E93-depleted adult insects preserve the prothoracic gland and molt again

Orathai Kamsoi and Xavier Belles
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Original submission

First decision letter

MS ID#: DEVELOP/2020/190066

MS TITLE: E93-depleted adult insects preserve the prothoracic gland and molt again

AUTHORS: Orathai Kamsoi and Xavier Belles

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 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 clearly shows that the adult-specifying transcription factor E93 in insects also plays a major role in eliciting the death of the prothoracic glands that secrete ecdysone that causes molting. This death normally occurs after the adult molt in hemimetabolous insects and during metamorphosis in holometabolous insects. What they show here for the cockroach Blattella germanica is that the suppression of E93 by RNAi on the last day of the final nymphal instar does not interfere with the formation of a normal adult except that the prothoracic glands do not die and consequently the adult undergoes an molt (which normally does not happen in insects). They further show that E93 normally initiates the cell death program in the prothoracic glands by interacting with the transcription factor β-FTZ-F1 that normally appears at the end of each molting cycle just before ecdysis when the ecdysteroid titer falls. β-FTZ-F1 then activates the cell death program in the glands (that β-FTZ-F1 was necessary for this activation was known from previous work of this laboratory). Here they show that this interaction between E93 and β-FTZ-F1 in the final instar seems to be specific to the prothoracic gland. Blattella is ideal for this study since it is very sensitive to RNAi and the critical time periods for E93 to determine the adult molt and to initiate prothoracic gland degeneration are sufficiently well-separated so that the latter can be disrupted without disrupting the former.
E93 and β-FTZ-F1 have previously been implicated in initiating cell death programs in the salivary glands, midgut and fat body of Drosophila and the fat body of the silkworm Bombyx mori. This is the first paper to show that E93 is involved in the cell death of the prothoracic glands in any insect.

Comments for the author

The study is carefully done and well controlled. Statistical tests are appropriate although I am surprised by the lack of significance for the change in nubbin expression in the wing of the molting adult after E93 RNAi. There appears to be a large difference between it and the control but the large SEM error bar suggests a very high variability among the 3 replicates so the difference is not significant. I find this lack of effect very interesting since nubbin is one of the genes specifically studied by Uyehara et al. (2017) in the wing discs of Drosophila and shown to be activated during the adult molt by E93's causing the opening of the chromatin in its enhancer. They should discuss this discrepancy.
Several points that need attention before publication:
1) line 79: I think that "Histolysis of cells and tissues….is a necessary part of metamorphosis…" is a more appropriate way of stating this concept rather than "is consubstantial…"
2) line 87: One should denote that in Drosophila, one has USP as the EcR partner, not RXR as in most insects including Blattella, as they are somewhat different in their properties.
3) lines 156-58: Do the prothoracic glands show proliferation in the early part of N4 and N5 instars as well or only in N6? The lack of this proliferation in the adult without E93 should be discussed in the Discussion.
4) lines 258-9: Did you ever try injection of α-ecdysone? In early Bombyx final instar larvae, feeding α-ecdysone causes repeated molting whereas feeding 20E does not [Tanaka, Y., and Takeda, S. (1993). Naturwissenschaften 80,131–132]. Likely this is due to the slower conversion of α-ecdysone to 20E.
5) lines 311-12: Is this rapid decay of the Ftz-F1 RNAi effect seen for any other transcription factors or in any other RNAi experiments done in Blattella? What happens if more RNAi is injected?
6) lines 321-23: is there any other system known where there is an organ or tissue-specific effect of a transcription factor or co-activator? More discussion is needed on this point.
7) line 356: One should be able to test this hypothesis quite quickly. It would make a better paper if this experiment could be done before publication although it is not essential.
8) Line 361: The epidermis expresses cuticular genes, not the cuticle which is inert as far as gene expression. This change should be made throughout the paper when talking about cuticular genes.
9) line 365: …cuticular protein expression…
10) line 380: In the discussion about adult cells molting, the authors should also mention the paper of Krishnakumaran and Schneiderman, [J. Exp. Zool. 157, 293-306 (1964)] which lays out the developmental capabilities of the adult moth epidermis. In this case, the adults were caused to

molt by parabiosis with developing pupae so had ecdysteroids in the hemolymph over a prolonged period of time.

