Nutrition and PI3K/Akt signaling are required for p38-dependent regeneration

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

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

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

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

Reviewer 1

Advance summary and potential significance to field

The submitted paper is an analysis of the Akt/Ask1 activation of p38 and JNK in the contexts of undamaged and damaged imaginal discs, examining how nutrient sensing via insulin signaling might alter this mechanism. The authors build upon previous work (Santabarbara-Ruiz 2019) that showed Ask1 is activated in dying and regenerating cells of genetically ablated wing discs, and its behavior is modified by Akt1 in regenerating cells to induce p38 and JNK expression.

In this extension of the work, the authors have examined this model further. The experiments demonstrate that combined expression of Pdk1 and Akt in undamaged wing discs activates P38 downstream of Ask1, and requires the highly conserved S83 residue of Ask1. By contrast this same experiment shows JNK signaling is not activated by Pdk1/Akt/Ask1 expression. A kinase domain mutant of Ask1 does not activate either P38 or JNK signaling, suggesting this domain is necessary for P38 activation. Since PI3K is known to alter Akt behavior, the authors speculate that Ask1 might be a link between nutrient sensing and its impact on regeneration.
Using genetic ablation experiments it is shown that nutrient restriction inhibits regeneration, which can be rescued by expression of a kinase upstream of P38, Lic.
Finally, expression of the Ask1 S83 mutant that does not activate P38 fails to regenerate (in normal nutrition conditions), which again can be rescued by Lic.
This work is an important addition to our understanding of how nutritional sensing might influence regeneration via the Akt/Ask1 proteins regulation of P38 however the work presented is only a moderate advance.

Comments for the author

- The experiments in Figures 2 and 3 demonstrate regenerative ability of wings following genetic manipulations. To more strongly support their conclusions the authors should show the imaginal discs from these experiments to illustrate the expression and activation of Ask1 (p-Thr), and P38 (p-P38) in regenerating discs. For example, although expression of Lic is shown to upregulate p38 in the pouch (Sup Figure 2), these additional data would confirm Lic activates P38 in the regenerating disc, as well as demonstrating the cells in which this occurs, and that this is the reason for the altered regeneration phenotype. These experiments may also reveal why Lic expression in standard food (figure 2) significantly limits regeneration alone.
- The work in this paper suggests that P38 is activated downstream of Ask1 (Figure 1D), but JNK is not (Figure 1H). This finding is surprising in light of the authors previous paper in which it is shown that Ask1 activates both P38 and JNK (shown by Mmp1 expression) in regenerating discs. The current work showing p-JNK activity is performed in undamaged discs, and shows no changes in JNK signaling by any manipulation performed. In damaged discs with increased JNK (HepWT expression, Figure 2 and 3) regeneration is either unaffected or inhibited.
Therefore it is important for the authors to address these results more clearly in the context of their previous work.
- In the experiments of Figures 2 and 3, the wing size and percent of regenerated wings is measured in the 29'C conditions (ablated and expressing the transgene) and the 17'C condition (unablated and not expressing the transgene).
The essential control experiment, in which the transgenes are expressed in unablated discs, was not performed. This experiment is very important to interpret the results presented.
- In Figure 1D the authors show that P38 is activated by the combination of Pdk1/Akt/Ask. They also show that the phosphorylation of the kinase domain (detected by p-Thr) is important for this activation (Figure 1K). However, in Supplemental Figure 1G, the p-Thr antibody does not detect Ask1, suggesting this domain is not phosphorylated. The authors should clarify why they don't see any p-Thr phosphorylation, despite seeing P38 activation in this experiment.
- It would be helpful to include controls to demonstrate the detection of p-JNK and p-P38 in wild type wing discs. Although the authors do show discs with tissue that is not genetically manipulated (the anterior compartments of discs in Figure 2A-B) p-JNK could be altered at a distance in these experiments, while p-P38 is not shown in a wild type disc in the paper.

