Sirtuin-1 regulates organismal growth by altering feeding behavior and intestinal morphology in planarians

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MS TITLE: Sirtuin Signaling Regulates Organismal Growth by Altering Feeding Behavior and Stem Cell Differentiation in Planarians.

AUTHORS: Benjamin Ziman, Peter Karabinis, and Nestor Oviedo
ARTICLE TYPE: Research Article

I am very sorry for the delay, but it took an unusually long time to get reviewers for your manuscript.
We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers raise a number of substantial criticisms that prevent me from accepting the paper at this stage. They suggest, however, that a revised version might prove acceptable, if you can address their concerns. If you think that you can deal satisfactorily with the criticisms on revision, I would be pleased to see a revised manuscript. We would then return it to the reviewers.

Please 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. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

In this paper by Ziman, Karabinis and Oviedo, the authors examine the homology and functional roles of Sirtuin family of protein deacetylases in the planarian species Schmidtea mediterranea. Given the known role of Sirtuins in cellular metabolism and aging and the unique biology of planarians in which they can precisely regulate cell number and body proportion in response to food intake, such an analysis seems likely to prove informative.
As this has not been done thoroughly before, this paper represents an important contribution to the fields of planarian biology, aging and metabolism.
In performing this study, the authors make several important discoveries: 1) that planarians have a comparable number of Sirtuin homologs to humans, different from other common model organisms, 2) that only one of these homologs appears to have a significant phenotype upon RNAi depletion, 3) that depletion of the Smed Sirtuin 1 homolog leads to an interesting phenotype in which the worms fail to respond to normal feeding cues.
Further, the authors make good use of two available drugs that target this enzyme, in two distinct ways, to validate the sirtuin-1(RNAi) phenotype and provide insight into the mechanisms through which it operates.
Overall, this paper is worthy of publication and provides several interesting insights and experiments that researchers in our field will find valuable. However, I do have a few questions and suggestions that I believe will strengthen these contributions.

Comments for the author

MAJOR points:
- is sirtuin-1 expression induced upon feeding and/or resveratrol treatment? If so, in which cell types? Given the RNAi phenotype and drug treatment results, it seems possible. I realize that Sirtuin-1 "induction" may not be at the level of gene expression, but it is worth checking by in situ hybridization. If so, it could tell you which cell types respond most strongly.
- in the experiments described in Figure 5, do you see an increase in the expression of these genes upon resveratrol treatment? Based on your hypothesis, one would expect this.
- also in relation to Figure 5, in which cell types do these genes increase expression upon stimulation? Do additional (presumably neuronal) cells turn on expression or do the cells already expressing these genes increase the rate of gene expression? You could test this again, by in situ hybridization in "stimulated" worms vs control.
- I am unconvinced by the "loss of intestinal lineage" argument. If you take a WT worm and starve it to achieve similar shrinking and then perform the same intestinal staining and counting, do you see loss of gut branches? In other words, is this loss simply an indirect effect due to the process of de-growth? Whether caused by starvation or sirt-1(RNAi)?
- on this point, I might be more convinced if the change in gut gene expression were measured at a time point EARLIER than when sirt-1(RNAi) worms show decreased size.
That would suggest the intestinal differentiation program is effected in a potentially mechanistic manner.
There are two major findings I think are under-explored and may greatly contribute to the overall findings:
1. what happens to all the extra neoblasts in the sirt-1(RNAi) worms if they're not dying?
Does loss of sirt-1(RNAi) create a piwi-1+/H3P+ pool of quiescent stem cells? This question could be informed by BrdU staining -- do cells take up BrdU at 15 and 55 days post RNAi?
Do they contribute to differentiated tissues?
- is it possible they are turning into protonephridia? This cell type marker appears relatively increased when accounting for the decreased size in the sirt-1(RNAi) worms. Also, egr-5(RNAi) worms show both increased H3P and increased protonephridia (Tu et al eLife 2015).
2. why do sirt-1(RNAi) worms show decreased feeding and resveratrol worms increased?
Other than the experiment in Figure 5, this is not explored. As discussed above, more can be done in this experimental strategy to ask in which specific cell types the increase of gene expression upon food "stimulation" occurs. Also, expression should be assayed after resveratrol treatment.
MINOR points:
- in S3A and S3B, you cannot see the data for two concentrations of each drug. Perhaps make some lines "dotted" or "dashed" to make it more clear that they are layered on top of the control. Also,

you should be clear in the text that the higher concentrations kill the animals but that "middle" concentrations have a lesser effect of head regression.
- In figure 3A, the schematic is confusing. Move the word "soak" above the time point at which they are soaked and include a legend as in 3D.
- the data in S3C should be in the main paper. Also, it appears the table has two rows inverted. It should show that there is "ns" effect in row 1 (NAM vs NAM + sirt1(RNAi)) and a significant effect in row 2 (NAM vs control), correct?
- it is not surprising that NAM has a stronger effect than sirt-1(RNAi), as hydrolysis of NAD is needed for other cellular functions, but this should be addressed in the text.

Reviewer 2

Advance summary and potential significance to field

Sirtuins have been enzymes of great interest in the past decade, for their roles in aging and in other aspects of cellular health. Because Sirtuin knockdown/knockout leads to poor health of some model organisms, the authors argue that better models are needed to carefully study links between sirtuins and metabolism and body growth. This paper uses planarians as a model and carries out the first analysis of sirtuin function in planarians, focusing on a potential role for Sirtuin-1 in regulation of organismal size.

There are several aspects of this paper that are quite strong. Sirtuin-1(RNAi), resveratrol treatment, and nicotinamide treatment were used in parallel studies, which was a nice validation of the drug treatment approach. The authors created clever approaches to measure the time that it took animals to approach food and to quantify the amount of food ingested per feeding. I also appreciate the approach that the authors took toward quantifying intestinal branching. Though this manuscript is overall a strong effort with some interesting results, several changes in writing are needed to improve or clarify aspects of the manuscript and several additional experiments are warranted to better support the central claims of the paper.