Reviewer 2

Advance summary and potential significance to field

This manuscript aims to understand the molecular origins of metamorphosis in insects. At the transition between the ametabolous and metabolous insects, adults lost their ability to moult and gained wings on their second and third thoracic segments. In the current manuscript, the authors present convincing evidence that shows that 1) the degeneration of the prothoracic gland is important for shutting down moulting in the adult, and 2) that the ecdysone response gene E93 plays an important role, together with FTZ-F1, in regulating the degeneration of this gland. Interestingly, injecting the active form of the hormone, 20-hydroxyecdysone, does not induce adult moulting. The authors correctly argue that this suggests that adult moults are more likely to be regulated by ecdysone itself, which is secreted by the prothoracic gland and converted to 20E elsewhere, or by some unknown systemic cue secreted by this gland. Taken together, the authors have provided exciting new evidence that hint at the origins of metaboly in insects. I have very few suggestions for improving this manuscript.

Comments for the author

Statistical analysis appears to be missing for the data in Figure 5C and E.

I didn't understand how Figure 5E demonstrates that wing discs showed signs of apolysis, as indicated in line 239.

Minor suggestions:
1) When first introduced on line 131 of the text, it would be useful to define N6D8 (6th instar day 8).

2) I think there's a typo in Figure 4. The corresponding text, lines 207-216, discussed knock down of ftz-f1, but the figure legend says the black bars are E93-depleted.

Reviewer 3

Advance summary and potential significance to field

The E93 gene is thought to be the master regulator of metamorphosis through its role in the MEKRE93 pathway. The Belles lab has had a significant role in characterizing and describing this pathway during insect metamorphosis. In this manuscript, the authors investigate the role of E93 during metamorphosis of the cockroach Blattella germanica, and test the role of E93 in the histolysis of the prothoracic gland (PG). The histolysis of the PG is thought to be a critical event in dictating the end of larval molts and the transition into metamorphosis, hence the significance of directly linking E93 and histolysis of PG. Indeed, the authors find that knockdown of E93 blocks histolysis of PG, and also results in the ability to molt again.

Comments for the author

Unfortunately, there are major concerns with the manuscript in its current form.

First and foremost, E93 does not regulate programmed cell death in Drosophila. This argument is used throughout the manuscript as hypothesis and confirmatory evidence, but it has been disproven quite emphatically. The body of work showing that E93 regulates cell death in Drosophila was based on a set of mutations that were later shown not to map to the E93 gene. Please refer to the paper from Ian Duncan's lab:
Duncan et al 2017, G3: 7(3): 789-799; doi: 10.1534/g3.116.037366G3. Genuine mutations in E93 do not disrupt tissue histolysis during Drosophila metamorphosis. Of course, this issue could be

addressed in a complete rewrite/reframing of the results presented in this manuscript; however, in its current form, it is inaccurate and misleading.

Second, the current study uses stage-specific knockdown of E93 for phenotypic analysis; however, the conclusions drawn are tissue-specific. This is concern because whole animal knockdown of E93 could disrupt histolysis of PG as a secondary/indirectly phenotype; moreover, as the authors themselves point out, and it is not possible to know if the effect on PG histolysis occurs as a result of loss of E93 in another tissue. Combined with the first concern raised above, there is little evidence to trust the conclusion that E93 has a direct role in histolysis of PG.

Having said that, the ability to trigger a new larval molt and prevent histolysis of PG are interesting phenotypes. I hope the authors find a way to salvage the story by casting the results in a completely different context.