Minor Comments

- The section describing the experiments in Figure 1B-D is somewhat unclear and could be written with more clarity to help the reader understand the work being done.
- In Figure 1D there are high levels of activated P38. The authors mention that high P38 activity kill cells (page 7). Can the authors comment on whether this would complicate their analysis?
- Page 7 “possibly because of a reduction of the insulin signal, and show a release of P-Thr Ask1 activity”. Is there evidence of the lower insulin signaling in dying cells or is this speculation? Use of the word “release” is confusing, and could indicate increase or decrease.
- Page 5 ”We propose here that this attenuation results in low levels of Ask1 activity but sufficient to trigger p38 function and to avoid the high Ask1 activity that can result in cell death”. This conclusion and the description of the supporting evidence are difficult to follow, as they describe an increase in staining but attenuation of activity. This section could be clarified for the reader.
- Some knowledge is presupposed but the authors, particularly the connection between PI3K and Akt, and Ask1 and Licorn. A simple pathway diagram or more background in the introduction would help the reader understand the field better and where this work is positioned within it.
- The authors conclude that increasing P38 activity rescues regeneration in nutrient deficient conditions. Page 7 “Altogether, these results show that p38 is able to rescue the effects of nutrient limitation and implies that p38 is highly sensitive and responsive to nutrients to drive regeneration”. However, they actually show Lic rescues regeneration, and that in undamaged discs a pouch with high Lic expression has high P38. The conclusion follows, but is not what the authors show.
- Can the authors include a detail of why they think HepWT expression inhibits regeneration?
- In the introduction it is stated that there is “an antagonism between p38 and JNK” but do not explain further. This could be a significant factor when interpreting the experiments presented in this paper, and should be elaborated on.
- At the beginning of the results section (Page 4) the authors mention Ask1 is inhibited by Thioredoxin (Trx), and oxidative stress inactivates Trx which leads to Ask1 activation. Since the experiments performed in undamaged discs (Figure 1) don’t have changes in ROS, can the authors comment on whether this mechanism might influence their results? For example is it assumed that the level of Ask1 expressed in these experiments is high enough to escape Trx regulation?

Reviewer 2

Advance summary and potential significance to field

In this manuscript Esteban-Collado et al. explore the function of PI3K/Akt signaling regulating the activity of p38 during regeneration. The authors propose that PI3K/Akt signalling is required for Ask1 to activate p38 but not JNK. They also show that the Ser83 residue of Ask1 is necessary for this function. The identification of the mechanisms by which ask1 discriminates between the activation of p38 and JNk signalling is key for understanding how Ask1-dependent regeneration program is activated. In my opinion, the data shown in this work are interesting and although I feel that some results presented here are still preliminary, I think that the overall contribution of this work is a useful one. I find several problems (highlighted below) that the authors should address.

Comments for the author

The author clearly shows that the over-expression of UAS-Pdk1:UAS-Akt1 and some forms of ask are sufficient to increase the levels of P-38. However, it is not clear whether this is a direct or an indirect effect. I see in figure 1D that the levels of Pp38 do not only increase in the domain of expression of patch-Gal4, but also in other regions of the anterior compartment. I wonder whether the over-expression of UAS-Pdk1:UAS-Akt1 and ask induces apoptosis, that in turn would activate Pp38, autonomously and non-autonomously. I think the authors should analyse whether cell death increases after the over expression of these transgenes, and any possible effects of apoptosis in the activation of P-p38 in these conditions.

Although the authors show that the levels of -JNK do not increase upon over-expression of UAS-Pdk1:UAS-Akt1, in my opinion the authors should confirm that this signalling pathway is not active using other JNK reporters, such as pucZ or TREGFP

The authors claim that " p38 is able to rescue the effects of nutrient limitation and implies that p38 is highly sensitive and responsive to nutrients to drive regeneration", however they do not analyse whether nutrient restriction affects the levels of P-pP38 during regeneration. In my opinion they should examine whether during regeneration, P-pP38 levels are altered under nutrient restriction conditions compared to standard food conditions.

The statistical analysis performed throughout the paper needs to be revised - one-way ANOVA is the appropriate test for majority of the experiments, rather than multiple t-tests to compare more than two groups.

Minor comments
The sentence "Pdk1, the gene that encodes the kinase downstream of the insulin pathway and PI3K that phosphorylates and activates the Akt1 kinase, was coexpressed with Akt using the UAS-Pdk1:UAS-Akt1 recombinant construct (hereafter Pdk1:Akt)" is confusing. It is not clear whether they over-expressed Pdk1 and PI3K or only Pdk1.

Reviewer 3

Advance summary and potential significance to field

Understanding the specific roles that stress response networks play in tissue regeneration remains a central and unsolved question un developmental biology. In this manuscript, Esteban-Collado and colleagues use Drosophila genetics to study the molecular connection between theMAP3 kinase Ask1 and the stress-response pathways p38 and JNK.

In the first part of the manuscript the authors used a gain of function approach to analyze how Ask1 controls p38. The authors show that expression of Ask1+Akt leads to p38 phosphorylation but, interestingly, it does not impact on JNK. The They conclude that p38 activation requires the Ser83 in Ask1. The authors also provide evidence presenting a cooperative interaction between the Ser83 and the kinase domain present in Ask1. In the second part of the study, the authors reveal that nutrients are necessary are required to maintain p38 activity in a context of tissue regeneration in the wing disc.

Comments for the author

The experimental approach used is elegant, the results shown are clear, and the conclusions obtained are supported by the results presented here.

