Ecdysone regulates the *Drosophila* imaginal disc epithelial barrier, determining the length of regeneration checkpoint delay

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MS TITLE: Ecdysone regulates the Drosophila imaginal disc epithelial barrier, determining the duration of regeneration checkpoint delay

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

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

Reviewer 1

Advance summary and potential significance to field

This manuscript explores regulation of the regeneration checkpoint, which is an organism-wide developmental delay induced by tissue damage in Drosophila imaginal discs. Previously, it was known that Ecdysone and Dilp8 were important regulators of this checkpoint. Using a combination of genetics and quantitative imaging, the findings of this manuscript move the field forward in an important way by providing a cellular mechanism for the cessation of regeneration checkpoint sensing. These results should be of broad interest to the organ regeneration field, as well as to developmental biologists interested in formation of epithelial barriers.

Comments for the author

Major comments:

*Ideally, the mutants that compromise the barrier would restore pre-barrier formation levels of permeability seen in WT. But in some cases, the mutant seems to exceed the WT pre-barrier permeability levels. This raises concerns about secondary phenotypes contributing to the observed phenotype. For example, do kune etc. mutants lead to elevated apoptosis, which then leads to an aberrant barrier? The authors should speak to this in some way, and any caveats should be frequently noted.

* The dextran assay is a very important part of the data- it would be good to present at least some of this (in image form) in the main figures rather than only supplemental.

*The manuscript is pitched as a regeneration story but only one main figure panel (7A) is devoted to this question. I suggest moving Fig S12 data to Fig 7. Additionally, if feasible, the authors should include some measure of regeneration (number of proliferative cells, amount of tissue restored) in the septate junction mutants.

*Does cessation of developmental mitoses coincide with acquisition of an impermeable barrier?
*The 20HE addition impact on barrier formation seems mild- does this induce premature septate junction localization? If so, this would strengthen claims about 20HE addition.

*Figs 6D-F lack image quantitation as per other similar figures (e.g. Fig5G)

Minor comments:
*The summary statement has a tight word count, but it's unclear what "becoming more restrictive" means, and regeneration checkpoint is not defined. This is an important stand alone statement, so I suggest revising along these lines.

*Abstract: Lines 30-31: this sentence is very hard to read and I couldn't quite interpret the meaning

*Line 33- restrictive to what? Impermeable to what? checkpoint delay of what?

*Various typos on lines 90, 158, 163, and 262.

Reviewer 2

Advance summary and potential significance to field

The manuscript "Ecdysone regulates the Drosophila imaginal disc epithelial barrier, determining the duration of regeneration checkpoint delay" by DaCrema et al. describes a novel approach to understanding the restrictions on the signaling that enables imaginal disc regeneration to proceed without interference from pupariation and metamorphosis. As such, it will be of great interest to the broad readership of Development, as the work has implications for both tissue regeneration as well as other long-range signals that might be impacted by animal maturation.

Before publication, the authors should address specific concerns. As many labs are partially or completely closed at the moment, this review will attempt to ask for clarifications that can be made using existing data, or with minimal additional experiments.

Comments for the author

Major concerns

What is the difference between the 92h and 116h barrier such that one requires Cora and the other does not? While the answer to that question may be beyond the scope of this study, more attention should be paid to the question in the results or discussion.

Why is barrier function intact in the Cora knockdown, when Nrx and Kune localization are lost – yet Nrx and Kune are required for barrier function? The possible explanation in the discussion – that Nrx and Kune are not completely lost - is not supported by data, but could be. Comparing S8B,D to 5E, is there a quantifiable difference in signal? Could Nrx or Kune be further reduced in the Cora knockdown background, to demonstrate that residual activity remains in the Cora knockdown and further reduction allows more dextran into the lumen? Perhaps Cora knockdown in a heterozygous mutant Kune or Nrx background?

The conclusion "The epithelial barrier regulates the end point of regeneration" (line 318 and Figure 7 title) is not supported by the data. The data indicate that compromising the barrier extends the time before pupariation after tissue damage. However, the duration of regenerative growth, as assessed by proliferation in the damaged disc, or extent of regenerative growth, as assessed by adult wing size, were not shown. Furthermore, the authors did not try compromising the barrier after tissue damage in more mature imaginal discs to see if doing so would enable regeneration in tissue that is losing competence for regenerative growth. Indeed, ecdysone signaling inhibits regeneration at least in part by silencing enhancers at multiple damage-responsive genes, so the extent to which the end of regeneration is controlled by Dilp8 and the onset of pupariation, rather than ecdysone signaling in the damaged epithelium itself, is an open question. The authors should add supporting experiments or reword their conclusion

The fluorescence intensity variation for the dextran experiments is of concern. How inconsistent was the barrier-impaired fluorescence from experiment to experiment? A supplemental figure showing the variation, and showing that the difference between experiment and control was consistent despite this variation, would be helpful.

Minor concerns
The results open with a supplemental figure – readers would appreciate seeing the FLAG-tagged Dilp8 image in the main Figure 1.