First revision

Author response to reviewers' comments

RESPONSE TO REVIEWERS
REVIEWER # 1.
Reviewer Advance summary and potential significance to field
This manuscript clearly shows that the adult-specifying transcription factor E93 in insects also plays a major role in eliciting the death of the prothoracic glands that secrete ecdysone that causes molting. This death normally occurs after the adult molt in hemimetabolous insects and during metamorphosis in holometabolous insects. What they show here for the cockroach Blattella germanica is that the suppression of E93 by RNAi on the last day of the final nymphal instar does not interfere with the formation of a normal adult except that the prothoracic glands do not die and consequently the adult undergoes an molt (which normally does not happen in insects). They further show that E93 normally initiates the cell death program in the prothoracic glands by interacting with the transcription factor ß-FTZ-F1 that normally appears at the end of each molting cycle just before ecdysis when the ecdysteroid titer falls. ß-FTZ-F1 then activates the cell death program in the glands (that ß-FTZ-F1 was necessary for this activation was known from previous work of this laboratory). Here they show that this interaction between E93 and ß-FTZ-F1 in the final instar seems to be specific to the prothoracic gland. Blattella is ideal for this study since it is very sensitive to RNAi and the critical time periods for E93 to determine the adult molt and to initiate prothoracic gland degeneration are sufficiently well-separated so that the latter can be disrupted without disrupting the former.
E93 and ß-FTZ-F1 have previously been implicated in initiating cell death programs in the salivary glands, midgut and fat body of Drosophila and the fat body of the silkworm Bombyx mori. This is the first paper to show that E93 is involved in the cell death of the prothoracic glands in any insect.
Reviewer 1 Comments for the author
The study is carefully done and well controlled. Statistical tests are appropriate although I am surprised by the lack of significance for the change in nubbin expression in the wing of the molting adult after E93 RNAi. There appears to be a large difference between it and the control but the large SEM error bar suggests a very high variability among the 3 replicates so the difference is not significant. I find this lack of effect very interesting since nubbin is one of the genes specifically studied by Uyehara et al. (2017) in the wing discs of Drosophila and shown to be activated during the adult molt by E93's causing the opening of the chromatin in its enhancer. They should discuss this discrepancy.
REPLY: This is a good point. The reviewer is right, the lack of significance (considering $p<0.050$) is due to the high SEM. The differences, however, are close to be significant ($p=0.058$), and this has been indicated in the new version, in Results (lines 259-260). Moreover, we have clarified (in the Discussion, new lines 367-371) the apparent discrepancy between Drosophila, in which, Uyehara et al. (2017), reported that nubbin is activated by E93 in the wing disc during metamorphosis, and our results in Blattella. The apparent contradiction is explained by the fact that we interfered E93 in on day 6 of the last nymphal instar, when the period of maximum expression of E93 in wing pads

has already elapsed (see Fig. 1). Thus, the metamorphic program in the wings was not altered, and wing genes, including nub, increased the expression.

Several points that need attention before publication:
1) line 79: I think that "Histolysis of cells and tissues….is a necessary part of metamorphosis…" is a more appropriate way of stating this concept rather than "is consubstantial…".
REPLY: We agree. Corrected as suggested.

2) line 87: One should denote that in Drosophila, one has USP as the EcR partner, not RXR as in most insects including Blattella, as they are somewhat different in their properties.
REPLY: Yes, the reviewer is right. Given that in this section we are speaking only about Drosophila, we have simply replaced RXR with usp (line 86).

3) lines 156-58: Do the prothoracic glands show proliferation in the early part of N4 and N5 instars as well or only in N6? The lack of this proliferation in the adult without E93 should be discussed in the Discussion.
REPLY: We have reported previously (Kamsoi and Belles, 2019) that prothoracic glands show proliferation in the early part of N5 and N6 instars (we did not check N4). According to the reviewer, we have stated this in lines 164-165 of the Results section, and we have commented the absence of proliferation in the E93-depleted adult in the Discussion (lines 307-311).

4) lines 258-9: Did you ever try injection of α-ecdysone? In early Bombyx final instar larvae, feeding α-ecdysone causes repeated molting whereas feeding 20E does not [Tanaka, Y., and Takeda, S. (1993). Naturwissenschaften 80,131–132]. Likely this is due to the slower conversion of α-ecdysone to 20E.
REPLY: Not in this work, but we had tried to induce a molt in the adult of Blattella with α-ecdysone in the past, when we had an abundant source of this compound from plant extracts. We were never successful, even using high doses. This time, we worked directly with 20E, considered the biologically active metabolite, which is, by the way, pretty stable.