I would suggest some minor corrections/modifications before the work is ready for publication. Specifically:

- The authors mention: "Upon oxidative stress, thioredoxin dissociates from the Ask1 thioredoxin-binding domain, Ask1 oligomerizes and its threonine-rich kinase domain phosphorylates to induce its activation (Fig.1A)". What specifically does Ask1 phosphorylate? Does it auto-phosphorylate itself? Does it phosphorylate other kind of substrates?

- The authors mention: "The YH_GVRESF sequence located in the N-terminal of Ask1 is highly conserved from sponges to humans and is located in the N-terminal part of Ask1 (Fig. 1A and Fig. S1A)". Redundant information is presented in the sentence. I would suggest to revise the writing.

- I would suggest to add fig labels (A. B, S, D…) to Figs 2 and 3 and refer to them in the main text. This would facilitate the understanding to the reader.

First revision

Author response to reviewers' comments

Comments to the Reviewers

General comment for the three reviewers:
We appreciate your positive comments and the suggestions to improve the text and presentation. We also appreciate the suggestions to improve our work including the proposed controls and experiments.
In this version, we have included several new figures and data to strengthen our model for the p38-dependant regeneration.

Reviewer 1
We thank Reviewer 1 for the comment '*this work is an important addition to our understanding of how nutritional sensing might influence regeneration via the Akt/Ask1 proteins regulation of P38*'
We have followed the suggestions of this reviewer with the hope to convince that the work is now a considerable advance.

Comment 1
The experiments in Figures 2 and 3 demonstrate regenerative ability of wings following genetic manipulations. To more strongly support their conclusions the authors should show the imaginal discs from these experiments to illustrate the expression and activation of Ask1 (p-Thr), and P38 (p-P38) in regenerating discs. For example, although expression of Lic is shown to upregulate p38 in the pouch (Sup Figure 2), these additional data would confirm Lic activates P38 in the regenerating disc, as well as demonstrating the cells in which this occurs, and that this is the reason for the altered regeneration phenotype. These experiments may also reveal why Lic expression in standard food (figure 2) significantly limits regeneration alone.

Since former Figure 1 has been now split in Fig 1 and 2, former Figure 2 and 3 are now Figure 3 and 4 respectively.
We have now included imaginal discs stained for P-p38 in both figures 3 and 4 of the genotypes
$sal^{E/Pv}$>*rpr, nub>GFP*
$sal^{E/Pv}$>*rpr, nub>*lic^{WT}
$sal^{E/Pv}$>*rpr, nub>*hep^{WT},
in standard food, in nutrient restriction food (Fig. 3) and in co-expression of $Ask1^{S83A}$ (Fig.4).

We are grateful for this comment since these images strengthen our conclusion and evidence that the rescue of regeneration by lic^{WT} during starvation is associated to P- p38.

As for P-Thr, we have previously studied the expression of P-Thr in regenerating discs, and found that is strongly activated in dying cells and very weak in living cells, which makes it very difficult to monitor in different genetic backgrounds since the differences are very subtle. Instead we have used P-JNK, which serves to monitor the discrimination between p38 and JNK. We include these observations in a new panel of P-JNK in imaginal discs of
$sal^{E/Pv}$>*rpr, nub>GFP,*
$sal^{E/Pv}$>*rpr, nub>*lic^{WT},
$sal^{E/Pv}$>*rpr, nub>*hep^{WT},
in standard food, nutrient restriction and in co-expression of $Ask1^{S83A}$ (Fig S3A-K).

We have introduced this comment concerning P-Thr and P-JNK in the discussion at the end of the paper (p.8):
"Dying cells lack P-p38, possibly because of a reduction of P-Akt levels in apoptotic cells (Franke et al., 2003), and therefore they are unable to attenuate P- Thr Ask1 activity. Indeed, P-Thr Ask1 has been found to be highly accumulated in the apoptotic zones of the disc (Santabárbara-Ruiz et al., 2019). We speculate that this could result in the activation of JNK in damaged cells, which then promotes apoptosis (Shlevkov and Morata, 2012). Although P-JNK is not abundant in dying cells, reporters of JNK signaling have been associated with apoptotic cells (Pinal et al., 2019)."

Comment 2
The work in this paper suggests that P38 is activated downstream of Ask1 (Figure 1D), but JNK is not (Figure 1H). This finding is surprising in light of the authors previous paper in which it is shown that Ask1 activates both P38 and JNK (shown by Mmp1 expression) in regenerating discs. The current work showing p- JNK activity is performed in undamaged discs, and shows no changes in JNK signaling by any manipulation performed. In damaged discs with increased JNK (HepWT expression, Figure 2 and 3) regeneration is either unaffected or inhibited.
Therefore it is important for the authors to address these results more clearly in the context of their previous work.