Comments for the author

Essential revisions:
1. Some of the central claims of this manuscript are undersupported.
a. “Sirtuin signaling is evolutionarily conserved in planarians” is not supported because the authors do not investigate signaling beyond the function of Sirtuin-1 itself. In the absence of further data on the pathway or molecular function of planarian Sirtuin-1, the authors should stick to claims regarding the role of Sirtuin-1 itself.
b. The claim of Sirt-1(RNAi) causing “impaired differentiation of intestinal lineage progenitors that resulted in reduced branching of the gut” is not well supported. A reduction of some differentiation markers does not necessarily mean that fewer cells are differentiating. Further, the Sirt1(RNAi) phenotype involves feeding behaviors that might indirectly affect intestine physiology. Any claim that Sirt-1 affects differentiation should be supported with cell counts and labeling – for example, showing decreased incorporation of BrdU/EdU+ cells into the intestine as per Forsthoefel, et al 2011. Furthermore, some of the effects on gut differentiation and intestinal branching could be secondary to changes in nutrition or feeding behavior. This possibility should be addressed in writing or through additional RNAi paradigms.
c. The claim that sirtuin regulates growth “by affecting neural inputs associated with feeding behavior and tissue turnover” is not supported. There are no studies designed to assess neural inputs and tissue turnover is not directly measured.
2. The authors should discuss limitations of the chemical approaches (resveratrol and nicotinamide).
Obviously higher concentrations of these chemicals harmed the planarians (head regression and movement defects), indicating some possibility of off-target effects.
3. There are three concerns with the feeding assays that can be fairly easily addressed.
a. The overnight feeding assay is a bit concerning for two reasons. First, leaving animals in liver paste could foul the water and make animals sick. Second, the assay cannot distinguish between effects of eating more or less and defecating more or less. The authors should address this complication and consider a shorter-term feeding assay to support their results.

b. The authors also show that animals take longer to approach food; this result could be complicated if the animals are slower to move in general. Quantification of animal speed in the absence of food should help address this concern.
c. Lastly, the authors examine transcriptional responses after feeding (30 minutes, by QPCR). It isn't obvious to me that this is the right time scale or type of response to measure in terms of the animal's primary response to feeding. I would expect that most of the direct response to food should be related to sensory functions (or neural transmission) and be very quick. It seems likely that the observed transcriptional responses could be quite indirect and therefore less informative than claimed.
4. Another possible interpretation of the H3P results is that cells have prolonged M phases, which doesn't seem outside the realm of possibility, given that other sirtuin homologs regulate Polo kinase and Chk2.
a. The authors should consider pulse chase experiments to examine the length of the cell cycle in sirtuin(RNAi) animals.
5. The Caspase-3 antibody should be validated with caspase-3(RNAi) to determine specificity.

Minor points:
1. A phylogeny of planarian sirtuins would be very helpful and would allow the reader to determine whether the 6 planarian sirtuins are paralogous to human sirtuins as the authors imply. This would also help to demonstrate that all 6 proteins are bona fide sirtuins.
2. The authors did not state whether they had attempted combinatorial sirtuin knockdown to examine potential redundancy in this gene family.
3. The manuscript would be strengthened by moving some data into supplemental material. I'd recommend moving Fig. 1B-C to supplement, as these are data not created by the authors. I also think some elements like efficacy of sirtuin-1(RNAi) could be moved to supplement. This can help the authors focus on the main narrative of their paper and streamline the manuscript to emphasize the most important parts of their story.
4. "Surface area measurements" is a bit misleading because it implies that the authors measured total surface area of the animal's epidermis. Rephrase?
5. The paradigms in Figure 3 are unclear in that it looks like drugs were administered only once. Is this correct?
6. Since the dye feeding experiments are a new innovation of this work, it would be helpful to know how the animal's pigment affects 620 readings. Were there any differences in absorbance between RNAi treatments without dye feeding?
7. The cell division and apoptosis results could be easily complicated by nutrition availability secondary to changes in feeding. This should be addressed in writing or by other paradigms for RNAi experiments.
8. The authors should clarify that autophagy is not yet well understood in planarians and that markers Atg5 8, 12 are not yet characterized in planarians.
9. The authors should specify in more detail which statistical methods were used. In particular, it isn't clear if some statistical analyses were repeated measures ANOVA, which might be more appropriate for time course experiments.
10. The model figure would be of bigger benefit if it did not combine planarian data with data from other organisms (liver changes).

First revision

Author response to reviewers' comments

Rebuttal
In this paper by Ziman, Karabinis, and Oviedo, the authors examine the homology and functional roles of the Sirtuin family of protein deacetylases in the planarian species Schmidtea mediterranea. Given the known role of Sirtuins in cellular metabolism and aging and the unique biology of planarians in which they can precisely regulate cell number and body proportion in response to food intake, such an analysis seems likely to prove informative. As this has not been done thoroughly before, this paper represents an important contribution to the fields of planarian biology, aging and

metabolism. In performing this study, the authors make several important discoveries: 1) that planarians have a comparable number of Sirtuin homologs to humans, different from other common model organisms, 2) that only one of these homologs appears to have a significant phenotype upon RNAi depletion, 3) that depletion of the Smed Sirtuin 1 homolog leads to an interesting phenotype in which the worms fail to respond to normal feeding cues. Further, the authors make good use of two available drugs that target this enzyme, in two distinct ways, to validate the sirtuin-1(RNAi) phenotype and provide insight into the mechanisms through which it operates. Overall, this paper is worthy of publication and provides several interesting insights and experiments that researchers in our field will find valuable. However, I do have a few questions and suggestions that I believe will strengthen these contributions.

Reviewer 1 Comments for the Author...

1) MAJOR points: - is sirtuin-1 expression induced upon feeding and/or resveratrol treatment? If so, in which cell types? Given the RNAi phenotype and drug treatment results, it seems possible. I realize that Sirtuin-1 "induction" may not be at the level of gene expression, but it is worth checking by in situ hybridization. If so, it could tell you which cell types respond most strongly.