The authors do not mention the possibility that some Dilp8 is released through the basal surfaces of the cells. While excluding this possibility is not essential for their model, evidence for or against such a possibility should be mentioned.

Figure 2 is called out before Figure 1, and Figure S8 is similarly called out of order. Fig. 5G is called out of order (Line 214). Data showing that each RNAi construct works should be moved to the first use of that construct. If keeping the experiments demonstrating RNAi efficacy in the same figure is desired, move the figure earlier.

Line 176 – reword to be more accurate - the authors see a progressive decrease in dextran in the lumen that suggests or indicates a decrease in barrier permeability

Figure 1 – the LacZ controls are not on the graph – presumably the mean of the controls would be at zero. What is the variation in pupariation timing among the controls and how does it compare to the variation in the experimental samples?

Figure 2 and materials and methods – How the dextran fluorescence was quantified is unclear. It would be helpful, in figure 2 or S3, to show an image of a lumen with the dextran fluorescence and the line drawn for quantification. It is unclear from S3 B, C. and D where such a line would be

drawn on these images, or whether cross-section images were used, and if so where the line would be drawn on them.

Figure 2B and 2C 92 hrs – the distribution looks bimodal – is this distribution consistent and might there be a reason for two populations at this time point?

Fig 2A and B. A higher n than 5, 6 or 7 would be preferable, but given the limitations on lab experiments may not be achievable.

Fig3A S6A, S8A, S10A – the arrow depicting the image location is confusing – it could be interpreted as pointing to the image location (the hinge folds). Is it correct that the stem of the arrow is the image location (the pouch) and the arrowhead indicates directionality? Better to use a dashed line or box with no arrowhead.

Fig 3 and beyond – all images need scale bars

Fig 3F,I,L – The normalization is confusing. Normalization is to the mean membrane intensity where? If normalization is to mean intensity all along the lateral membrane, wouldn't the intensity at the apical-lateral then be greater than one (much higher than the mean)?

Fig 3L – it's surprising, based on the images, that the Medial quantification difference is not greater.

Fig 5G is difficult to interpret at a glance (perhaps it needs more contrast) and seems subjective, especially given the low n for the imaging experiments and lack of quantification (compared to quantification in Figure 3). For example, there seems to be diffuse Kune signal at 92h in the NrxRNAi and the CoraRNAi but nothing noted in the diagram. The diagram is not necessary to support the main claim of the figure, which is that Kune and Nrx are mislocalized in CoraRNAi.

Fig 6A – It would be helpful to see this graph on the same Y axis scale as Fig 2C, for comparison.

Fig 6D/E/F – quantify apical Cora to determine whether there is a reduction of Cora at the septate junctions.

Fig 7 the line between Ecdysone and regeneration should be solid, as it has been shown that ecdysone signaling leads to epigenetic silencing of regeneration-responsive enhancers.

Fig S1 – The legend does not note what the white arrows are pointing to.

Fig S3 – why is the LacZ not restricted to the dorsal half of the disc?

Fig S3 – cross section images of B,C, and D as diagrammed in A would be helpful, especially to demonstrate that the signal in C is indeed in the lumen.

Fig S9A – the meaning of the orange arrows is not immediately clear. Perhaps draw an orange curve above the blue curve to conceptually explain the elevated ecdysone levels.

Experimental suggestions stemming from curiosity – requirement left to the discretion of the editor
Is there an experimental way to increase barrier strength at earlier time points to see if doing so prevents expression of Dilp8 from causing a delay in pupariation?

The hypothesis in the Fig 7 legend is interesting – that restoration of the barrier following regeneration helps end the regeneration checkpoint. It is unclear if the authors are considering "restoration of the barrier" to be completion of wound closure, or perhaps delayed maturation of the epithelial barrier at some point during regeneration. To clarify, the authors could examine dextran permeability over a time course during regeneration following bx>eiger ablation, to compare to normal development.

There is variation in apparent membrane permeability at 92 and 98 hr (2C and S5). Are there functional consequences to this variation? Perhaps variation in ability of a pulse of Dilp8 at these times to affect pupariation timing?

Corrections for the text
Line 34: add “the” between at and end Line 86 remove “the” before Dilp8 Line 87 add “the” before regeneration Line 89 epithelia should be epithelium. Also: peripodium or peripodial epithelium (not membrane)

Line 158 remove “exists”

Line 163 remove comma after indicates Line 206 – How was Cora detected, given that the paragraph details how Kune and Nrx were detected?

Line 335 Not a complete sentence Lines 378-379 Not a complete sentence Line 384 – tissue is not mutant for Cora Line 410 – add “a” before mature Line 441 add “serum” after goat Line 453 add a “t” in “Trion”

Line 463 remove extra 1000

Reviewer 3

Advance summary and potential significance to field

This study aims to investigate how Dipl8 controls the development and regenerative checkpoints in Drosophila wing imaginal disc. The authors discover that septate junction (SJ) maturation is dependent on EcR and correlates with wing growth. The authors also confirm that Dilp8 accumulates in the imaginal lumen, suggesting that epithelial SJs mediate its signaling. To test this, the authors use a fluorescein-dextran permeability assay. Knockdown of SJs, Kune, Nrx, or Cora causes permeability of the disc to 10kD-dextran and also a developmental delay in imaginal disc growth and regeneration. This study helps to answer a long-standing question in the field on how Dipl8 signals to regulate imaginal disc growth.
However, it remains unclear whether Dipl8-SJ regulation is direct. In other words is Dilp8-SJ regulation dependent on lumen Dilp8 or does SJ signal through another intermediate to regulate disc growth. Additional evidence is needed to conclude that epithelial SJ barrier regulates growth by limiting Dilp8 signaling to Drosophila brain and prothoracic gland. The following Major comments should be address to strengthen this conclusion.

