in $Myo1A^{ts}$>$Gish^{RNAi}$ guts, Rho-GFP was upregulated in ECs but not in progenitor cells.

*Figure 7A-C and E-F: same comment.*

We use Arm staining as an internal control for Fig. 7A-C and Phalloidin staining as an internal control for Fig. 7E-G. These signals do not show age-dependent change in their intensity.

Reviewer #3 (Comments to the Authors (Required)):

*In this paper, Li et al. provide compelling evidence to show that Gish/Ck1 maintains Drosophila midgut homeostasis during the aging process by restricting JNK pathway activity. Although it has been reported that the JNK pathway plays a fundamental role during midgut aging (by Heinrich Jasper's lab), how the activity of JNK is up-regulated upon aging is still somewhat unclear. This paper illustrates a part of one possible mechanism. Some major questions are raise here, however, that are not answered. For instance, why does Gish expression decline with aging?*

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*How does Rho1 activated the JNKKK, Slipper? Although the paper would be more satisfying with answers to these questions, it is nevertheless a well-written paper, and the data are substantial, good quality and presented clearly and logically. It should be a good candidate for JCB after a minor revision. Comments are listed below.*

We thank this reviewer for the positive comments.

*Major:*

*1. As the authors mention, they did a kinome screen in gut progenitors. It would be of great benefit for readers and the field if the authors could exhibit their screen results in a supplementary table.*

Response:

We did not present the screen results as a whole because for most kinases, we only tested one RNAi line. What make things worse is that a large fraction of RNAi lines we used (>50%) are KK lines from VDRC. A previous study using KK lines to screen Hippo pathway genetic modifiers revealed that many KK lines have a unanticipated insertion that could modify the tissue overgrowth phenotype caused by Yki overexpression (Vissers et al., Nat Commun 2016). Therefore, the screen we conducted could yield many false positive and false negative hits that could mislead the readers. For this reason, we focused on Gish for which we tested multiple independent RNAi as well as a genetic mutation and presented an in-depth study of how Gish regulates stem cell activity and tissue homeostasis.

*2. Base on Figure 2, it seems that GishRNAi upregulates not only JNK but also EGFR, JAK-STAT, and Wnt signaling. However, it remains unclear whether GishRNAi actives these pathways in parallel, or if GishRNAi specifically actives JNK first, and then EGFR, JAK-STAT, and Wnt signaling are stimulated later as a result of JNK activity or the stress of hyperplastic ISC division. The authors should make this limitation to their analysis more transparent in their conclusions. Alternately, they may wish to address this issue experimentally. A simple way to do this is to artificially block the proliferation of ISC at the same time as Gish knockdown (esgts>StringRNAi+GishRNAi), then examine the upregulation of EGFR, JAK-STAT, and Wnt ligands as shown in Fig.2A. If those upregulations diminish, it means the activation of EGFR, JAK-STAT, and Wnt signaling is a secondary effect of GishRNAi-driven ISC proliferation.*

Response:

A previous study has demonstrated JNK signaling activates EGFR and JAK-STAT pathway ligands (Jiang et al., 2009). We included this information in the revised text. We also conducted experiments to show that blocking JNK signaling by a dominant-negative Bsk ($Bsk^{DN}$) can at least partially inhibit the upregulation of cytokines and growth factor production caused by Gish RNAi (revised Fig. S4F-G).

*3. In Figure 2B&2C, it is hard to see in which cell types upd3-lacZ is upregulated (progenitors?*

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*ECs? or both?). Please show a zoom-in picture and describe the expression pattern.*

Response:

We have provided a zoom-in picture (see Inset in revised Fig. 2C) that reveals upd3-lacZ upregulation in both progenitors and ECs. We have added the following text in the revised MS. "Of note, Gish RNAi in progenitor cells also upregulated *upd3-lacZ* in many ECs adjacent to progenitor cell clusters (arrows in Fig. 2C). This non-cell-autonomous upregulation of *upd3-lacZ* is likely due to epithelial stress caused by ISC overproliferation (Patel et al., 2015)".

*4. To confirm that the regulation of JNK by Gish is conserved from ISCs/EBs to ECs, it would be great to repeat the type of experiments shown in Figure 3E-M using Myo1Ats driver.*

Response:

We coexpressed a dominant-negative Bsk ($Bsk^{DN}$) with Gish-RNAi in ECs and found that blocking JNK signaling can partially block ISC overproliferation caused by Gish inactivation in ECs (revised Fig. S4A-E).

*5. As noted above, some information on why Gish expression declines with aging would enhance the paper. We also request better data on this phenomenon.*

Response:
We have revised Fig. 7 extensively. e.g., we have repeated the experiments described in Fig. 7A-C using Arm staining as an internal control for immunostaining. We have included an experiment in which we showed that overexpression of Gish can suppress the elevated Rho-GFP expression in old guts (revised Fig. 7E-G'). We have provided better images to show that overexpression of Gish in progenitor cells repressed the levels of *puc-lacZ* in both progenitor cells and ECs (revised Fig. 7H-J"). Figuring out how or why Gish expression declines with aging is beyond the scope of this study. Nevertheless, we added the following the discussion:

"How Gish expression is downregulated in aging guts remains an open question but it appears to occur at the level of transcription although we cannot rule out the possibility that post-transcriptional regulation may also occur. The precise mechanism awaits further investigation".