We agree with the reviewer that we should address and clarify this confusion. First, the main difference between this work and the previous one, is that in this work we analyze the effects on JNK and p38 after activation of Akt signaling. This is important because it has helped us to discern from the response program that is clearly used for p38 (=Ask1+Akt), but not for JNK. We infer, that Ask1 control of JNK will require other factors.
To help the reader and with the aim to clarify possible confusions, we decided to address this issue in this paper as:

First results section pp 5 third para
'These results demonstrated that the Akt-dependent phosphorylation of Ask1 at Ser83 is canalized through p38 and not through JNK, suggesting that the Ask1- dependent activation of JNK requires factors other than Akt.'

In the second para of the introduction we stated that
'In this context, Ask1 acts upstream of p38 and JNK and is sensitive to reactive oxygen species (ROS)'

This issue has been also addressed in at the discussion (third paragraph page 8):
'p38 and JNK respond to damage differently. It is known that JNK is associated with cell death and that there is a mutual antagonism between p38 and JNK (Wagner and Nebreda, 2009). Dying cells lack P-p38, possibly because of a reduction of P-Akt levels in apoptotic cells (Franke et al., 2003), and therefore they are unable to attenuate P-Thr Ask1 activity. Indeed, P-Thr Ask1 has been found to be highly accumulated in the apoptotic zones of the disc (Santabárbara-Ruiz et al., 2019). We speculate that this could result in the activation of JNK in damaged cells, which then promotes apoptosis (Shlevkov and Morata, 2012).
Although P-JNK is not abundant in dying cells, reporters of JNK signaling have been associated with apoptotic cells (Pinal et al., 2019). As previously suggested, the attenuated form of Ask1 might not only be key for p38 activation, but also for maintaining low levels of JNK in regenerating cells (Santabárbara- Ruiz et al., 2019). Therefore, both MAPKs play a role in regeneration, p38 in a manner that is dependent on nutrients and PI3K/Akt, and JNK independently of them. In addition to the attenuated or low levels of Ask1, the MAP3 kinase Tak1 could operate independently of nutrients to activate JNK (La Marca and Richardson, 2020). '

Comment 3
In the experiments of Figures 2 and 3, the wing size and percent of regenerated wings is measured in the 29'C conditions (ablated and expressing the transgene) and the 17'C condition (unablated and not expressing the transgene). The essential control experiment, in which the transgenes are expressed in unablated discs, was not performed. This experiment is very important to interpret the results presented.

We apologize the reviewer for not having done these controls.
We have performed these controls for all conditions and are now in a new Figure S3. We hope that the interpretation is now more sustained.

In page 6 end of third paragraph:
The expression of any of these three transgenes driven by *nub-Gal4* in unablated discs did not affect the normal pattern of the wings, although a small reduction in size was detected for *UAS-lic^{WT}* and *UAS-hep^{WT}* in comparison to the control *UAS-GFP* (Fig. S3).

Comment 4
In Figure 1D the authors show that P38 is activated by the combination of Pdk1/Akt/Ask. They also show that the phosphorylation of the kinase domain (detected by p-Thr) is important for this activation (Figure 1K). However, in Supplemental Figure 1G, the p-Thr antibody does not detect Ask1, suggesting this domain is not phosphorylated. The authors should clarify why they don't see any p-Thr phosphorylation, despite seeing P38 activation in this experiment.

We also agree on the importance to raise this issue and clarify it.
We have demonstrated that the kinase domain is essential for p38 phosphorylation, indeed, and that the Ask1 S83A mutant results in high expression of P-Thr Ask1. Therefore, we deduce that low levels of P-Thr must be present, far below the threshold that triggers apoptosis. We apologize for having failed in commenting that in those discs, P-Thr associated to Pdk1:Akt , $Ask1^{WT}$ is present at very low levels and in patches. Now we have added an arrow in Figure S1G and modified the text in Page 5 last paragraph as:
"Ask1 activity was determined with an anti-P-Thr Ask1 antibody, a phospho- threonine antibody that targets the core of the Ask1 catalytic domain. This antibody showed increased staining in the discs co-expressing *Pdk1:Akt* and $Ask1^{S83A}$ compared to the *Pdk1:Akt*, $Ask1^{WT}$ and *Pdk1:Akt*, $Ask1^{K618M}$ discs (Fig. S1F-I, K). The co-expression of *Pdk1:Akt* and $Ask1^{WT}$ resulted in a very weak staining, although this was not statistically significant, suggesting that the ectopic expression of Akt attenuated P-Thr Ask1 levels (Fig. S1G)."

Comment 5
It would be helpful to include controls to demonstrate the detection of p-JNK and p-P38 in wild type wing discs. Although the authors do show discs with tissue that is not genetically manipulated (the anterior compartments of discs in Figure 2A-B) p-JNK could be altered at a distance in these experiments, while p- P38 is not shown in a wild type disc in the paper.