Comments for the author

Major Comments:
1. The genetic loss of SJs was recently shown to affect tissue growth via Hippo-Yki and epithelial differentiation (Khadilkar and Tanentzapf. Development, 2019 and Lee et al. PLOS Genetics 2020). This study does not address alternative roles for the SJs in regulating wing imaginal disc growth that could impinge on the interpretation of their data. Additional evidence is needed conclude that the SJ barrier is limiting lumen Dilp8 signaling. To do so, the authors should test whether Dilp8 accumulation in lumen is dependent on SJs shown in Figure S1. Also how does RNAi of the SJs affect Hippo-Yki signaling? Is epithelial differentiation or proliferation affected when SJs are knocked down in the wing disc?

2. Authors show that SJ matures with imaginal disc development. What happens when SJs are ectopically expressed early at 92h? Is SJ expression sufficient to restrict Dilp8 signaling and limit disc growth?

3.Representative images should be shown in each main Figure, not just the graphical quantification. It is difficult to interpret what is actually being measured in these figures. Representative images in Figure S3 (permeability assay)

should be included in the main figures as well for Figure 2, 4, and 6. There should also be representative images of the developmental delay in Figures 1, 7A and S2. A description of developmental delay assay should be included in methods section as well.

First revision

Author response to reviewers' comments

Below we include our specific responses (in red) to reviewer comments:

Reviewer 1 Comments for the Author: Major comments:

*Ideally, the mutants that compromise the barrier would restore pre-barrier formation levels of permeability seen in WT. But in some cases, the mutant seems to exceed the WT pre-barrier permeability levels. This raises concerns about secondary phenotypes contributing to the observed phenotype. For example, do kune etc. mutants lead to elevated apoptosis, which then leads to an aberrant barrier? The authors should speak to this in some way, and any caveats should be frequently noted.

In response to this reviewer's comment, we realize that we have failed to effectively communicate the barrier activity we see at are earliest timepoints. This isn't "pre-barrier" activity, rather, we think that these early discs exhibit an intermediate level of barrier permeability. This conclusion is based on our comparison to a) physically damaged discs, or b) discs lacking the critical barrier component Kune-Kune. We have tried to better communicate our interpretation of these results through revisions to the text (lines 168-180)

In response to the reviewer's suggestion, we observe that Kune-Kune mutants produce a moderate increase in apoptosis in the wing discs (data included in supplemental figure S4). This does raise the issue that the tissue damage in the Kune mutant may also contribute to the disruption in barrier function and we have included that caveat in the text (lines 163-167)

However, we think that this caveat does not impact our main conclusions since those conclusions rely on the Kune mutant primarily as a means of disrupting the wing disc epithelial barrier and are not focused on the mechanism of that disruption.

* The dextran assay is a very important part of the data- it would be good to present at least some of this (in image form) in the main figures rather than only supplemental.

We agree with the reviewer and have included examples of these images in figures 2, 4, and 6.

*The manuscript is pitched as a regeneration story but only one main figure panel (7A) is devoted to this question. I suggest moving Fig S12 data to Fig 7. Additionally, if feasible, the authors should include some measure of regeneration (number of proliferative cells, amount of tissue restored) in the septate junction mutants.

We agree that this was a limitation in our previous manuscript. Unfortunately, it's difficult to examine the regenerative outcomes of barrier disruption in adult tissues because loss of Kune (or other barrier components) in the wing disc leads to a severely deformed adult tissue with which interpretations of regeneration become impossible. Additionally, our attempts to transiently downregulate Kune or Nrx to see if we could extend the regenerative period but still allow for normal morphogenesis of the tissue were largely unsuccessful. We think this might be due to the relatively low turnover of these proteins at the septate junction, requiring extended RNAi expression to see a meaningful effect on protein levels.

However, we can look at regenerative gene expression within the damaged imaginal tissues. Therefore, it is possible to determine whether the extended delay produced by kune mutant discs

(Fig. 8A) is accompanied by continued regenerative gene expression. We see that the expression of Dilp8 and Wingless, two genes that reflect regenerative activity in the tissue, persist throughout the extended checkpoint delay in Kune mutant wing discs (The image data and quantification is now included in Fig. 8 and FigS18). This observation is consistent with our model that the extended checkpoint delay is produced by the failure of the barrier to sequester dilp8. However, we now also show that this is associated with extended regenerative activity. We describe these results and our interpretation in the text, lines 347-368

We also see that the morphology of the regenerating kune mutant discs is altered towards the end of the checkpoint period, which may reflect either dysregulated regenerative growth, the effect of Kune on the hippo signaling pathway, or additional growth resulting from the longer checkpoint delay. We discuss this observation as well in these interpretations in the text lines 364-368.