*6. As noted above, the paper would be better with some information about how Rho1 activates JNKKK. They cite (Neish 2010) but don't say anything about the mechanism. In fact, that paper doesn't have much on mechanism besides a Rho1:Slpr physical interaction and genetic epistasis, and the work was done mostly in wing discs, so there is more to be done on this topic. The authors should at the very least add better discussion of what exactly is known about the Rho:Slpr interaction. Better would be to add new data on this topic, to advance from where Neish et al left off.*

Response:

We have added the following in the discussion:

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"How Rho1 inhibits JNK pathway remains an open question. A previous study revealed that Rho1 physically interacts with Slipper (Slpr)/JNK kinase kinase (JNKKK) regardless its GDP/GTP-binding state and that Rho1 promotes Slpr cortical localization (Neisch et al., 2010). Therefore, it is possible that plasma-membrane-associated Rho1 promotes JNK pathway activation by increasing the local concentration of Slpr/JNKKK at the plasma membrane. The precise biochemical mechanism by which Rho1 activates Slpr/JNKKK awaits further investigation".

*7. The paper has a number of typos/grammatical mistakes that need correction.*

Response:

We have corrected the typos/grammatical mistakes.

*8. Regarding Figure3, S4 and results part "Loss of Gish leads to JNK pathway activation'. It is unclear in which cells puc-lacZ is upregulated following esg>gishRNAi and Myo>gishRNAi. The authors claim that the effect on JNK activity is direct rather than a secondary effect of ISC over-proliferation, however in Fig3B,3D and S4B (3days) puc-lacZ seems to me to be upregulated both in the progenitors (ISC+EB) and the differentiated epithelial cells (enterocytes). Thus, the Gish RNAi would have both cell-autonomous and non-cell autonomous effects. Given this, we think the authors should modify their conclusions, and also provide much clearer descriptions of which cell types upregulate puc-lacZ after the various treatments. This can be easily determined by co-staining with cell identity markers, a standard procedure in this field. If the authors wish to show that the effect is direct (cell autonomous) they should provide earlier time-point and quantification data or an an experimental treatment that seperates cell autonomous and non-autonomous effects. This could be done using clonal expression, or by blocking cell divisions triggered by GISH, or by epistasis with other things that activate JNK.*

Response:

We thank the reviewer for pointing this out. We agree with the review that puc-lacZ is upregulated both cell-autonomous and non-cell autonomously. We have now included an inset in Fig.3B' to indicate the non-cell autonomous upregulation of puc-lacZ caused by Gish KD in progenitor cells. We have rewritten this part as the following:

"Careful examination of *puc-lacZ* expression in $esg^{ts}$>*GFP+*$Gish^{RNAi}$ guts revealed that JNK pathway activation also occurred ECs that are GFP negative (arrows in Fig. 3B'), which could explain the non-cell-autonomous activation of *upd3-lacZ* in $esg^{ts}$>*GFP+*$Gish^{RNAi}$ guts (Fig. 2B). However, Gish RNAi in progenitor cells for a shorter period of time (3 days at $29^{0}$C) did not significantly increased ISC proliferation and resulted in increased *puc-lacZ* expression primarily in $GFP^{+}$ progenitor cells (Fig. S3A-C), suggesting that cell-autonomous JNK activation could be a direct effect of Gish inactivation while the non-cell-autonomous JNK activation observed in prolonged Gish RNAi guts is likely due to epithelial stress caused by ISC overproliferation similar to what has described by a previous study (Patel et al., 2015)".

Minor:

*1. Based on Fig.1, the authors found that Gish acts as a general repressor for JNK activity in the*

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*whole ISC-EB-EC lineage. What about the function of Gish in EEs?*

Response:

We did not examine the role of Gish in EEs. It would be interesting to determine what is the consequence of Gish inactivation in EEs in the future.

*2. In Figure 4N, what does the grey column represent? (pucRNAi?)*

We corrected this.

*3. In Figure 7I, it seems that overexpression of Gish in esg+ cells significantly repressed the levels of puc-lacZ in ECs, but not in ISCs/EBs. Please discuss the meaning of this result.*

Response:

We have now provided better images to show that overexpression of Gish in progenitor cells repressed the levels of *puc-lacZ* in both progenitor cells and ECs (revised Fig. 7H-J"), and stated "Gish overexpression in ISCs/EBs, not only suppressed puc-lacZ in progenitors but also in ECs. The non-cell-autonomous suppression of *puc-lacZ* in ECs is likely due to the reduced ISC proliferation in these guts".

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January 10, 2020

RE: JCB Manuscript #201909103R

Dr. Jin Jiang
Department of Molecular Biology
University of Texas Southwestern Medical Center at Dallas 6000 Harry hines Blvd.
Dallas, TX 5390

Dear Dr. Jiang:

Thank you for submitting your revised manuscript entitled "A CK1-Rho1-JNK signaling axis regulates tissue homeostasis and intestinal stem cell activity in adult Drosophila midgut". We would be happy to publish your paper in JCB pending final revisions necessary to meet our formatting guidelines (see details below).

- Please provide a short eTOC blurb
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"The kinase Gilgamesh (Gish)/CK1g regulates tissue homeostasis and stem cell activity in the adult Drosophila midgut"
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- Provide figures as separate, editable files according to the instructions for authors on JCB's website, paying particular attention to the guidelines for preparing images at sufficient resolution for screening and production
- Provide full methods for immunoprecipitation and western blot analysis
- Add conflict of interest statement to Acknowledgements section
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