Engrailed, Suppressor of fused and Roadkill modulate the *Drosophila* GLI transcription factor Cubitus interruptus at multiple levels

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MS title: Engrailed, Suppressor of fused and Roadkill modulate the Drosophila GLI transcription factor, Cubitus interruptus at multiple levels

Authors: Nicole Roberto, Anne Plessis, and Robert Holmgren
Article type: Research Article

Dear Reviewer,

Thank you for reviewing the above paper. After careful consideration, we have decided to reject this manuscript.

Reviewer 1

Advance summary and potential significance to field

Hedgehog morphogen forms concentration gradient for pattern formation. Feedback regulations are required to maintain robust Hedgehog signaling. In this manuscript, Roberto et al. used Drosophila genetics to show that Engrailed, Su(fu) and Rdk modulate Ci and target gene expression, and Su(fu) and Rdk interact to participate in competition between two forms of Ci: CiA vs. CiR.

Comments for the author

Overall, there is not much surprise for the reported interplay among Engrailed, Su(fu) and Rdk, and the phenotypes are not very strong. First, the regulation of Ci and ptc by Hh activated En is expected. Intensive research demonstrated that En inhibits Ci expression at the posterior compartment as well as the most posterior region in the anterior compartment along the A-P boundary. Second, given the well-known function of Rdx on full length Ci degradation in both flies and vertebrates, and Su(fu) on shuttling and stabilization of full length Ci, it is not hard to make a genetic connection between these two regulators and the competition between CiA and CiR. As the

wing phenotypes are not strong, direct biochemical evidence are needed to reinforce their conclusion.

Minor points:
1. Altered distance between veins 3 and 4 shown in adult wings need to be quantified.
2. The orientation of the wing discs and eye discs is different, and scale bars are also missing in most figures.
3. There are many typos in the manuscript and the writing needs to be re-organized to make it more reader-friendly.

Reviewer 2

Advance summary and potential significance to field

The authors provide a significant advance in the understanding of the genetic network that controls the range of action of Hh in the Drosophila wing imaginal disc. Hh action during wing development is a classical paradigm to understand morphogen action. Specifically the authors re-evaluate the function of En in the anterior compartment as a key regulator of the shape of Hh gradient by downregulating Ci and ultimately decreasing the levels of the receptor Patched (Ptc). Next, they show interesting genetic interactions between suppressor of Fused (su(fu)) and the E-3 ligase Roadkill (Rdx). They propose an attractive model where Su(Fu) and Rdx tune the balance between the repressor and activator form of the Gli homologue Ci. This is especially exciting as both En and Rdx are Hh targets, which makes this genetic network even more interesting.

Comments for the author

The manuscript describes an interesting gene regulatory network. While the signaling implications are important to understand how Hh activity gradient is shaped, the paper could also provide more information on the dynamics of cell identity: One key message is the regulation by feedback loops, so timing matters! I do understand that wing imaginal discs are not suitable for live imaging, but with minimal efforts the authors could use some markers of different life-span to visualize the dynamics of cell identity-long lasting Bgal, or GFP and RFP with different life-spans. Specifically, are the en cells induced in the anterior compartment added sequentially? When is the roadkill feedback loop kicking in? In other words is there a short wave of Hh signaling that makes the comparison with the eye disc even more pertinent? To summarize this point I thing that bringing in the cellular scale would greatly widen the scope of the paper and give a broader developmental dimension to a very interesting signaling-oriented paper.

Specific comments:
Figure 2: Markers' accumulation is the main criteria to qualify En effect on Hh pathway activity and as such curves reporting Ptc, Ci and DPP levels along the antero- posterior axis would greatly help visualize the biological effect of En LOF clones. In addition, it is important to show that the increase in Ptc expression is meaningful in term of Hh range of action, so I would suggest bringing the SRF/bs data from Figure S2 to the main figure S2. The authors should also include the DPP-lacZ data that are mentioned at line 126, but not shown, in figure 2. Another piece of data that would strengthen the authors' conclusion would be to present En and Ci expression in a wild type disc at a high magnification so to show that anterior Engrailed correlates with a lower Ci detection. Even more telling would be the analysis of Ci vs En in discs of different ages: Early we would not expect En induction and Ci should be the strongest at the boundary. Only when En gets induced would Ci expression decrease. This would help bring up the dynamic nature of this genetic network and greatly improve the study by emphasizing the function of the feed-back loop.