*Does cessation of developmental mitoses coincide with acquisition of an impermeable barrier?

We examined this question and found that mitosis is still observed when the barrier is fully formed. This data has been added to Figure S6 and referenced in the main text lines 187-190.

*The 20HE addition impact on barrier formation seems mild- does this induce premature septate junction localization? If so, this would strengthen claims about 20HE addition.

This is a very helpful suggestion. We examined the localization of coracle in animals fed 20HE and found that indeed 20HE feeding accelerates the localization of coracle to the apical site of the SJ. Therefore, we can conclude that ecdysone signaling in the wing disc is both necessary and sufficient for the re-localization of coracle to the SJ. This data has been added to figure 7 and included in the main text lines 305-316,

*Figs 6D-F lack image quantitation as per other similar figures (e.g. Fig5G)

We have included the quantifications for all of the lateral localization data in either the primary figure or as part of an associated supplemental figure.

Minor comments:

*The summary statement has a tight word count, but it's unclear what "becoming more restrictive" means, and regeneration checkpoint is not defined. This is an important stand alone statement, so I suggest revising along these lines.

*Abstract: Lines 30-31: this sentence is very hard to read and I couldn't quite interpret the meaning

*Line 33- restrictive to what? Impermeable to what? checkpoint delay of what?

*Various typos on lines 90, 158, 163, and 262.

We have made revisions and edits as per the reviewer's suggestions

Reviewer 2 Advance Summary and Potential Significance to Field:
The manuscript "Ecdysone regulates the Drosophila imaginal disc epithelial barrier, determining the duration of regeneration checkpoint delay" by DaCrema et al. describes a novel approach to understanding the restrictions on the signaling that enables imaginal disc regeneration to proceed without interference from pupariation and metamorphosis. As such, it will be of great interest to the broad readership of Development, as the work has implications for both tissue regeneration as well as other long-range signals that might be impacted by animal maturation.

Before publication, the authors should address specific concerns. As many labs are partially or completely closed at the moment, this review will attempt to ask for clarifications that can be made using existing data, or with minimal additional experiments.

Reviewer 2 Comments for the Author: Major concerns

What is the difference between the 92h and 116h barrier such that one requires Cora and the other does not? While the answer to that question may be beyond the scope of this study, more attention should be paid to the question in the results or discussion.

Why is barrier function intact in the Cora knockdown, when Nrx and Kune localization are lost – yet Nrx and Kune are required for barrier function? The possible explanation in the discussion – that Nrx and Kune are not completely lost - is not supported by data, but could be. Comparing S8B,D to 5E, is there a quantifiable difference in signal? Could Nrx or Kune be further reduced in the Cora knockdown background, to demonstrate that residual activity remains in the Cora knockdown and further reduction allows more dextran into the lumen? Perhaps Cora knockdown in a heterozygous mutant Kune or Nrx background?

We do agree that this is an interesting and somewhat unexpected observation that we would we plan to explore in further studies. We have taken the approach suggested by the author and examined there is remaining Kune localizing to the lateral membrane in the absence of Coracle. To determine whether there still might be residual Kune localizing to either the apical or medial lateral membranes when Cora is targeted with RNAi, we compared the residual Kune localization in CoraRNAi vs. KuneRNAi. Interestingly, in 92- hour discs we can detect more Kune in the apical and medal lateral membranes of cora^{RNAi} expressing tissues than *kune*^{RNAi} expressing tissues. In contrast, we see no significant difference in detectable Kune levels in either the apical or medal lateral membrane at 116 hours (This data is now included in a new Fig. S12 and described in lines 253-264). This result is consistent with our hypothesis that residual Kune localization within the lateral membrane may contribute to the barrier function we see in early Cora mutant discs, whereas in late discs, Kune localization is entirely dependent on Coracle.

This observation does not address the mechanism that might contribute to this residual activity. One possibility that a fraction of the Kune in the lateral membrane in 92-hour discs does not depend on Coracle to produce barrier activity. However, we can't exclude the possibility that these observations could be due to differences in the dynamics of Coracle and Kune knockdown at different stages of development. We include this caveat in our discussion of these observations (lines 415-426)

The conclusion "The epithelial barrier regulates the end point of regeneration" (line 318 and Figure 7 title) is not supported by the data. The data indicate that compromising the barrier extends the time before pupariation after tissue damage. However, the duration of regenerative growth, as assessed by proliferation in the damaged disc, or extent of regenerative growth, as assessed by adult wing size, were not shown. Furthermore, the authors did not try compromising the barrier after tissue damage in more mature imaginal discs to see if doing so would enable regeneration in tissue that is losing competence for regenerative growth. Indeed, ecdysone signaling inhibits regeneration at least in part by silencing enhancers at multiple damage-responsive genes, so the extent to which the end of regeneration is controlled by Dilp8 and the onset of pupariation, rather than ecdysone signaling in the damaged epithelium itself, is an open question. The authors should add supporting experiments or reword their conclusion

Similar to our response to the comments from Reviewer 1 above, we agree that this is a limitation of the previous version of the manuscript. We are constrained in our ability to assess adult regenerative phenotypes when we disrupt the wind disc barrier for the reasons also described above so the experiments proposed by the reviewer are not technically feasible. However, we have added data looking at the expression of Dilp8 and wingless, genes that reflect regenerative activity in the disc and at both of which are responsive to the ecdysone signal that constrains regeneration, which demonstrate that the extended regenerative checkpoint delay produced by Kune mutant discs is accompanied by persistent expression of wingless and Dilp8. This data is now included in Figure 7.