We apologize for not having included those controls. We have them now in Figure 1 B and Figure 2A for P-p38 and P-JNK respectively.

Minor Comments

- *The section describing the experiments in Figure 1B-D is somewhat unclear and could be written with more clarity to help the reader understand the work being done.*

We have re-writen the entire paragraph (page 4 second paragraph). We hope that is now more clear.

- *In Figure 1D there are high levels of activated P38. The authors mention that high P38 activity kill cells (page 7). Can the authors comment on whether this would complicate their analysis?*

To clarify this issue, we have now added a panel with discs of the 3 main genotypes used, with Dcp1 (=an effector caspase in *Drosophila*) in order to monitor cell death (new Figure S2 A, B, C). Dead cells are absent or just few ones when present.
Therefore, we discard that p38 could be the result of dying cells or that the p38 induced by Ask1+Akt induces cell death. In the second paragraph of page 5 we modified the text as:

'We found that P-p38 did not significantly increase in the *ptc* stripe of cells when *Pdk1:Akt* was co-expressed with *GFP* (Fig. 1D). Instead, P-p38 significantly increased when *Pdk1:Akt* was co-expressed with $Ask1^{WT}$ (Fig. 1E and G). P-p38 was highly concentrated in the *ptc>* stripe, although some labeling could also be observed in the cells anterior to the stripe. It is conceivable that this anterior staining resulted from the persistence of the early *ptc-Gal4* expression in the anterior compartment rather than a non-autonomous effect from dying cells, since no or only a few dead cells (positive for caspase Dcp1) were found (Fig. S2A-C) (Bosch et al., 2016; Evans et al., 2009). By contrast, P-p38 did not increase when *Pdk1:Akt* was co-expressed with the mutant $Ask1^{S83A}$, neither in the *ptc>* stripe nor in the anterior compartment (Fig. 1F and G). Therefore, we concluded that the Ser83 of the YH_GVRE**S**F sequence is essential for p38 activation in PI3K/Akt signaling.'

- *Page 7 "possibly because of a reduction of the insulin signal, and show a release of P-Thr*

Ask1 activity". Is there evidence of the lower insulin signaling in dying cells or is this speculation? Use of the word "release" is confusing, and could indicate increase or decrease.

We have changed insulin by Akt because it is more in the context of the manuscript. In fact, it is known in the literature that Akt activity is downregulated after pro-apoptotic insults.
We have change the sentence to (third paragraph pp 8):
"Dying cells lack P-p38, possibly because of a reduction of P-Akt levels in apoptotic cells (Franke et al., 2003), and therefore they are unable to attenuate P-Thr Ask1 activity."

- *Page 5 "We propose here that this attenuation results in low levels of Ask1 activity but sufficient to trigger p38 function and to avoid the high Ask1 activity that can result in cell death". This conclusion and the description of the supporting evidence are difficult to follow, as they describe an increase in staining but attenuation of activity. This section could be clarified for the reader.*

We have modified this sentence as (end of first paragraph page 6 :
"We propose here that this attenuation results in low levels of Ask1 activity that are still sufficient to activate p38. This is important for cell survival, as strong stimulation of Ask1 can result in apoptosis (Kuranaga et al., 2002). "

And the entire preceding text of this paragraph (page 5-6) has been modified.

- *Some knowledge is presupposed but the authors, particularly the connection between PI3K and Akt, and Ask1 and Licorn. A simple pathway diagram or more background in the introduction would help the reader understand the field better and where this work is positioned within it.*

A diagram (simplified) has been now introduced in Figure 1A'.

- *The authors conclude that increasing P38 activity rescues regeneration in nutrient deficient conditions. Page 7 "Altogether, these results show that p38 is able to rescue the effects of nutrient limitation and implies that p38 is highly sensitive and responsive to nutrients to drive regeneration". However, they actually show Lic rescues regeneration, and that in undamaged discs a pouch with high Lic expression has high P38. The conclusion follows, but is not what the authors show.*

With the introduction in this version of the staining of anti-P-p38 in all genotypes of standard food and nutrient restriction, it provides evidences that *licorn* rescue is associated to p38 phosphorylation (and not to P-JNK). Figures 3, 4 and S4.
In addition we have modified the sentence to (page 7 end paragraph 4):
'Altogether, these results showed that lic^{WT}, but not hep^{WT}, was able to revert the effects of nutrient limitation, indicating that the p38 pathway is highly sensitive and responsive to nutrients to drive regeneration.'

Thanks for the comment.

- *Can the authors include a detail of why they think HepWT expression inhibits regeneration?*

Again, thanks to the experiments of anti-P-p38 now included in this version (Fig. 3B, C, D, I, J, K), we now see that the hep^{WT} discs lack the P-p38, thus, the response necessary for regeneration.