In figure 4, Su(Fu) clones are giving clear results on Ptc and dpp expression. Still, it appears that the intensity of the Ci staining may not be well calibrated between the panels. The authors could either try to use the same PMT settings between the panels, or take an area as a reference. For example Ci expression in the most anterior cells of the wing pouch. I would expect the global intensity in B3 to decrease.

In Figure 5 I would have appreciated to see Rdx wild type expression domain compared with Ci expression. Ci in Ptc-Gal4, UAS-Rdx RNAi could be included, and the data from figure S3 could be inserted here.

In figure S1 I agree with the fact that heterozygous flies do not display a distance phenotype between L3 and L4. Still it is not clear to me how much of the expression is decreased as these genes are highly regulated and if so if the decrease in expression is significant. The authors should insert a reference that proves the point. If no reference is available, the authors should generate clones and show that twin spots in ptc/+, smo/+ and Hh/+ have a stronger (ideally twice stronger) signal using anti-ptc, anti-smo and anti-Hh antibodies.

Discussion: It is not clear whether the authors suggest that the cooperative effect regarding full-length Ci is sufficient to explain DPP patterning or if both the affinity of the repressor form on one hand and the affinity of the full- length on the other are required.

Minor Comments:
82: En "anterior expression is not understood": The authors should discuss the model according to which anterior En directly inhibits DPP expression (Strigini and Cohen, Park et al).

121: "As can been seen" should read "As can be seen"
309: "moved away": unclear
505: "." Missing after "boundary" so (B3) appears to refer to that statement whereas it announces the next image.
523: 35C should read 35°C

Reviewer 3

Advance summary and potential significance to field

In this manuscript, the Holmgren Lab carried out series of genetics experiments to determine the deeper mechanisms for Ci regulation by multiple regulators, including En, Rdx, and Su(fu). Understanding how Ci is differentially regulated will provide great insight into the mechanism of how thresholds of Hh signaling activity is propagated in vivo. Previous study from this group showed Su(fu) enters the nucleus with the full-length Ci but not the repressor form of Ci. This study moves one step further to determine whether Su(fu) protects full- length Ci from degradation by Rdx in the nucleus. And, the hypothesis is very interesting, that Rdx and Su(fu) shift competition between wild type full-length Ci and the truncated repressor form of Ci. Most of the genetics data are promising; however, there are concerns regarding data explanation and the mechanisms of Ci regulation.

Comments for the author

In this manuscript, the Holmgren Lab carried out series of genetics experiments to determine the deeper mechanisms for Ci regulation by multiple regulators, including En, Rdx, and Su(fu). Understanding how Ci is differentially regulated will provide great insight into the mechanism of how thresholds of Hh signaling activity is propagated in vivo. Previous study from this group showed Su(fu) enters the nucleus with the full-length Ci but not the repressor form of Ci. This study moves one step further to determine whether Su(fu) protects full-length Ci from degradation by Rdx in the nucleus. And, the hypothesis is very interesting, that Rdx and Su(fu) shift competition between wild type full-length Ci and the truncated repressor form of Ci. Most of the genetics data are promising; however, there are concerns regarding data explanation and the mechanisms of Ci regulation.