We have changed title of figure 7 and the section to reflect a more limited conclusion: "The epithelial barrier regulates the duration of the regeneration checkpoint". In the discussion (lines 386-398), we address the possible mechanisms by which barrier function could influence the duration of regenerative activity. Our model is consistent with the mechanism proposed by

reviewer 2: by delaying the ecdysone signaling that determines the duration of the regenerative checkpoint, we delay the silencing of enhancers that regulate the expression of regeneration genes. However further investigation would be needed to deter whether this or other mechanisms link barrier activity and regenerative activity.

The fluorescence intensity variation for the dextran experiments is of concern. How inconsistent was the barrier-impaired fluorescence from experiment to experiment? A supplemental figure showing the variation, and showing that the difference between experiment and control was consistent despite this variation, would be helpful.

We agree that this would be helpful and have included this data in Supplemental Figure S4

Minor concerns

The results open with a supplemental figure – readers would appreciate seeing the FLAG-tagged Dilp8 image in the main Figure 1.

We have moved this image and data to Figure1 and included additional images and data that demonstrate that the localization of dilp8 in the disc lumen is dependent on Kune activity in the disc

The authors do not mention the possibility that some Dilp8 is released through the basal surfaces of the cells. While excluding this possibility is not essential for their model, evidence for or against such a possibility should be mentioned.

We have included this possibility in the text lines 130-133.

Figure 2 is called out before Figure 1, and Figure S8 is similarly called out of order. Fig. 5G is called out of order (Line 214). Data showing that each RNAi construct works should be moved to the first use of that construct. If keeping the experiments demonstrating RNAi efficacy in the same figure is desired, move the figure earlier.

We have made sure that the figures are now correctly called out in order and that demonstrations of RNAi efficacy are presented in the supplemental information and referenced when the RNAi construct is first used

Line 176 – reword to be more accurate - the authors see a progressive decrease in dextran in the lumen that suggests or indicates a decrease in barrier permeability

We have changed the text to reflect this suggestion

Figure 1 – the LacZ controls are not on the graph – presumably the mean of the controls would be at zero. What is the variation in pupariation timing among the controls and how does it compare to the variation in the experimental samples?

We have included this data in the supplemental figure 1

Figure 2 and materials and methods – How the dextran fluorescence was quantified is unclear. It would be helpful, in figure 2 or S3, to show an image of a lumen with the dextran fluorescence and the line drawn for quantification. It is unclear from S3 B, C. and D where such a line would be drawn on these images, or whether cross-section images were used, and if so where the line would be drawn on them.

We have added those images to the supplemental figure 3 illustrating where the fluorescence quantification occurs in the images and have made clarifying edits to the methods section as well.

Figure 2B and 2C 92 hrs – the distribution looks bimodal – is this distribution consistent and might there be a reason for two populations at this time point?

Occasionally some of these data will appear to distribute bimodally. However, when we run a

D'Agostino and Pearson test of normality on these data (fully acknowledging that these tests aren't perfect and that there may be too low an n for these tests to be truly meaningful) we see that both sets of data pass a normality test (2B: K2=5.572 p=0.0563, 2C: K2=2.117 p=.3470). Therefore, we think it is unclear whether they reflect true bimodal populations.

Fig 2A and B. A higher n than 5, 6 or 7 would be preferable, but given the limitations on lab experiments may not be achievable.

We agree that this would be ideal, but these quantitative barrier assays are quite labor intensive, and with the limitations in our research capacity we have had to prioritize other experiments.

Fig3A S6A, S8A, S10A – the arrow depicting the image location is confusing – it could be interpreted as pointing to the image location (the hinge folds). Is it correct that the stem of the arrow is the image location (the pouch) and the arrowhead indicates directionality? Better to use a dashed line or box with no arrowhead.

The arrow demonstrates the cross-sectional slice taken for the images from left (arrow base) to right (arrow head) as the images are presented in the figure. We have added text in the legends of these figures that clarify this

Fig 3 and beyond – all images need scale bars

We have added scale bars to images to illustrate the scale for all the images.

Fig 3F,I,L – The normalization is confusing. Normalization is to the mean membrane intensity where? If normalization is to mean intensity all along the lateral membrane, wouldn't the intensity at the apical-lateral then be greater than one (much higher than the mean)?

The data are normalized to the mean apical intensity at the 116-hour timepoint (the second column of each graph). We have made changes to the figure legend to make clarify this.