In page 7 paragraph 4 we now describe:
'We also tested whether JNK could restore regeneration. JNK signaling was induced moderately by the expression of the wild-type form of the *Drosophila* JNKK, *hemipterous* (hep^{WT}) (Uhlirova and Bohmann, 2006). The expression of hep^{WT}, but not of *GFP* or lic^{WT}, resulted in an increase of P-JNK (Fig. S4A-G). The activation of hep^{WT} did not result in P-p38 around the apoptotic zone of the discs (Fig. 3C, J) and did not reverse the defects in regeneration resulting from nutrient restriction (Fig.

3E, F, L, M). Altogether, these results showed that *lic^{WT}*, but not *hep^{WT}*, was able to revert the effects of nutrient limitation, indicating that the p38 pathway is highly sensitive and responsive to nutrients to drive regeneration. ‘

- *In the introduction it is stated that there is “an antagonism between p38 and JNK” but do not explain further. This could be a significant factor when interpreting the experiments presented in this paper, and should be elaborated on.*

Yes, indeed, the antagonism between p38 and JNK is important for the interpretation. We have introduced a detailed explanation, examples and referencesAt the end of the second paragraph on the introduction section we state:
“How increased p38 phosphorylation can coexist with tolerable, presumably low, levels of JNK remains unclear.”

Ant then, in the following paragraph :
“There are many observations that support an antagonism between p38 and JNK, mainly reinforced by the finding that p38 can negatively regulate JNK activity in mammalian cells (Wagner and Nebreda, 2009). For example, the inhibition of p38 in mammalian myoblasts, epithelial cells and macrophages leads to the activation of JNK (Cheung et al., 2003; Perdiguero et al., 2007). Moreover, Jun- deficient hepatocytes show increased p38α phosphorylation (Stepniak et al., 2006). Therefore, we speculated that the mechanism that activates p38 during *Drosophila* regeneration operates concomitantly with a reduction of JNK. As MAP3 kinases are key regulators of MAPK activity, we decided to investigate how Ask1 discriminates between p38 and JNK and particularly how Ask1 fuels p38-dependent regeneration.”

At the end of the paper, (page 8 3rd para) we again mention that:

“p38 and JNK respond to damage differently. It is known that JNK is associated with cell death and that there is a mutual antagonism between p38 and JNK (Wagner and Nebreda, 2009).”

However, antagonism is not all. Other signals are necessary to divert Ask to JNK. A pargraph that ends by:

“Therefore, both MAPKs play a role in regeneration, p38 in a manner that is dependent on nutrients and PI3K/Akt, and JNK independently of them. In addition to the attenuated or low levels of Ask1, the MAP3 kinase Tak1 could operate independently of nutrients to activate JNK (La Marca and Richardson, 2020). “

- *At the beginning of the results section (Page 4) the authors mention Ask1 is inhibited by Thioredoxin (Trx), and oxidative stress inactivates Trx which leads to Ask1 activation. Since the experiments performed in undamaged discs (Figure 1) don’t have changes in ROS, can the authors comment on whether this mechanism might influence their results? For example is it assumed that the level of Ask1 expressed in these experiments is high enough to escape Trx regulation?*

Indeed, we assume that the excess of monomers are functional in the absence of Trx inactivation. We previously found that the ectopic activation of *Ask1^{WT}* results in moderate increase of Ask1 activity in the absence of extra thioredoxin inactivation, as deduced by staining with phospho-Ask1 antibodies (Santabárbara-Ruiz et al., 2019). We raised this issue in the present version as: (second para of results section)

“We previously demonstrated that the ectopic activation of wild-type Ask1 (*UAS-Ask1^{WT}*) resulted in a moderate increase in Ask1 activity in the absence of extra thioredoxin inactivation (Santabárbara-Ruiz et al., 2019). Ectopic expression of *Ask1^{WT}* in wing imaginal discs using the *patched-Gal4* driver (hereafter *ptc>*) did not increase phosphorylated p38 (P-p38) levels beyond basal values (Fig. 1B-C).”

Reviewer 2
Comment 1. *The author clearly shows that the over-expression of UAS-Pdk1:UAS-Akt1 and some forms of ask are sufficient to increase the levels of P-38. However, it is not clear whether this is a*

direct or an indirect effect. I see in figure 1D that the levels of Pp38 do not only increase in the domain of expression of patch-Gal4, but also in other regions of the anterior compartment. I wonder whether the over- expression of UAS-Pdk1:UAS-Akt1 and ask induces apoptosis, that in turn would activate Pp38, autonomously and non-autonomously. I think the authors should analyse whether cell death increases after the over expression of these transgenes, and any possible effects of apoptosis in the activation of P-p38 in these conditions.