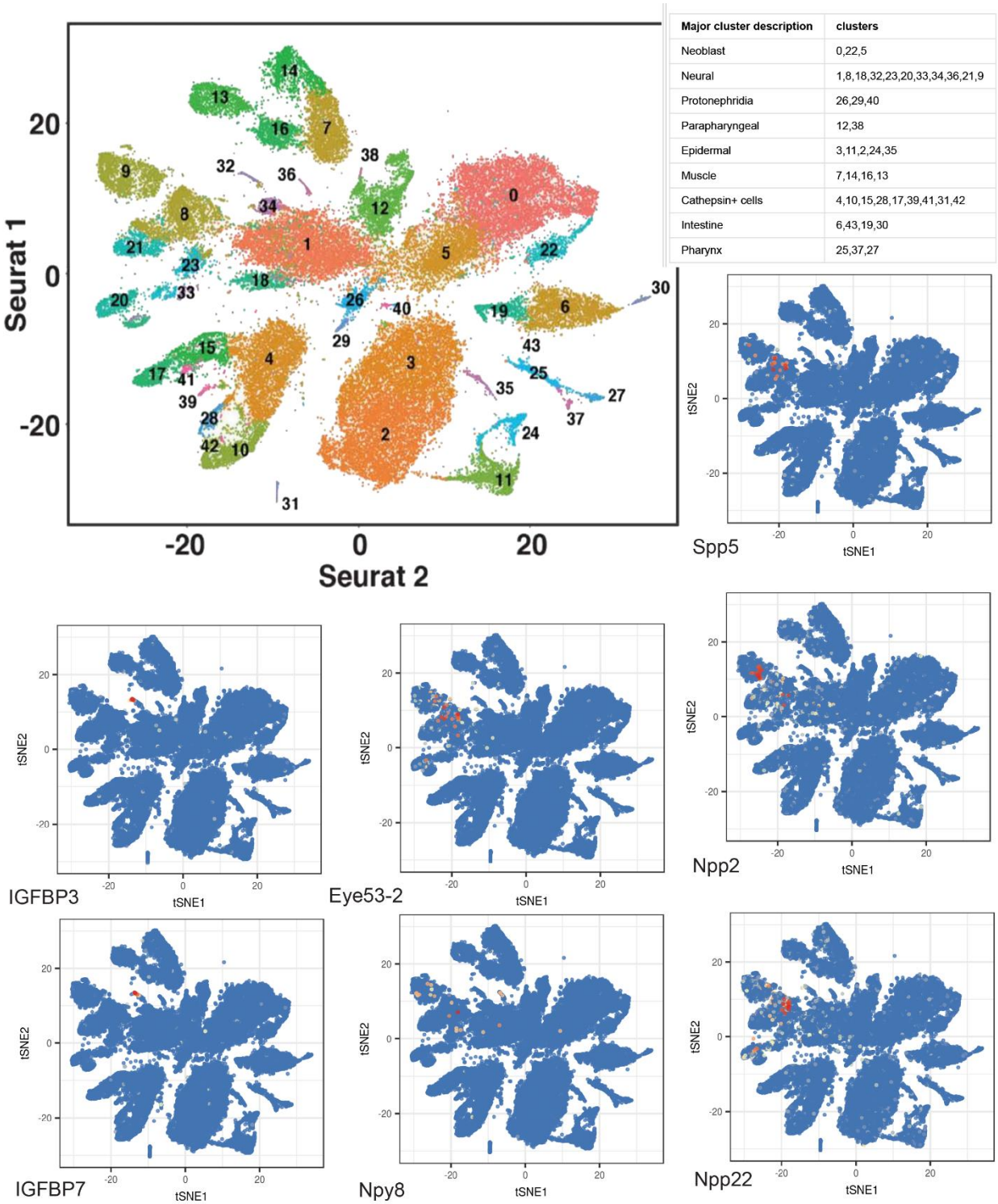
In response to the Reviewer's request, we did perform additional experiments involving gene expression levels upon feeding and Resveratrol (RESV) treatment. These results are now represented in two new figures (Figure S2F and S3D). From these experiments, we determined that upon feeding, Sirt-1 expression goes up as early as 6hrs and is gradually increased after 12 and 24 hrs post-feeding (36%; 64%; 60%, respectively). The specific time points post-feeding were chosen based on when the mitotic burst is expected to occur after feeding. On the other hand, RESV treatment does not induce Sirtuin-1 gene expression, but rather it reduces by 14%. This is consistent with other findings demonstrating that RESV acts at the post-translational level.

Regarding the identity of cells responding to Sirt-1(RNAi), we are technically limited based on the diffuse pattern of Sirt-1 expression (Figure 1E, S2H). However, the in silico analysis performed from single-cell database, suggest that Sirt-1 expression does not appear restricted to a specific cellular cluster, but rather scattered across various cell types including muscle, neural, protonephridia, epidermal, neoblasts, among others (Figure S2H). Together, these results suggest that different cell types may be responding to Sirt-1(RNAi). In the future, we plan to resolve this by single-cell sequencing; nonetheless, this is out of the scope of the current manuscript.

2) in the experiments described in Figure 5, do you see an increase in the expression of these genes upon resveratrol treatment? Based on your hypothesis, one would expect this. - also in relation to Figure 5, in which cell types do these genes increase expression upon stimulation? Do additional (presumably neuronal) cells turn on expression or do the cells already expressing these genes increase the rate of gene expression? You could test this, again, by in situ hybridization in "stimulated" worm's vs control. –

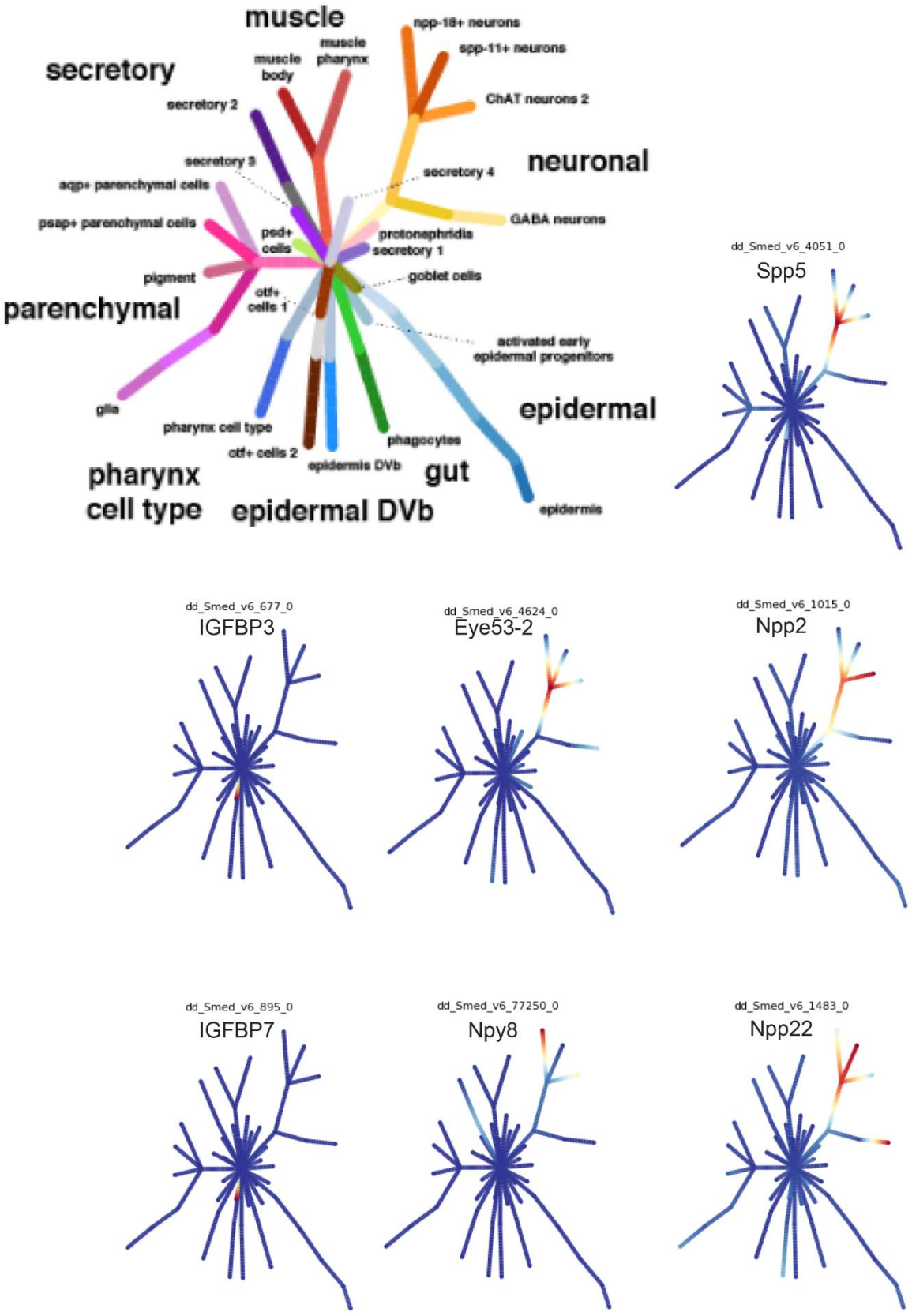
We did the experiment with RESV treatment and found an increase in the expression of all the genes tested compared to the RNAi. To accommodate the request from the Reviewer#2, these data is now in Figure S5B. The new Figure 4B includes gene expression analysis at earlier time points after stimulation. This way, we suggest that the primary feeding response of transcriptomic changes take place earlier upon stimulation.