Fig 3L – it's surprising, based on the images, that the Medial quantification difference is not greater.

We have chosen an image is a better representative of the data for this figure

Fig 5G is difficult to interpret at a glance (perhaps it needs more contrast) and seems subjective, especially given the low n for the imaging experiments and lack of quantification (compared to quantification in Figure 3). For example, there seems to be diffuse Kune signal at 92h in the NrxRNAi and the CoraRNAi but nothing noted in the diagram. The diagram is not necessary to support the main claim of the figure, which is that Kune and Nrx are mislocalized in CoraRNAi.

We have removed figure 5G and provided quantification for this figure in Figs. S9-S11 to assist with the interpretation of this data

Fig 6A – It would be helpful to see this graph on the same Y axis scale as Fig 2C, for comparison.
We agree and have changed the Y axis range of 6A accordingly
Fig 6D/E/F – quantify apical Cora to determine whether there is a reduction of Cora at the septate junctions.

We have included this quantification in the figure, which is now Figure 7

Fig 7 the line between Ecdysone and regeneration should be solid, as it has been shown that ecdysone signaling leads to epigenetic silencing of regeneration-responsive enhancers.

In this case, the hatched line just suggests that there are steps in between that haven't been fully characterized and that the genetic interaction we are illustrating is not likely to be direct. This is also true for Lgr3 regulation of ecdysone and the epithelial barrier maturation effect on regeneration and dilp8 signaling. We have also included an explanation of this in the figure legend to clarify how we are using that symbolism.

Fig S1 – The legend does not note what the white arrows are pointing to.

This image was moved to Figure 1 and an explanation of the arrows is included in the figure legend.

Fig S3 – why is the LacZ not restricted to the dorsal half of the disc?

The LacZ is restricted to the dorsal half of the wing disc, (see the bright staining at the edge of each disc). The lacZ reporter is localized to the nucleus, which is at a more basal focal plane that that being used for these images. We have added this explanation to the text of the figure legend.

Fig S3 – cross section images of B,C, and D as diagrammed in A would be helpful, especially to demonstrate that the signal in C is indeed in the lumen.

In Figure S3, we have included representative images of cross sections of dextran infiltration and exclusion (from different discs) to help visualize this signal

Fig S9A – the meaning of the orange arrows is not immediately clear. Perhaps draw an orange curve above the blue curve to conceptually explain the elevated ecdysone levels.

We have removed this figure as we judged it wasn't necessary for understanding the ecdysone feeding experiment.

Experimental suggestions stemming from curiosity – requirement left to the discretion of the editor

Is there an experimental way to increase barrier strength at earlier time points to see if doing so prevents expression of Dilp8 from causing a delay in pupariation?

This is an excellent idea and we have been trying to figure out ways to do this experiment, but to this point we have been unsuccessful. We have tried overexpressing some components (in particular, Cora) to see if we can enhance barrier function, but our initial experiments suggest that we ended up having little effect and even disrupting barrier activity slightly, suggesting that the functional complex may be sensitive to stoichiometry and that overexpression of some components may produce a dominant-negative effect. We hope to explore these experiments more thoroughly in further studies.

The hypothesis in the Fig 7 legend is interesting – that restoration of the barrier following regeneration helps end the regeneration checkpoint. It is unclear if the authors are considering "restoration of the barrier" to be completion of wound closure, or perhaps delayed maturation of the epithelial barrier at some point during regeneration. To clarify, the authors could examine dextran permeability over a time course during regeneration following bx>eiger ablation, to compare to normal development.

Again, we agree with reviewer two that the relationship between regeneration and the restoration of the epithelial barrier is interesting and we hope to explore this relationship in subsequent studies.

There is variation in apparent membrane permeability at 92 and 98 hr (2C and S5). Are there functional consequences to this variation? Perhaps variation in ability of a pulse of Dilp8 at these times to affect pupariation timing?

We don't actually see a significant difference in the membrane permeability at 92 at 98 hours, but we do see a reduction in membrane permeability between 98 and 104 hours. Is this what the reviewer was referring to? If so, indeed this is around the time (about 104h from our previous work) that we start see a limitation in the ability of damage to produce a dilp8-induced delay. We do think both are related as both are likely the result of increasing ecdysone levels in the larva as it approaches pupariation.

Corrections for the text

Line 34: add “the” between at and end Line 86 remove “the” before Dilp8 Line 87 add “the” before regeneration
Line 89 epithelia should be epithelium. Also: peripodium or peripodial epithelium (not membrane)
Line 158 remove “exists”
Line 163 remove comma after indicates
Line 206 – How was Cora detected, given that the paragraph details how Kune and Nrx were detected?
Line 335 Not a complete sentence Lines 378-379 Not a complete sentence Line 384 – tissue is not mutant for Cora Line 410 – add “a” before mature
Line 441 add “serum” after goat Line 453 add a “t” in “Trion” Line 463 remove extra 1000

We have made the suggested corrections to the text

Reviewer 3 Advance Summary and Potential Significance to Field:
This study aims to investigate how Dipl8 controls the development and regenerative checkpoints in Drosophila wing imaginal disc. The authors discover that septate junction (SJ) maturation is dependent on EcR and correlates with wing growth. The authors also confirm that Dilp8 accumulates in the imaginal lumen, suggesting that epithelial SJs mediate its signaling. To test this, the authors use a fluorescein-dextran permeability assay. Knockdown of SJs, Kune, Nrx, or Cora, causes permeability of the disc to 10kD-dextran and also a developmental delay in imaginal disc growth and regeneration. This study helps to answer a long-standing question in the field on how Dipl8 signals to regulate imaginal disc growth.