Major points:
1. En RNAi shows a partial loss of Hh phenotype, indicated by the narrower LV3-4 (Fig. 1C); however, the authors are trying hard to claim a negative role for En to be involved in Hh signaling. This is a major problem. Looking into the details of the data, Fig. 2A is problematic. Can the authors show a smaller clone near the A/P border but not covering the whole ventral compartment? This is because some non-representative wild type discs exhibit variation of ptc-lacZ staining in ventral vs dorsal. Fig. 2B, it is a problem to compare Ci expression using the cells on the D/V boundary. Can the authors show a representative clone not covering the whole dorsal and/or

ventral compartments? This major concern can also be addressed by using clonal analysis to express UAS-En-RNAi and examine Ci and Ptc (or ptc-lacZ) expression. To further determine the expression of Ptc regulated by En, the authors can use a luciferase reporter assay that will be a direct supplement to Fig. 2A2.
2. The dual roles of Su(fu) in regulating Ci has been published by this group (Sisson et al. 2006). Can the authors make it clearer what new findings is in this manuscript? This can be addressed by re-writing the statements, but I think it is very critical for the reader to easily pick up the new information. It is about Su(fu) competing with Rdx for full-length Ci binding, correct? Some experiments to address protein interactions using protein pull-down assay should be included, especially to test the hypothesis of "Rdx preferentially targets full-length Ci".
3. There is another major concern in Fig. S3D. Specifically, the overgrowth between LV2-3 indicates a gain of Hh phenotype, which is contradictory to a previous finding in which the overexpression of Rdx causes loss of Hh signaling activity (Zhang et al., Dev Cell 2006). Can the authors examine the expression of Ci and ptc-lacZ in this experiment? Did the authors examine different transgenic lines?
4. Fig. 6. What would be the phenotypes when Rdx is overexpressed under such conditions?
5. It is very complicated to bring Fu kinase to the story, because Fu has both direct (phosphorylates Ci) and indirect (phosphorylates Su(fu) to regulate Ci) roles in the regulation of Ci. I would suggest removing Fu related data. The authors can surely include some discussions in the Discussion Section.

Minor points:
1. The authors should have introduced the eye disc in the introduction section, so that to compare the pattern of Hh responsiveness in wing disc.
2. Not sure whether it would be possible for the authors to perform some cultured cell experiment to examine the localization of the proteins, which would provide some clearer data to support the hypothesis.
3. Regarding the references, many other papers were published around this area of research. I am not sure why specific papers are cited but not others.
4. Line 78, "(Hh signaling cells)", should be "Hh producing cells"
5. Line 134, add "(Fig. 2B2)" at the end of the sentence.
6. Line 157, add a period after the sentence.
7. Recommend to simply label the genotype on each figure. The current version is very hard to read. The authors have indicated the genotypes in adult wing figures, which is good.
8. The orientation of the wing discs should be consistent. For example, in Fig. 2, top panel has the ventral compartment on the bottom, but lower panel has the ventral compartment on the top.
9. Figure S2, it is hard to see Bs change. Show the data in different channels. Fig. S3 should also have separate channels.
10. Fig. S5 needs some changes. Can the authors show higher magnification of the images? Can the authors use different color combinations to show the co-localization of the proteins?

Response to reviewers
In response to reviewer 1 that the results were to be expected, we would say that in retrospect that might be the case, but it is not reflected in the literature. In the case of reduced Ci protein levels adjacent to the compartment boundary, papers continue to propose that this is the consequence of Hh activation of Ci proteolysis eg. the introduction in Little, JC et al. Elife 2020. In the case of Su(fu), the focus of the literature is very much on its negative regulatory effects eg. Han, Y, Wang B. et al. Dev. Cell 2019. We would disagree with reviewer 1's statement that the wing phenotypes are not strong. In animals heterozygous for ciCe2, overexpression of rdx or loss of Su(fu) leads to a complete fusion between wing veins 3 and 4 and dramatic attenuation of ptc and dpp expression. As for the effect of anterior en expression, while the effect is modest on wing vein patterning, it represents a novel way of modulating the output of a morphogen gradient in a specific tissue.

In response to the minor points of reviewer 1, we have quantified the differences between control wings and wings lacking anterior expression of en and inv, added scale bars, and reorganized the text.

The most difficult issue to address is the one raised by reviewer 2 about the temporal aspects of these processes.