Regarding the cell types expressing the genes in the stimulation assay, we agree with the Reviewer that neuronal cells are expressing these genes. This is further supported by previous publications demonstrating spatial distribution with ISH and the single-cell prediction associated with the CNS. For example, NPY's, Npp's, and Spp's, IGFBP's; ILP, ILR; 1020HH, and FLI-1 are all found in the CNS (Plass et al. 2018; Rozanski et al. 2018; Miller and Newmark, 2012; Inoue et al. 2004; Roberts-Galbraith et al. 2016). Additionally, we confirmed the neural expression of those genes by in silico analysis shown below.



Major cluster description	clusters
Neoblast	0,22,5
Neural	1,8,18,32,23,20,33,34,36,21,9
Protonephridia	26,29,40
Parapharyngeal	12,38
Epidermal	3,11,2,24,35
Muscle	7,14,16,13
Cathepsin+ cells	4,10,15,28,17,39,41,31,42
Intestine	6,43,19,30
Pharynx	25,37,27

T-SNE plot of single cells displaying *Smed-Sirt-1* expression among major clusters of neoblasts and differentiated cells. Gene expression levels are based on the scale bar top right (red is high, and blue is low). The single-cell gene expression was obtained from the cell type transcriptome atlas database, provided by the Reddien Lab at the Whitehead Institute for Biomedical Research (Fincher et al., 2018).



Two-dimensional t-SNE showing cell type populations. Gene expression levels are based on red is high, and blue is low. Data was obtained using resources published by the Rajewsky lab at the Berlin Institute for Medical Systems Biology (Plass et al. 2018)

3) I am unconvinced by the "loss of intestinal lineage" argument. If you take a WT worm and starve it to achieve similar shrinking and then perform the same intestinal staining and counting, do you see loss of gut branches? In other words, is this loss simply an indirect effect due to the process of de-growth? Whether caused by starvation or sirt-1(RNAi)? - on this point, I might be more convinced if the change in gut gene expression were measured at a time point EARLIER than when sirt-1(RNAi) worms show decreased size. That would suggest the intestinal differentiation program is effected in a potentially mechanistic manner

We do see lack of intestinal branches (IPBL) in the experimental group as early as 15 days post-RNAi. During this period, there is no feeding, and the animals are about the same size as controls (Figure 6I, J, K). Therefore, we argue that the loss of IPBL is not necessarily due to the reduction in animal length but rather to a manifestation of Sirt-1 downregulation.

4) There are two significant findings I think are under-explored and may greatly contribute to the overall findings: 1. what happens to all the extra neoblasts in the sirt-1(RNAi) worms if they're not dying? Does loss of sirt-1(RNAi) create a piwi-1+/H3P+ pool of quiescent stem cells? This question could be informed by BrdU staining -- do cells take up BrdU at 15 and 55 days post RNAi? Do they contribute to differentiated tissues? - is it possible they are turning into protonephridia? This cell type marker appears relatively increased when accounting for the decreased size in the sirt-1(RNAi) worms. Also, egr- 5(RNAi) worms show both increased H3P and increased protonephridia (Tu et al eLife 2015).

We performed multiple experiments involving cell cycle with flow cytometry and gene expression analyses with qPCR and WISH. The results revealed that Sirt-1 downregulation leads to transcriptomic defects characterized by a reduction in progenitor's markers of most lineages (e.g., gut, pharynx, parapharyngeal, muscle, and epidermal). Conversely, as the phenotype progresses, markers for differentiated tissues tend to reduce the expression except for protonephridia, epidermal and, epithelial tissues that showed no changes or increases, respectively (Figure 6A, B). Whole-mount *in situ* hybridization (WISH) confirmed expression in protonephridia is not reduced after *Smed-Sirt-1(RNAi)*. These results also suggest there may be dysregulation in the expression of markers corresponding to different segments of protonephridia (Figure 6C). To test this assumption, we measured levels of expression for genes found in protonephridia tubules (proximal and distal) and collecting ducts (Figure 6D, E). The results demonstrate there was a tendency for upregulation in markers for the proximal tubules, which is consistent with the possibility of *Smed-Egr5* downregulation. Loss of *Smed-Egr5* function induces the proliferation of neoblasts accompanied by an increase in the expression of protonephridia markers. We found that *Smed-Sirt-1(RNAi)* animals displayed a 22% and 27% decrease in the expression of *Smed-Egr5*, 15, and 55 days after the first injection, respectively (Figure 6F). Altogether, *Smed-Sirt-1* may regulate tissue-specific transcriptomic dynamics in the protonephridia.