However, it remains unclear whether Dipl8-SJ regulation is direct. In other words, is Dilp8-SJ regulation dependent on lumen Dilp8 or does SJ signal through another intermediate to regulate disc growth. Additional evidence is needed to conclude that epithelial SJ barrier regulates growth by limiting Dilp8 signaling to Drosophila brain and prothoracic gland. The following Major comments should be address to strengthen this conclusion.

Reviewer 3 Comments for the Author: Major Comments:
1. The genetic loss of SJs was recently shown to affect tissue growth via Hippo- Yki and epithelial differentiation (Khadilkar and Tanentzapf. Development, 2019 and Lee et al. PLOS Genetics 2020). This study does not address alternative roles for the SJs in regulating wing imaginal disc growth that could impinge on the interpretation of their data. Additional evidence is needed conclude that the SJ barrier is limiting lumen Dilp8 signaling. To do so, the authors should test whether Dilp8 accumulation in lumen is dependent on SJs shown in Figure S1. Also, how does RNAi of the SJs affect Hippo-Yki signaling? Is epithelial
differentiation or proliferation affected when SJs are knocked down in the wing disc?

These are valuable suggestions from our third reviewer. First, we have done the additional experiments that they suggested and demonstrated that the luminal localization of dilp8 is dependent on the SJs. We have included these images and quantification of this data in the main Figure 1

We also agree that the we need to address whether the genetic interaction we see between the septate junction proteins and dilp8 signaling potentially reflects a role for Hippo-Yki signaling, especially to eliminate the possibility that the hippo pathway may be activating dilp8 expression when the SJs are disrupted. We think that the best experiment to address this is in figures 2B and S2B, where we demonstrate that the genetic interaction between kune and Dilp8 occurs even in the absence of the endogenous copies of dilp8. This means that Kune must be genetically interacting with the UAS-regulated transgenic copy of Dilp8, which would be unlikely to transcriptionally regulated by the hippo pathway. We address this in the text lines 119-129.

2. Authors show that SJ matures with imaginal disc development. What happens when SJs are ectopically expressed early at 92h? Is SJ expression sufficient to restrict Dilp8 signaling and limit disc growth?

This would be an interesting experiment. Aside from ecdysone feeding (which we have shown

previously does limit regeneration), we haven't found a way to accelerate the maturation of the SJs. We have found that overexpression of Cora, for instance, actually slightly disrupts the barrier. Therefore, we are still working on approaches to address this question and would like to include those experiments in subsequent studies examining the relationship between barrier activity and regenerative activity in the disc.

3. Representative images should be shown in each main Figure, not just the graphical quantification. It is difficult to interpret what is actually being measured in these figures. Representative images in Figure S3 (permeability assay) should be included in the main figures as well for Figure 2, 4, and 6. There should also be representative images of the developmental delay in Figures 1, 7A, and S2. A description of developmental delay assay should be included in methods section as well.

We have added representative images for the permeability assay for figures 2, 4, and 6.
The developmental delay assay is not image based, so there are no images to present. However, we have included a description of this assay in the methods section.

Second decision letter

MS ID#: DEVELOP/2020/195057

MS TITLE: Ecdysone regulates the Drosophila imaginal disc epithelial barrier, determining the duration of regeneration checkpoint delay

AUTHORS: Danielle DaCrema, Rajan Bhandari, Faith Karanja, Ryunosuke Yano, and Adrian Halme

Thank you for your patience. 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 overall evaluation is quite positive, and each reviewer is enthusiastic about your study. As you will see, Reviewer #2 requests some specific changes in how the results are presented and interpreted that they feel will improve the manuscript and should not require further experimentation. Please address these comments in your revised manuscript and detail them in a separate point-by-point response. If you do not agree with any of their suggestions explain clearly why this is so.

Reviewer 1

Advance summary and potential significance to field

This manuscript explores regulation of the regeneration checkpoint, which is an organism-wide developmental delay induced by tissue damage in Drosophila imaginal discs. Previously, it was known that Ecdysone and Dilp8 were important regulators of this checkpoint. Using a combination of genetics and quantitative imaging, the findings of this manuscript move the field forward in an important way by providing a cellular mechanism for the cessation of regeneration checkpoint sensing. These results should be of broad interest to the organ regeneration field, as well as to developmental biologists interested in formation of epithelial barriers.

Comments for the author

The authors responded appropriately to my previous review comments.