We thank the reviewer for raising this point. We addressed this issue and we have now done some extra experiments to clarified it. Briefly, we have studied apoptosis in these discs and found that the activation of the transgenes does not cause apoptosis, or if it does, occurs in few isolated cells. Rather, it is more likely that this is an effect of the persistence the ectopic expression from the earliest stages. The *ptc-Gal4* driver in young discs operates in the entire A compartment, and persistence of its expression has been documented (Bosch et al., 2016; Evans et al., 2009).
We have added a panel with discs of the 3 main genotypes used, with DCP1 (an effector caspase in *Drosophila*), to monitor cell death (new Figure S2 A, B, C). Dead cells are absent or if they do, there are just few per disc.
Therefore, the P-p38 localization in *Pdk1:Akt Ask1^{WT}* discs must come from the genetic manipulation. This is now commented in the text page 5, 2nd paragraph (note that now this issue corresponds to Fig 1E):
"Instead, P-p38 significantly increased when *Pdk1:Akt* was co-expressed with *Ask1^{WT}* (Fig. 1E and G). P-p38 was highly concentrated in the *ptc>* stripe, although some labeling could also be observed in the cells anterior to the stripe. It is conceivable that this anterior staining resulted from the persistence of the early *ptc-Gal4* expression in the anterior compartment rather than a non-autonomous effect from dying cells, since no or only a few dead cells (positive for caspase Dcp1) were found (Fig. S2A-C) (Bosch et al., 2016; Evans et al., 2009). "

Comment 2. Although the authors show that the levels of -JNK do not increase upon over-expression of UAS-Pdk1:UAS-Akt1, in my opinion the authors should confirm that this signalling pathway is not active using other JNK reporters, such as pucZ or TREGFP

We have now included a figure with Mmp1, another reporter of JNK. Mmp1 is a widely used reporter as it is know to operate downstream of JNK. The results confirmed the observations with P-JNK. We have now made a composition of images taken from the different genotypes and stained for Mmp1 and also DCP-1, as a marker for apoptosis, and included in Figure S2 A,B,C,D.

Comment 3. *The authors claim that " p38 is able to rescue the effects of nutrient limitation and implies that p38 is highly sensitive and responsive to nutrients to drive regeneration", however they do not analyse whether nutrient restriction affects the levels of P-pP38 during regeneration. In my opinion they should examine whether during regeneration, P-pP38 levels are altered under nutrient restriction conditions compared to standard food conditions.*

We have addressed this comment in depth and included images of all conditions in Figures 3 and 4 (former Figure 2 and 3). Note that in addition to the P-p38 staining during regeneration (Figs 3, 4), we also included the P-JNK staining in figure S4. Perhaps the most relevant and exciting result is that the recovery after *lic* expression in nutrient restriction, is complemented with the increase of P-p38 that is very similar to the 'normal' increase that occurs in standard food (compare Fig. 3B with J).

We have now included imaginal discs stained for P-p38 in both figures 3 and 4 of the genotypes
sal$^{E/Pv}$>rpr, nub>GFP
sal$^{E/Pv}$>rpr, nub>lic^{WT}
sal$^{E/Pv}$>rpr, nub>hep^{WT},
in standard food, in nutrient restriction food (Fig. 3) and in co-expression of *Ask1^{S83A}* (Fig.4).

We are grateful for this comment since these images strengthen our conclusion and evidence that the rescue of regeneration by *lic^{WT}* during starvation is associated to P- p38.

Comment 4. *The statistical analysis performed throughout the paper needs to be revised - one-way ANOVA is the appropriate test for majority of the experiments, rather than multiple t-tests to compare more than two groups.*

We thank the reviewer for this suggestion, which indeed is more appropriate. We have now used the one way ANOVA in our statistical analysis. The material and methods now reads:
”In all the statistical analysis (wing areas and mean pixel intensity graphics), error bars indicate standard deviation of the mean. To make statistical comparisons, we used one-way analysis of variance (ANOVA) followed by Tukey post-hoc test to make pair comparisons between each group using IBM SPSS statistics.”

Minor comments

- *The sentence “Pdk1, the gene that encodes the kinase downstream of the insulin pathway and PI3K that phosphorylates and activates the Akt1 kinase, was coexpressed with Akt using the UAS-Pdk1:UAS-Akt1 recombinant construct (hereafter Pdk1:Akt)” is confusing. It is not clear whether they over-expressed Pdk1 and PI3K or only Pdk1.*

We have modified the entire paragraph with the hope to make it clear. The sentence has now been re-written as (second paragraph results section):
“We used *ptc-Gal4* to drive the expression of three transgenic combinations and tested them for p38 activation, as follows: (1) In the control experiments, we ectopically expressed *Akt* and *GFP*. To activate Akt, we used the *UAS- Pdk1:UAS-Akt1* recombinant construct (hereafter *Pdk1:Akt*). This transgene allows co-expression of Akt and *phosphoinositide-dependent kinase-1* (*Pdk1*), the gene encoding the kinase downstream of the insulin pathway that phosphorylates and activates Akt1 (Fig. 1A’). This construct ensures the ectopic expression of both genes and the activation of Akt without killing the cell. In this transgenic combination, we co-expressed *Pdk1:Akt* with *UAS-GFP* as a neutral transgene.”