5) lines 311-12: Is this rapid decay of the Ftz-F1 RNAi effect seen for any other transcription factors or in any other RNAi experiments done in Blattella? What happens if more RNAi is injected?
REPLY: Yes, we have observed this in some other genes. For example, after RNAi, mRNA levels of the lipophorin receptor in the fat body recover very rapidly normal levels (Ciudad L, Belles X, Piulachs MD. 2007. Structural and RNAi characterization of the German cockroach lipophorin receptor, and the evolutionary relationships of lipoprotein receptors. BMC Mol. Biol. 8:53). We think that this fast recovery occurs in genes with efficient feedback mechanisms of regulation, which can readily counteract depletion of mRNA levels with higher rates of transcription (see Bellés, X. 2010. Beyond Drosophila. RNAi in vivo and functional genomics in insects. Annu.Rev. Entomol. 55: 111–128.). In these cases, it is not possible to reach a stable depletion with high doses of dsRNA (or even with repeated injections of dsRNA). In the new version of the manuscript, we have added the reference "Belles, 2010" (line 331), which compiles these cases.

6) lines 321-23: is there any other system known where there is an organ or tissue-specific effect of a transcription factor or co-activator? More discussion is needed on this point.
REPLY: The sentence in lines 321-323 was misspelled. It is not that the FTZ-F "coactivating effect" on E93 is specific to the PG. What is specific is the high co-expression of FTZ-F1 and E93 on the PG only at a particular stage (last day of the last nymphal instar), which allows the above coactivating effect in the PG. We have rewritten the sentence with those premises (now in lines 337-339).

7) line 356: One should be able to test this hypothesis quite quickly. It would make a better paper if this experiment could be done before publication although it is not essential.
REPLY: Following the reviewer's suggestion, we have performed new DAPI staining experiments, which empirically confirm that there are more DAPI signal in the veins of dsE93-treated insects than in controls. This is shown in the new figure S2, and associated new texts in the Results section (lines 174-176) and in the Discussion (line 376).

8) Line 361: The epidermis expresses cuticular genes, not the cuticle which is inert as far as gene expression. This change should be made throughout the paper when talking about cuticular genes.

REPLY. The observation is exact. We have specified that cuticular genes are expressed by epidermal cells (not by the cuticle), checking all the manuscript.

9) line 365: …cuticular protein expression…
REPLY: Corrected as indicated.

10) line 380: In the discussion about adult cells molting, the authors should also mention the paper of Krishnakumaran and Schneiderman, [J. Exp. Zool. 157, 293-306 (1964)] which lays out the developmental capabilities of the adult moth epidermis. In this case, the adults were caused to molt by parabiosis with developing pupae so had ecdysteroids in the hemolymph over a prolonged period of time.
REPLY: Certainly, this is an important reference, which has been added in the new version of the manuscript along with an associated new text (lines 398-400). Those results would fit with the hypothesis we mentioned shortly after, proposing that "the occurrence of a PG factor that makes the epidermal cells competent to respond to ecdysteroids and produce a new cuticle, a putative factor whose production would be interrupted with the histolysis of the PG". After reading the Krishnakumaran and Schneiderman paper, we believe that the above hypothesis is the most plausible. Therefore, we have simplified the final conclusion of the discussion in this regard.