Reviewer 2

Advance summary and potential significance to field

See prior review

Comments for the author

I appreciate the effort made by the authors to address the reviewers' concerns and conduct additional experiments in the current environment in which access to labs is limited. While the review of these additions was made challenging by the fact that many of the figures and text line numbers mentioned in the response to the reviewers were incorrect, I will comment based on what I think are the correct changes.

Overall, the manuscript and figures are significantly improved and easier for the reader to follow and assess. The addition of representative images and added explanations in the figure legends are appreciated. My remaining concerns revolve around presentation and interpretation of the existing data:

Figure 8 B-E were added to confirm that the tissue's response to damage is sustained past the time when pupariation normally occurs when the EB is disrupted, as assessed by Wg and Dilp8 expression. However, there is no indication that Wg and Dilp8 expression are not sustained past pupariation when the EB is NOT disrupted. This experiment uses continuous expression of Eiger to induce continuous damage, and expression of regeneration genes is not reduced before pupariation as they are when damage is transiently induced. Thus, the conclusion drawn from this experiment must be carefully worded. For example, see lines 402-403, which imply that the barrier normally functions to limit expression of these regeneration genes, which has not been demonstrated.

The orange lines in Figure S3 were initially confusing, likely because they are labeled "measurement area" in the figure, so I expected them to encircle an area, when in fact they are along the fold used for measuring fluorescence. Perhaps changing the word "area" would be helpful.

The equation for median pupariation assumes a steady rate of animals pupariating. Therefore, perhaps it would be better to say that the median pupariation time was estimated rather than determined.

Reviewer 3

Advance summary and potential significance to field

This study answers the long-standing question of how Dipl8 signals to regulate imaginal disc growth. The authors discover that septate junction maturation is dependent on EcR and correlates with wing growth. This revision now more clearly demonstrates that Dilp8 accumulation in the imaginal lumen is dependent on separate junction protein, Kune. Also permeability of epithelial barrier during developmental checkpoints correlates with changes in junctional protein expression and localization. Overall, this study shows that imaginal disc epithelial barrier is required to limit Dilp8 signaling thereby regulating the developmental and regenerative imaginal disc checkpoints. This is a significant and novel contribution to our understanding of development.

Comments for the author

The revisions were adequately addressed no further revision is requested.

Second revision

Author response to reviewers' comments

1) "Figure 8 B-E were added to confirm that the tissue's response to damage is sustained past the time when pupariation normally occurs when the EB is disrupted, as assessed by Wg and Dilp8 expression. However, there is no indication that Wg and Dilp8 expression are not sustained past pupariation when the EB is NOT disrupted. This experiment uses continuous expression of Eiger to induce continuous damage, and expression of regeneration genes is not reduced before pupariation as they are when damage is transiently induced. Thus, the conclusion drawn from this experiment must be carefully worded. For example, see lines 402-403, which imply that the barrier normally functions to limit expression of these regeneration genes, which has not been demonstrated."

We do acknowledge that this is a limitation in our observations and therefore have eliminated the text that the reviewer identified as suggesting that the epithelial barrier normally functions to limit the expression of Wg and Dilp8 and tried to focus on the more supportable observation that this extended delay can continue to support regenerative signaling. In particular, then end of the results section describing Figure 8 now reads (lines 361-374):

However, when we measure dilp8 and Wingless expression in the extended delay period of Bx>kuneRNAi; eiger larvae, we see that the expression of these regenerative markers not only persists during the extended delay, but both are substantially upregulated during this period (Fig. 8C,E; Fig. S18E,F). In addition, we see substantial overgrowth of the regenerating wing disc (Fig. S18D, compare 188h AED with earlier timepoints or with S18A). It is unclear from our analysis whether the increased dilp8 and Wingless expression and tissue overgrowth that we observe results from dysregulated regenerative activity in the disc or the extended growth period.
Together, these results demonstrate that disruption of the EB in damaged imaginal discs produces an extended larval period that can support persistent regenerative gene expression. This demonstrates that a fully-functional EB can limit the duration of damage-induced checkpoint delay, likely through the sequestration of Dilp8 within the imaginal disc lumen.

Additionally, we have removed the text in the first discussion section: "How is the end of regeneration determined?" that draws a connection between barrier function and regenerative gene expression. We think that these changes should satisfy this concern raised by reviewer 2.

2) "The orange lines in Figure S3 were initially confusing, likely because they are labeled "measurement area" in the figure, so I expected them to encircle an area, when in fact they are along the fold used for measuring fluorescence. Perhaps changing the word "area" would be helpful."

We have relabeled this line "measurement region". Hopefully this will help allay any confusion.

3) The equation for median pupariation assumes a steady rate of animals pupariating. Therefore, perhaps it would be better to say that the median pupariation time was estimated rather than determined.

We have altered the text in the methods section to describe this measurement as an estimated median pupariation time.

Third decision letter

MS ID#: DEVELOP/2020/195057

MS TITLE: Ecdysone regulates the Drosophila imaginal disc epithelial barrier, determining the duration of regeneration checkpoint delay

AUTHORS: Danielle DaCrema, Rajan Bhandari, Faith Karanja, Ryunosuke Yano, and Adrian Halme
ARTICLE TYPE: Research Article

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