(1) In the case of En regulation of ci, we have added figure S2 in which ptc null mutant clones were generated. In these clones, there is a distribution of effects from elevated levels of both Ci and En to elevated levels of only En where it has presumably shut off of ci leading to a transformation into a posterior fate. These distinct outcomes can be seen in an individual clone. It doesn't have a temporal dimension, but it does provide a snapshot of the process.
(2) In the case of Rdx feed- back loop, we tried to examine a potential role for Rdx in clearing full-length Ci at the margins of Hh signaling. Our approach was to express a couple of different UAS-RNAi lines targeting rdx in the dorsal compartment of the wing and compare the distribution of ptc-lacZ and dpp-lacZ between the dorsal and ventral regions. Loss of rdx might be expected to lead to a broader distribution of the target genes if one of its roles is to clear full-length Ci.
We did not observe an effect and this may well have been a consequence of perdurance.
To have the best chance of observing a difference, one might need to use embryos in which MS2 loop sequences have been inserted into the 3'UTR of wg. Live imaging could be used to follow WT and embryos zygotically mutant for rdx. Such an experiment is presently beyond our capabilities.

As requested by Reviewer 2 we have used the Bar function in ImageJ to plot the distributions of Ci, ptc-lacZ, ci-lacZ and dpp- lacZ. We have incorporated figure S2 into the main figures as figure 2 along with bar plots of Bs and dpp-lacZ.

To answer to the last comment of reviewer 2 on Fig 2 concerning En and Ci expression, instead of following Ci expression over time, we manipulated the domain of anterior En. We expressed different forms of Smo to show a precise correlation between anterior En and attenuated Ci (figure S1).

Concerning the comment of reviewer 2 on previous figure 4 now figure 5, the staining for Ci was just used to distinguish between eyD/+ and ciCe2/+ discs. This secondary for Ci unfortunately had a high background so the changes in Ci levels in the clones are not obvious. The effects of Su(fu) on Ci levels have been well established so we did not feel that the Ci background issue detracted from the main points of the figure.

Concerning the comment of reviewer 2 on figure5 and S3, we decided not to include an image of rdx1 (rdx-lacZ), which is present in a narrow stripe along the compartment boundary in the anterior compartment as it does not correlate with the domain where rdx is functioning. Expression of rdx-lacZ is completely eliminated in ciCe2/+ heterozygotes; nonetheless in these heterozygotes rdx has a profound effect on the expression of Hh target genes in the absence of Su(fu). Figure S3 was provided largely to show the efficacy of the UAS- RNAi-rdx line. To address UAS- Rdx overexpression phenotypes, we have inserted a new Figure 3 in which ap-GAL4 is used to over express UAS- rdx in the dorsal compartment of ciCe2/+ heterozygotes and the expression of ptc-lacZ assayed. This also addresses the major point 3 of reviewer 3.

We have removed figure S1 since as reviewer 2 points out there could be compensation mechanisms that boost the expression of the single functional gene in the heterozygotes.

In the discussion as asked by reviewer 2, we make it clear that protein-protein interactions are one way in which the competition between full-length Ci and the Ci repressor for DNA binding sites could be modulated.

We have also corrected the minor comments of reviewer 2.

In response to major point 1 of reviewer 3 we have replaced the images in what was Figure 2 with new images with different clones in what is now figure 3 and have included graphs using the ImageJ Bar function.

We chose to put the data on Ci and Su(fu) nuclear import in supplemental figures as we have in part addressed this question earlier as noted by reviewer 3 in major point 2. In figure S7 we specifically compare the distributions of full-length Ci and Su(fu) relative to CiCe2, which has not been previously done. We have made this explicit in the text. In part we have included this as the role of Su(fu) translocating into the nucleus with full-length Ci continues to be underappreciated.

With regards to the UAS-rdx- myc construct, this is the same line that was used in Kent et al. 2006. In wild type wing discs, we observed the previously reported down regulation of both ptc-lacZ and dpp-lacZ after over expression of UAS- rdx-myc in the dorsal compartment. We have added a new figure 3 in which UAS- rdx-myc is over expressed in a ciCe2/+ background that results in dramatic downregulation of ptc-lacZ.
We have added some discussion addressing the different results in WT and a ciCe2/+ backgrounds. In wild type attenuation of ptc expression allows Hh to extend further into the anterior compartment expanding the domain between LV3 and 4. Attenuation of dpp expression decreases the areas of the other parts of the wing.