REVIEWER # 2.
Reviewer 2 Advance summary and potential significance to field
This manuscript aims to understand the molecular origins of metamorphosis in insects. At the transition between the ametabolous and metabolous insects, adults lost their ability to moult and gained wings on their second and third thoracic segments. In the current manuscript, the authors present convincing evidence that shows that 1) the degeneration of the prothoracic gland is important for shutting down moulting in the adult, and 2) that the ecdysone response gene E93 plays an important role, together with FTZ-F1, in regulating the degeneration of this gland. Interestingly, injecting the active form of the hormone, 20-hydroxyecdysone, does not induce adult moulting. The authors correctly argue that this suggests that adult moults are more likely to be regulated by ecdysone itself, which is secreted by the prothoracic gland and converted to 20E elsewhere, or by some unknown systemic cue secreted by this gland. Taken together, the authors have provided exciting new evidence that hint at the origins of metaboly in insects. I have very few suggestions for improving this manuscript.
Reviewer 2 Comments for the author
Statistical analysis appears to be missing for the data in Figure 5C and E.
I didn't understand how Figure 5E demonstrates that wing discs showed signs of apolysis, as indicated in line 239.
REPLY: Statistical analysis was done in all instances, except in cases when there are virtual 0 values (UD = under the limit of detection, where it is not really necessary. In the legend we explain that significant differences are indicated with asterisks. Then, the absence of asterisks at the top of bars (like the case of nub in panel D, or in various cases in figures 3 and 4) indicate that there are no significant differences between samples.
In line 239 we indicated "Remarkably, wings also showed signs of molting (apolysis) (Fig. 2E)". Perhaps "apolysis" is not the best word to define what is shown in Figure 2E, that is, the formation of a new set of veins in the adult wing of E93-depleted insects. Thus, we have reformulated the sentence as follows: "Remarkably, wings also showed signs of molting, indicated by the formation of new veins under the old ones (Fig. 2E)". (now in lines 251-252).

Minor suggestions:

1) When first introduced on line 131 of the text, it would be useful to define N6D8 (6th instar, day 8).
REPLY: We agree. N6D8 is now defined (in line 139).

2) I think there's a typo in Figure 4. The corresponding text, lines 207-216, discussed knock down of ftz-f1, but the figure legend says the black bars are E93-depleted.
REPLY: Good eye. The reviewer is right. Black bars indicate FTZ-F1-depleted. This has been corrected in a new version of figure 4.

REVIEWER # 3.
Reviewer 3 Advance summary and potential significance to field
The E93 gene is thought to be the master regulator of metamorphosis through its role in the MEKRE93 pathway. The Belles lab has had a significant role in characterizing and describing this pathway during insect metamorphosis. In this manuscript, the authors investigate the role of E93 during metamorphosis of the cockroach Blattella germanica, and test the role of E93 in the histolysis of the prothoracic gland (PG). The histolysis of the PG is thought to be a critical event in dictating the end of larval molts and the transition into metamorphosis, hence the significance of directly linking E93 and histolysis of PG. Indeed, the authors find that knockdown of E93 blocks histolysis of PG, and also results in the ability to molt again.
Reviewer 3 Comments for the author
Unfortunately, there are major concerns with the manuscript in its current form.
1) First and foremost, E93 does not regulate programmed cell death in Drosophila. This argument is used throughout the manuscript as hypothesis and confirmatory evidence, but it has been disproven quite emphatically. The body of work showing that E93 regulates cell death in Drosophila was based on a set of mutations that were later shown not to map to the E93 gene. Please refer to the paper from Ian Duncan's lab: Duncan et al 2017, G3: 7(3): 789-799; doi: 10.1534/g3.116.037366G3. Genuine mutations in E93 do not disrupt tissue histolysis during Drosophila metamorphosis. Of course, this issue could be addressed in a complete rewrite/reframing of the results presented in this manuscript; however, in its current form, it is inaccurate and misleading.