Additional experiments with flow cytometry suggest that Sirt-1(RNAi) alters cell cycle dynamics by possibly reducing the number of cells by 10% in G1 and extending the time of the G2/M phases by 7% (Figure S6). Altogether, the results show that Sirt-1(RNAi) interferes with the proper expression of most lineage markers and cell cycle dynamics. We speculate about a possible scenario in which lineage differentiation towards protonephridia and epithelia are favored in the absence of the Sirt-1 function.

5) why do sirt-1(RNAi) worms show decreased feeding and resveratrol worms increased? Other than the experiment in Figure 5, this is not explored. As discussed above, more can be done in this experimental strategy to ask in which specific cell types the increase of gene expression upon food "stimulation" occurs. Also, expression should be assayed after resveratrol treatment.

Our results show that Sirt-1(RNAi) acts to inhibit its function, whereas RESV enhances the Sirt-1 function as anticipated. This is consistent with results disrupting/enhancing the Sirt-1 function in other model organisms. Regarding the identity of cells mediating these responses, we believe that neural cells are the targets, as explained in the comments above (please see #2). This is included in

the result section of Figure 5. We also found that the treatment with RESV increases the expression of most genes downregulated by Sirt-1(RNAi) while enhancing the expression of some genes upregulated in the control group.

6) MINOR points: - in S3A and S3B, you cannot see the data for two concentrations of each drug. Perhaps make some lines "dotted" or "dashed" to make it more clear that they are layered on top of the control. Also, you should be clear in the text that the higher concentrations kill the animals but that "middle" concentrations have a lesser effect of head regression. - In figure 3A, the schematic is confusing. Move the word "soak" above the time point at which they are soaked and include a legend as in 3D. - the data in S3C should be in the main paper.

As requested by the Reviewer, we added dotted lines for the concentrations that were overlapping in Figure S3A. We added clarifications in the text of the result section about the titration and effects of the drug. We modified Figure 3A (now Figure 2A) as requested by the Reviewer. The legend for Figure 3A was also edited as recommended. Additionally, we moved Figure S3C (now Figure 2E) to the main paper.

7) Also, it appears the table has two rows inverted. It should show that there is "ns" effect in row 1 (NAM vs NAM + sirt1(RNAi)) and a significant effect in row 2 (NAM vs control), correct? -

Yes, that was a mistake and it has been corrected.

8) It is not surprising that NAM has a stronger effect than sirt-1(RNAi), as hydrolysis of NAD is needed for other cellular functions, but this should be addressed in the text.

Done, we have addressed the effects of NAM in the discussion as recommended.

Reviewer 2 Advance Summary and Potential Significance to Field...

Sirtuins have been enzymes of great interest in the past decade, for their roles in aging and in other aspects of cellular health. Because Sirtuin knockdown/knockout leads to poor health of some model organisms, the authors argue that better models are needed to carefully study links between sirtuins and metabolism and body growth. This paper uses planarians as a model and carries out the first analysis of sirtuin function in planarians, focusing on a potential role for Sirtuin-1 in regulation of organismal size. There are several aspects of this paper that are quite strong. Sirtuin-1(RNAi), resveratrol treatment, and nicotinamide treatment were used in parallel studies, which was a nice validation of the drug treatment approach. The authors created clever approaches to measure the time that it took animals to approach food and to quantify the amount of food ingested per feeding. I also appreciate the approach that the authors took toward quantifying intestinal branching. Though this manuscript is overall a strong effort with some interesting results, several changes in writing are needed to improve or clarify aspects of the manuscript and several additional experiments are warranted to better support the central claims of the paper.

Reviewer 2 Comments for the Author...

Essential revisions: 1. Some of the central claims of this manuscript are undersupported.

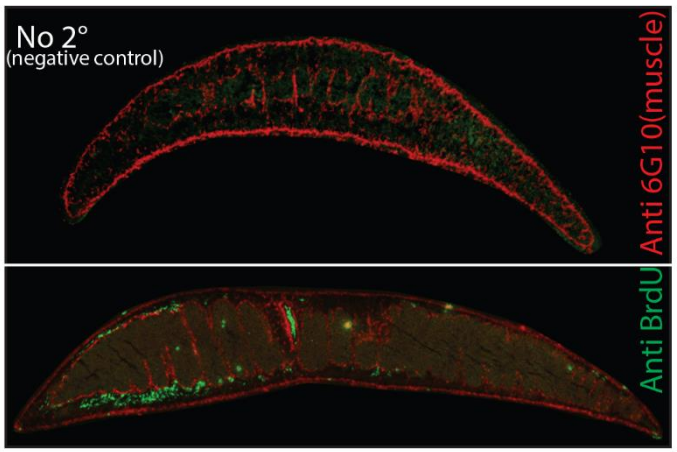
9) a. “Sirtuin signaling is evolutionarily conserved in planarians” is not supported because the authors do not investigate signaling beyond the function of Sirtuin-1 itself. In the absence of further data on the pathway or molecular function of planarian Sirtuin-1, the authors should stick to claims regarding the role of Sirtuin-1 itself.

We agree with the Reviewer, we have removed the words “Sirtuin signaling” and replaced it with “Sirtuin-1”.

10) b. The claim of Sirt-1(RNAi) causing “impaired differentiation of intestinal lineage progenitors that resulted in reduced branching of the gut” is not well supported. A

reduction of some differentiation markers does not necessarily mean that fewer cells are differentiating. Further, the Sirt1(RNAi) phenotype involves feeding behaviors that might indirectly affect intestine physiology. Any claim that Sirt-1 affects differentiation should be supported with cell counts and labeling – for example, showing decreased incorporation of BrdU/EdU+ cells into the intestine as per Forsthoefel, et al. 2011. Furthermore, some of the effects on gut differentiation and intestinal branching could be secondary to changes in nutrition or feeding behavior. This possibility should be addressed in writing or through additional RNAi paradigms.

We agree with the Reviewer and eliminated our claims regarding the role of Sirt-1 in differentiation, and also modified the title of the manuscript to account for these changes. We followed the Reviewer's recommendation and attempted to the BrdU experiments but no avail. Specifically, we performed the protocol as stated in Forsthoefel et al., 2011, and the results were inconsistent across different experiments. We were particularly unsure about the BrdU signal, and despite several attempts troubleshooting, we concluded our protocol needs more optimization (see below). To comply with the timeline of the resubmission, we decided to change our claim about differentiation and continue working on refining the BrdU protocol.



Sagittal cross-sections with anti-BrdU and Anti 6G10(muscle). Animals were fed BrdU and fixed 24 hours later. All steps were followed, as noted in Forsthoefel et al., 2011.