We have added discussion of Fu in both the introduction and the discussion to put figure S6 now figure 6 in better context (major point 6 of referee 3). It is true that the Fu kinase has multiple functions, but it is well established that loss of Fu leads to the cytoplasmic retention of Ci and data to date puts Rdx function in the nucleus. In fu94; Su(fu) double mutants, Ci shuttles into the nucleus where it can be targeted by Rdx.

Reviewer 3 minor points:

We have added a discussion of the eye disc in the introduction.

We have examined the distribution of full-length Ci, Ci repressor and Su(fu) in tissue culture cells. Su(fu) colocalizes with full-length Ci. In the case of Ci repressor, it is nuclear while Su(fu) is primarily cytoplasmic. The effect is not nearly as dramatic as in wing discs. We assume this is a consequence of overexpression in the tissue culture cells allowing the single Su(fu) binding site on Ci repressor to effectively recruit some Su(fu) and bring it into the nucleus.

Figure S2 is now figure 2 with separate channels and bar graphs.

For figure S5 now figure S7, we have colored the text labels of the individual channels to identify the channels in the merge.

The typos have been corrected.

Original submission

First decision letter

MS ID#: DEVELOP/2021/200159

MS TITLE: Engrailed, Suppressor of fused and Roadkill modulate the Drosophila GLI transcription factor, Cubitus interruptus, at multiple levels

AUTHORS: Nicole Roberto, Isabelle Becam, Anne Plessis, and Robert Holmgren

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. Referees 1 and 2 are satisfied with the revisions to your study. Referee 3 also acknowledges that you have strengthened your argument and that the data are of interest to the field. This referee suggests that the study would benefit from assays that provide some indication of the dynamics of Hh signaling and in particular assess the state of Hh signaling early, before the feedback comes into play. I agree with the referee that this would help build the argument you are making. I hope you are able to add these data to your manuscript. I will be happy receive a revised version of the manuscript. Your revised paper may be re-reviewed by one or more of the original referees, and acceptance of your manuscript will depend on your addressing satisfactorily the reviewers' concerns.

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

In this manuscript, the Holmgren Lab carried out series of genetics experiments to determine the deeper mechanisms for Ci regulation by multiple regulators, including En, Rdx, and Su(fu). Understanding how Ci is differentially regulated will provide great insight into the mechanism of how thresholds of Hh signaling activity is propagated in vivo. Previous study from this group showed Su(fu) enters the nucleus with the full-length Ci but not the repressor form of Ci. This study moves one step further to determine whether Su(fu) protects full-length Ci from degradation by Rdx in the nucleus. And, the hypothesis is very interesting, that Rdx and Su(fu) shift competition between wild type full-length Ci and the truncated repressor form of Ci. Most of the genetics data are promising; however, there are concerns regarding data explanation and the mechanisms of Ci regulation.

Comments for the author

My concerns have been addressed.

Reviewer 2

Advance summary and potential significance to field

The revised manuscript ‘Engrailed, Suppressor of fused and Roadkill modulate the Drosophila GLI transcription factor, Cubitus interruptus at multiple levels’ by Roberto et al. provides significant improvement over previous version and addressed my major concerns.

Comments for the author

The revised manuscript ‘Engrailed, Suppressor of fused and Roadkill modulate the Drosophila GLI transcription factor, Cubitus interruptus at multiple levels’ by Roberto et al. provides significant improvement over previous version and addressed my major concerns. Before I recommend publication in Development, the manuscript needs careful proof-reading and editing as there are many phrasing and spelling errors. In addition, some references are incomplete and the style does not conform to Development guideline.