Reviewer 3
We are grateful to the Reviewer for the comment *The experimental approach used is elegant, the results shown are clear, and the conclusions obtained are supported by the results presented here*.

Minor changes
The authors mention: “Upon oxidative stress, thioredoxin dissociates from the Ask1 thioredoxin-binding domain, Ask1 oligomerizes and its threonine-rich kinase domain phosphorylates to induce its activation (Fig.1A)”. What specifically does Ask1 phosphorylate? Does it auto-phosphorylate itself? Does it phosphorylate other kind of substrates?

We agree this comment, which indeed was unclear. We have introduced in the text (first paragraph introduction section):
“Upon oxidative stress, thioredoxin dissociates from the thioredoxin-binding domain of Ask1, enabling Ask1 to oligomerize and its threonine-rich kinase domain to autophosphorylate, leading to its activation and function as a MAP3 kinase (Fig. 1A) (Liu et al., 2000; Nishida et al., 2017; Saitoh et al., 1998). ”

- *The authors mention: “The YH_GVRESF sequence located in the N-terminal of Ask1 is highly conserved from sponges to humans and is located in the N- terminal part of Ask1 (Fig. 1A and Fig. S1A)”. Redundant information is presented in the sentence. I would suggest to revise the writing.*

This redundancy has been corrected. Now the sentence reads (first sentence results section):
“The YH_GVRESF sequence located in the N-terminal region of Ask1 is highly conserved from sponges to humans (Fig. 1A and Fig. S1A).”

- *I would suggest to add fig labels (A. B, S, D…) to Figs 2 and 3 and refer to them in the main text. This would facilitate the understanding to the reader.*

Many thanks for this suggestion. We have labeled the figures accordingly (note that figures 2 and 3, are now 3 and 4 respectively).

Second decision letter

MS ID#: DEVELOP/2020/197087

MS TITLE: Nutrition and PI3K/Akt signaling are required for p38-dependent regeneration

AUTHORS: José Esteban Collado, Montserrat Corominas, and Florenci Serras

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

The reviews are all positive and I am happy to say that we would like to publish your manuscript in Development, asking first that you make minor revisions in writing/presentation in response to Reviewer #1's suggestions. I do not expect that this will require much time and hope that you agree, but if you do not agree with reviewer suggestions please explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

The paper by Estaban-Collado et al. examines how the activation of p38 and JNK by Akt/Ask1 influences regeneration of damaged imaginal discs, and demonstrates that nutrient sensing via insulin signaling is an important input into this mechanism. The revisions included by the authors in this submission strengthen the work significantly.

Comments for the author

The authors have made changes to the text and figures that have addressed all of this reviewer's original criticisms at great length. I have only very minor comments that could be considered to provide further clarity to the reader:

- Figure 1A could specifically label the Ser 38 residue, since it is a focal point of the paper.
- Figure 1C the genotype label should include GFP
- Page 5 "The catalytically inactive ASK1K61M mutant acts as a dominant negative form…". This paragraph could use a clearly stated conclusion that this form of the protein results in neither JNK nor P38 phosphorylation, the current wording obscures the message..
- The images of the wing blades in Figures 3,4 and Sup 3 are quite small and not very clear when printed.

Reviewer 2

Advance summary and potential significance to field

The results presented in this manuscript are important for understanding the mechanism whereby Ask activates p38, but not JNK signalling , during tissue repair and regenerative growth

Comments for the author

The authors have addressed satisfactorily all my concerns. My recommendation is to accept the manuscript.

Reviewer 3

Advance summary and potential significance to field

The authors have addressed all my concerns and, in my view, the paper is ready for publication in its current version.

Comments for the author

-

Second revision

Author response to reviewers' comments

Answer to Reviewer 1

We would like to thank Reviewer 1 for the suggestions.
Fig 1A. We have labeled Ser 83
Fig 1C. We have included GFP in the genotype
Page 5. We have ended the paragraph with the sentence “In conclusion, the inactive form of the Ask1 protein results in neither p38 nor JNK phosphorylation.“
All the wing blades shown in figures 3, 4 and Supplemental 3 are enlarged.

Third decision letter

MS ID#: DEVELOP/2020/197087

MS TITLE: Nutrition and PI3K/Akt signaling are required for p38-dependent regeneration

AUTHORS: José Esteban Collado, Montserrat Corominas, and Florenci Serras
ARTICLE TYPE: Research Report

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