11) c. The claim that sirtuin regulates growth "by affecting neural inputs associated with feeding behavior and tissue turnover" is not supported. There are no studies designed to assess neural inputs and tissue turnover is not directly measured.

We agree with the Reviewer and have removed the phrase "neural inputs associated with feeding behavior and tissue turnover."

12) 2. The authors should discuss limitations of the chemical approaches (resveratrol and nicotinamide). Obviously higher concentrations of these chemicals harmed the planarians (head regression and movement defects), indicating some possibility of off-target effects.

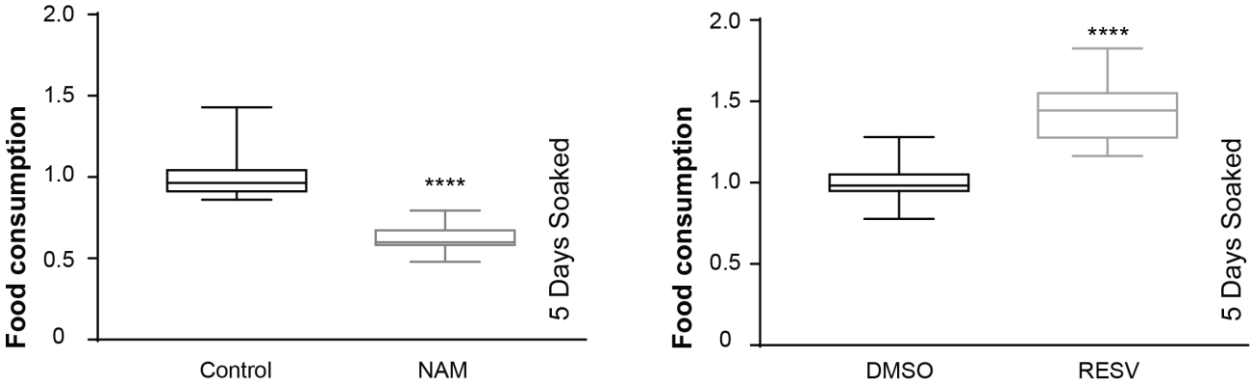
The reviewer is correct that these drugs could have some potent off-target effects. We have addressed this in the discussion (please see the third paragraph).

13) 3. There are three concerns with the feeding assays that can be fairly easily addressed. a. The overnight feeding assay is a bit concerning for two reasons. First, leaving animals in liver paste could foul the water and make animals sick

Second, the assay cannot distinguish between effects of eating more or less and defecating more or less. The authors should address this complication and consider a shorter- term feeding assay to support their results.

In response to the Reviewer's concern, we did perform experiments using shorter feeding times (i.e., 1 hour) and found similar results to overnight feedings, as shown in Figure 3H-I. These results are included below and since they were no different from the data reported in the paper. We

included a statement in the Methods section about the length of the feeding. It is worth noting that upon overnight feedings, we do not observe abnormal behavior or signs of sickness, including external tissue damage in any of the experiments included in this manuscript or previous work from our lab (Thiruvalluvan et al. 2018). We do concede that it is challenging no matter what method is used to determine the amount of food ingested or expelled per animal.



Changes in food consumption among pharmacologically soaked animals. Animals were soaked with either Nicotinamide (NAM) or Resveratrol (RESV) for five days before feeding. Data was obtained using at least 20 animals with two biological replicates. ****p<0.0001; Unpaired t-test.

14) b. The authors also show that animals take longer to approach food; this result could be complicated if the animals are slower to move in general. Quantification of animal speed in the absence of food should help address this concern.

To address this concern, we performed: 1) video recordings and 2) immunostainings to visualize cilia on the ventral surface of the animals. First, we did not observe any visible impairment in the movement of Sirt-1(RNAi) animals. In addition to this qualitative observation, we used video recordings and traced the distance and the length of time it took for animals to move across a delimited surface. Both control and experimental animals displayed similar gliding dynamics and covered in the same range of time the distance recorded. The results are included in the supplement Figure S4A. Second, Using the anti-acetylated tubulin antibody, we compared patterns of the signal in the ventral surface where the locomotion cilia are located. We did not observe apparent differences between control and Sirt-1(RNAi) Figure S4B. These data strongly support the idea that differences in time to reach food in Sirt-1(RNAi) animals are not due to physical impairment in locomotion.

15) c. Lastly, the authors examine transcriptional responses after feeding (30 minutes, by QPCR). It isn't obvious to me that this is the right time scale or type of response to measure in terms of the animal's primary response to feeding. I would expect that most of the direct response to food should be related to sensory functions (or neural transmission) and be very quick. It seems likely that the observed transcriptional responses could be quite indirect and therefore less informative than claimed.

We agree with the Reviewer's request, and in response, we evaluated gene expression analysis in a shorter time scale (i.e., 3-5 mins). As suggested, the transcriptomic response is different from the previous observation. This experiment showed differences in the expression of genes associated with feeding behavior between control and Sirt-1(RNAi). This suggests that the stimulation assay could be used to determine transcriptomic changes immediately after food exposure. Interestingly, these genes (e.g., ALS and npy3) were increased upon stimulation in the control group, further enhanced upon RESV treatment, and decreased in Sirt-1(RNAi) animals. The reasons for such changes are not readily evident, and future experiments will be needed to address the mechanisms. Since we were able to detect transcriptional changes at an early time point, we decided to display the new results in Figure 4B and move to supplement the previous data with the late changes after feeding (Figure S5A, B). Also, the text was modified in the results and discussion section to account for the new data.

16) 4. Another possible interpretation of the H3P results is that cells have prolonged M phases, which does not seem outside the realm of possibility, given that other sirtuin homologs regulate Polo kinase and Chk2. a. The authors should consider pulse chase experiments to examine the length of the cell cycle in sirtuin(RNAi) animals.

Yes, the Reviewer is correct. We measured the length of the cell cycle in control and Sirt-1(RNAi) animals with flow cytometry using our improved protocol (Peiris et al., 2016a; Peiris et al., 2016b). We found that Sirt-1(RNAi) animals appeared to have about 10% fewer cells in the G1 phase, 1.5% more cells in the S phase, and 7% more cells in the G2-M phase. These results are included in the new supplemental Figure S6. The results are consistent with the increased number of H3P positive cells in Sirt-1(RNAi) animals. Together, the increased number of cells in the G2/M phases may suggest that Sirt-1(RNAi) animals have a prolonged M phase. This possibility, along with the new data, is included in the manuscript results and discussion sections.

17) 5. The Caspase-3 antibody should be validated with caspase-3(RNAi) to determine specificity.