Reviewer 3

Advance summary and potential significance to field

As stated in the previous round of rewiews, this manuscript provides significant advances in the understanding of the genetic network that controls the range of action of Hh in the Drosophila wing imaginal disc. Both Engrailed and Rdx mediated feedback are important to shape the Hh gradient, leading to striking effects on disc patterning. Thus this paper presents a very nice gene regulatory network that controls the patterning in a classical example of morphogen.

Comments for the author

As mentioned in the first round of reviews, this manuscript presents very interesting data about feedback mechanisms that control Hh range of action. I am glad the authors improved their figures by providing a graphic view of reporter gene expressions that facilitates the reading of the data. My main concern remains the lack of perspective regarding the dynamics of the process: the manuscript could benefit from a state of Hh signaling early, before the feedback gets into play, and one afterwards. At minima, the authors could include stainings that show the evolution of patterning with time: For example en-Gal4 UAS-stable GFP and antii-Engrailed, the same with DPP-gal4 and stable vs labile fluorescent protein. This is important so that the "signaling data" translates into "developmental information". Along this line of thoughts, I was uncomfortable with the sentence "While it is still the case that this domain corresponds to the region of the highest level of Hh signaling", line 298. The point of the paper is to present how Hh signaling gets dampened in this region, and the data clearly point out a decrease in Ci expression. Thus this is no longer a domain of High Hh signaling at late larval stages. To the contrary, I believe that the authors should clearly state that the feedback reshapes Hh gradient, transforming the initial unidirectional gradient into a double gradient centered in the middle of the L3-L4 domain. This is what I find very exciting about the paper. Hence for the sake of accuracy, the authors should name the domains according to their positions (ie proximal to AP boundary) and not to the strength of Hh signaling that varies with time. And this is why a more precise description of the dynamics is important.

An interesting outcome is to provide a simpler model to explain the differential patterning of targets regulated by imperfect Ci binding sites (dpp) and perfect consensus sites (Ptc): A stronger signal will act on imperfect binding sites, and a weaker signal will be sufficient to activate perfect consensus binding sites. The cooperative effect of CiR would be needed to repress Ptc in the middle region, but the activation of DPP no longer requires a special cooperative mechanism of the full length and can be explained by the higher strength of Hh signaling at this location and at that later stage of development. Therefore I do not see the need to propose a Ci Full length cooperative mechanism in the discussion. On the other hand, in a context where Ci Full length determines central patterning it is important for the system to robustly assess its presence, and that is where the Rdx/Su(fu) regulation, that is specific to the full length becomes very interesting: Ci signaling would therefore require constant Hh signaling to prevent Ci full length specific degradation.

Minor comments:
157 : of en finely tunes the Hh gradient or/and transduction by controlling the levels its receptor Ptc.
Should read :
of en finely tunes the Hh gradient or/and transduction by controlling the levels of its receptor Ptc.
211-212 : loss of Su(fu) in this genetic context, leads to Should read :
loss of Su(fu) in this genetic context leads to
266 : To further exam the role Should read :
To further examine the role

First revision

Author response to reviewers' comments

We have obtained flies containing the UAS-TransTimer from Norbert Perrimon. This construct encodes a destabilized GFP followed by RFP. We have used it to examine the dynamics of ci and ptc expression. In the case of ci, it is stably expressed throughout the anterior compartment in mid-third instar and as expected, down regulated along the compartment boundary in late third instar larvae (new Fig. 4). In the case of ptc away from the compartment boundary, levels of destabilized GFP decrease relative to RFP showing that ptc expression is being down regulated. This effect is lost when ptc-GAL4 is also driving UAS-RNAi-rdx (new Fig 6). This result is consistent with the hypothesis that Rdx functions in part to remove full-length Ci from cells that are no longer receiving the Hh signal.

We have also added references to address the concerns of reviewer 2.

Second decision letter

MS ID#: DEVELOP/2021/200159

MS TITLE: Engrailed, Suppressor of fused and Roadkill modulate the Drosophila GLI transcription factor, Cubitus interruptus, at multiple levels

AUTHORS: Nicole Roberto, Isabelle Becam, Anne Plessis, and Robert Holmgren
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.