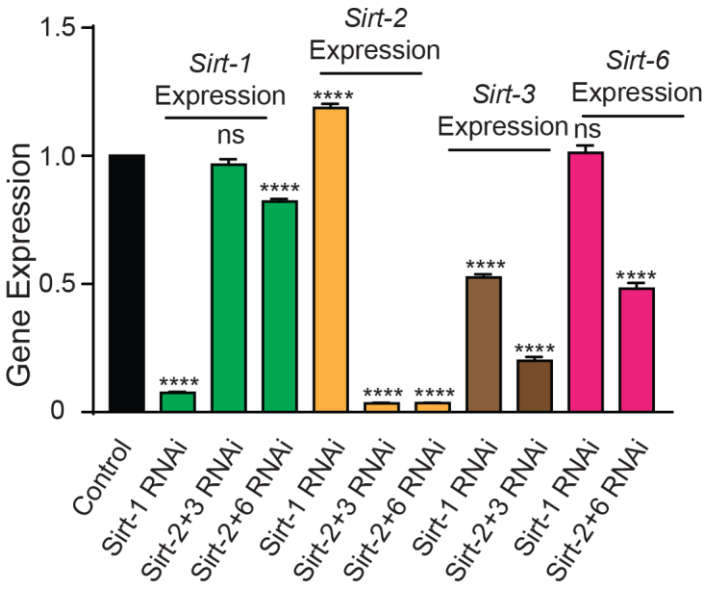
We appreciate the concerns of the reviewer with the specificity of the Caspase-3 antibody. This antibody has been previously validated in *Schmidtea mediterranea*. Specifically, we have shown that the intensity of the signal corresponding to caspase-3 increases in areas where cell death is known to occur (Thiruvalluvan et al., 2018). We also contacted Abcam and confirmed that this epitope is conserved in *Schmidtea mediterranea*.

18) Minor points: 1. A phylogeny of planarian sirtuins would be very helpful and would allow the reader to determine whether the six planarian sirtuins are paralogous to human sirtuins as the authors imply. This would also help to demonstrate that all six proteins are bona fide sirtuins.

We created a phylogenetic tree showing both human and planarian Sirtuins 1-6. The results suggest the proteins reported in the manuscript are bona fide Sirtuins. The new data is displayed in Figure S1A. Specifically, we found that except for Sirtuin-2 (only 42% conservation with humans), all other Sirtuins (1, 3-6) are well conserved between humans and planarians. Future studies will address the apparent difference between the planarian Sirt-2 and its human counterpart. We gave less weight on this as Sirt-2 did not lead to any noticeable phenotype and did not alter the focus of this manuscript on Sirt-1.

19) 2. The authors did not state whether they had attempted combinatorial sirtuin knockdown to examine potential redundancy in this gene family.

We did combinations of RNAi to determine if these would produce gross morphological defects, which we did not observe. To answer this question quantitatively, we performed qPCR to measure changes in gene expression. We did two groups of combinatorial RNAi. The first was the nuclear sirtuins (2,6). Since Sirtuin-1 is also nuclear, we reasoned this could provide some clarity. We found that upon Sirt-2,6(RNAi), we had a decrease in the expression of Sirtuin-1.
Additionally, we found that upon Sirt-1(RNAi), we had an increase in Sirtuin-2 expression. Next, we performed combinatorial RNAi on the closely related planarian sirtuins (2,3). We did not find any alterations in Sirtuin-1 expression in Sirt-2,3(RNAi) animals. However, we found a decrease in the expression of Sirtuin-3 in Sirt-1(RNAi) animals. While we found compensation to be occurring possibly, we did not find any of these Sirtuins (2,3,6) to influence growth, as observed with Sirt-1(RNAi). The experiments suggest there is some degree of redundancy. However, future experiments would be implemented with additional permutations and biochemical characterizations to carefully dissect the extent of the compensatory roles in the planarian Sirtuin function.



Gene Expression levels of Sirtuin markers, in RNAi animals 15 days after the 1st injection. Gene expression is represented as fold change normalized to control. Data were obtained from triplicates per experiment using at least two biological replicates. ****p<0.0001; one-way ANOVA.

20) 3.The manuscript would be strengthened by moving some data into supplemental material. I'd recommend moving Fig. 1B-C to supplement, as these are data not created by the authors. I also think some elements like efficacy of sirtuin-1(RNAi) could be moved to supplement. This can help the authors focus on the main narrative of their paper and streamline the manuscript to emphasize the most important parts of their story.

We agree with the Reviewer and moved Figures 1B, C to the Supplement as requested. The efficacy of Sirtuin-1(RNAi) Figures 2B, D are now Figures S2A, D. The new arrangement merges the original Figure 1A with Figure 2 (now Figure 1E).

21) 4. "Surface area measurements" is a bit misleading because it implies that the authors measured total surface area of the animal's epidermis. Rephrase?

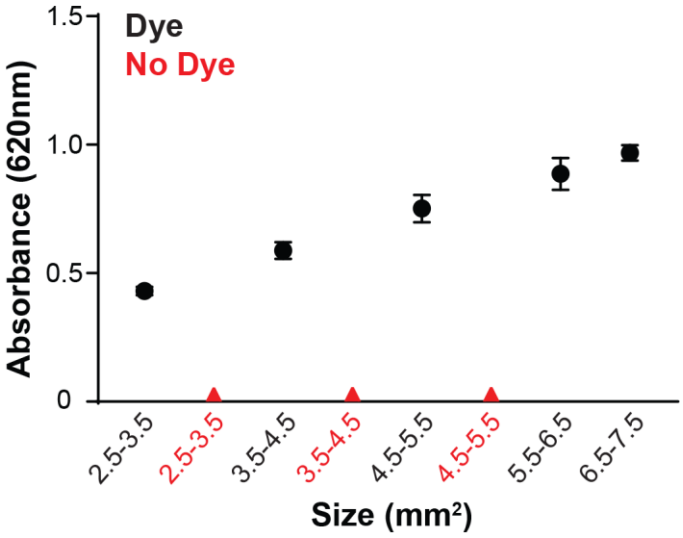
We have rephrased the "Surface Area" to now read as the "Δ Animal Area."

22) 5. The paradigms in Figure 3 are unclear in that it looks like drugs were administered only once. Is this correct?

The drugs were administered every other day for the length of the time course. To help clarify this, we placed an arrow to indicate the length of soaking with drugs. The figure legends and methods should also state this to help avoid any confusion.

23) 6. Since the dye feeding experiments are a new innovation of this work, it would be helpful to know how the animal's pigment affects 620 readings. Were there any differences in absorbance between RNAi treatments without dye feeding?

The Reviewer is correct. In an attempt to determine whether the 620nm readings influenced by the pigment in animals of different sizes (2.5-3.5, 3.5-4.5, 4.5-5.5 mm^2), we performed the experiment without the addition of dye. The results suggest the pigmentation in animals of different sizes was negligible compared to the presence of the dye (see below). Therefore, we do not believe that pigment influences the results in our measurements. Additionally, we did not find any difference in the pigment between control and Sirt-1(RNAi) animals.



24) 7. The cell division and apoptosis results could be easily complicated by nutrition availability secondary to changes in feeding. This should be addressed in writing or by other paradigms for RNAi experiments.

Yes, we agree that nutrition can influence cell division and apoptosis. We added text in the material and methods section to clarify our analysis at different time points. Briefly, in our experiments, we included both starved animals (15 days RNAi) and fed animals (55 days RNAi). Additionally, feed animals (55 days RNAi) were starved for five days before any evaluations of cell division/death. We found that at specific time points, the trend in cell division/death was consistent in both starved and fed animals.

25) 8. The authors should clarify that autophagy is not yet well understood in planarians and that markers Atg5, 8, 12 are not yet characterized in planarians.

We agree with the reviewer that autophagy is not yet well understood in planarians. We have added to the results section that these are markers necessary for autophagosome formation in other models, and these are interpretations of autophagy.

26) 9. The authors should specify in more detail which statistical methods were used. In particular, it isn't clear if some statistical analyses were repeated measures ANOVA, which might be more appropriate for time course experiments.

In response to the Reviewer's observation, we have added the corresponding details to the methods section.

27) 10. The model figure would be of bigger benefit if it did not combine planarian data with data from other organisms (liver changes).

Yes, we agree. The figure was modified accordingly.

Second decision letter

MS ID#: JOCES/2019/239467

MS TITLE: Sirtuin-1 Regulates Organismal Growth in Planarians by Altering Feeding Behavior and Intestinal Morphology.

AUTHORS: Benjamin Ziman, Peter Karabinis, Paul G Barghouth, and Nestor Oviedo
ARTICLE TYPE: Research Article

Please accept my apologies for the unusual delay in my decision. The reason is that due to the COVID outbreak my past days have been hectic, preparing the shutdown of the labs and organizing on-line teaching.

We have now reached a decision on the above manuscript.

To see the reviewers' reports and a copy of this decision letter, please go to: https://submit-jcs.biologists.org and click on the 'Manuscripts with Decisions' queue in the Author Area. (Corresponding author only has access to reviews.)

As you will see, the reviewers recognize that most of their initial concerns have been addressed in your revised manuscript. However, both still raised comments that will require amendments to your manuscript. I hope that you will be able to carry these out, because I would like to be able to accept your paper.

Please 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. Please attend to all of the reviewers' comments. If you do not agree with any of their criticisms or suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The authors have done a thorough job of addressing the questions and concerns I raised within the limitations of their system and time available. I recommend the revised manuscript for publication.

Comments for the author

My one comment is that I do not fully agree with the changes made in response to a comment by reviewer #2 regarding the time point at which to measure changes in gene expression after liver stimulation. Although transcription from DNA to mRNA by Pol II may be completed for most genes within 3-5 minutes, the assay used here (qPCR on cDNA) largely measures completely processed, steady-state transcripts (spliced, polyadenylated, etc). Thus 3-5 minutes will most accurately assay for changes in those genes that are transcribed and processed rapidly. I would advocate for moving the data from the 30 minute time point into the main figures/text, as together the two time points give a more complete picture of the changes that occur after liver stimulation.

Reviewer 2

Advance summary and potential significance to field

This manuscript characterizes a planarian sirtuin and shows that it has roles in growth through changes in feeding behavior and intestinal morphology.

Comments for the author

The authors have submitted a very thoughtful and thorough revision of this manuscript. They have taken care of nearly all of the prior critiques; the manuscript has been improved over the prior

submission with the inclusion of new data, further quantification and changes in writing. At this point, I recommend that the manuscript be published in the Journal of Cell Science.

One minor change that I would recommend is an indication of which QPCR results are significantly different after drug treatment. The graph shows increases or decreases in transcript abundance but not whether the changes are significantly different.

Second revision

Author response to reviewers' comments

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Reviewer 1 Comments for the author
My one comment is that I do not fully agree with the changes made in response to a comment by reviewer #2 regarding the time point at which to measure changes in gene expression after liver stimulation. Although transcription from DNA to mRNA by Pol II may be completed for most genes within 3-5 minutes, the assay used here (qPCR on cDNA) largely measures completely processed, steady-state transcripts (spliced, polyadenylated, etc). Thus 3-5 minutes will most accurately assay for changes in those genes that are transcribed and processed rapidly. I would advocate for moving the data from the 30 minute time point into the main figures/text, as together the two time points give a more complete picture of the changes that occur after liver stimulation.

In response to the comments by the Reviewer, we placed together in Fig. 4 the gene expression data for both time points (5 and 30 mins) as suggested. The text corresponding to figure legends and the calls for each figure were adjusted accordingly.

Reviewer 2 Advance summary and potential significance to field
This manuscript characterizes a planarian sirtuin and shows that it has roles in growth through changes in feeding behavior and intestinal morphology.

Reviewer 2 Comments for the author
The authors have submitted a very thoughtful and thorough revision of this manuscript. They have taken care of nearly all of the prior critiques; the manuscript has been improved over the prior submission with the inclusion of new data, further quantification and changes in writing. At this point, I recommend that the manuscript be published in the Journal of Cell Science.

One minor change that I would recommend is an indication of which QPCR results are significantly different after drug treatment. The graph shows increases or decreases in transcript abundance but not whether the changes are significantly different.

The statistical analysis was performed, and the results are now included as part of the supplementary fig 5 (Fig S5). The Fig S5 includes two tables with the list of genes and the comparison for each of the conditions in the experiment. The figure legend was adjusted accordingly to reflect the significance when appropriate.

We thank both Reviewers for the insights and the time invested. We believe the incorporation of the suggestions improved the quality of the article.

Third decision letter

MS ID#: JOCES/2019/239467

MS TITLE: Sirtuin-1 Regulates Organismal Growth in Planarians by Altering Feeding Behavior and Intestinal Morphology.

AUTHORS: Benjamin Ziman, Peter Karabinis, Paul G Barghouth, and Nestor J Oviedo
ARTICLE TYPE: Research Article

I am happy to tell you that your manuscript has been accepted for publication in Journal of Cell Science, pending standard ethics checks.