REPLY: Indeed, Duncan et al. (2017) reported that mutant alleles of E93 used in earlier studies by Carl Thummel and his group (Baehrecke et al., 1995) were alleles of a nearby gene isocitrate dehydrogenase 3b (idh3b). Some of the original E93 alleles do indeed map to idh3b (but possibly, others not). Therefore, the role of E93 in Drosophila salivary gland degradation is not clear at present. In any case, however, the body of evidence out there, not only from Drosophila but also in other insects, strongly supports a role for E93 in adult specific development in general, as well as in associated programmed cell death (PCD) processes. Some of the studies, like two of Sheng Li group, one on E93 and fat body PCD in Drosophila (Liu et al., 2014) and another one on E93 and fat body PDC in the silkworm Bombyx mori (Liu et al., 2015) use E93 RNAi approaches, unequivocally showing the involvement of E93 in the PCD processes in these tissues at metamorphosis. We think that more work is needed to clarify the role of E93 in salivary gland PDC in Drosophila. However, we consider that this additional work will not lead to a big change in our understanding of E93 and PCD.
What we did in the new version of the manuscript (new text in the introduction, lines 108-115) is to take a prudent view, citing Duncan paper (which we didn't do in the first version, and that wasn't a good idea), and stating that further studies are needed to clarify E93 function in Drosophila salivary glands PCD at metamorphosis, although the body of evidence, not only from D. melanogaster but also in other insects, in a number of cases using approaches other than the E93 alleles, such as E93 RNAi, supports a role for E93 in adult specific development in general, as well as in associated PCD processes.

2) Second, the current study uses stage-specific knockdown of E93 for phenotypic analysis; however, the conclusions drawn are tissue-specific. This is concern because whole animal knockdown of E93 could disrupt histolysis of PG as a secondary/indirectly phenotype; moreover, as the authors themselves point out, and it is not possible to know if the effect on PG histolysis occurs as a result of loss of E93 in another tissue. Combined with the first concern raised above, there is little evidence to trust the conclusion that E93 has a direct role in histolysis of PG.
REPLY: The concern appears to be logical given that, in principle, systemic RNAi affects all tissues. However, the key point is in the first section of the Results "Tissue-specificity of E93 expression", and the associated figure 1. The entire experimental strategy is based on the peculiar expression of E93 in the PG, which justifies that we can focus our conclusions on the PG. Note that, as described in the manuscript, we injected the dsRNA targeting E93 on day 6 of the last nymphal instar (two days before metamorphosis). That is, when the expression of E93 has already declined in all the tissues relevant for metamorphosis (CC-CA, epidermis, wing pads), while the expression in the PG is yet to come (a sudden and high expression in day 8). Thus, with this approach we practically only affect the expression and effects of E93 in PG at the time prior to metamorphosis and degradation of PG, which is what we intend (and achieve). The phenotypes obtained show that after the imaginal molt, the E93-depleted insects have an external aspect similar to the controls (see figure

2A), and only the PG is affected, as it did not disintegrates, in contrast with the controls (figure 2B).

3) Having said that, the ability to trigger a new larval molt and prevent histolysis of PG are interesting phenotypes. I hope the authors find a way to salvage the story by casting the results in a completely different context.
REPLY: Well, we do not trigger a new larval molt, but a new adult molt. In any case, and according to the two previous replies, we honestly consider that we can keep the general structure of the manuscript as it was in the first submitted version of the manuscript.

Final comment
We have uploaded a formatted PDF of this Response to Revierews as Supplementary Information, thus reviewers can see also this thext there.

Second decision letter

MS ID#: DEVELOP/2020/190066

MS TITLE: E93-depleted adult insects preserve the prothoracic gland and molt again

AUTHORS: Orathai Kamsoi and Xavier Belles

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 positive and we would like to publish a revised manuscript in Development, provided that the referees' comments can be satisfactorily addressed. Please attend to all of the reviewers' comments in your revised manuscript and detail them in your point-by-point response. If you do not agree with any of their criticisms or suggestions explain clearly why this is so. In particular, I think it would help to acknowledge that the CC-CA expression could mean that there is an indirect role for E93 through this tissue, as pointed out by Reviewer 2. With respect to the issue of the role of E93 in Drosophila, I think that the revision you have provided is the minimum, but i also think it would strengthen the paper and help readers understand the full context, to consider acknowledging the first point that Reviewer 1 makes.

As these revisions are quite minor, if you choose to implement revisions and send back the MS, I will review the changes myself rather than having them sent out for a third round of review externally.

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.

Reviewer 1

Advance summary and potential significance to field

This manuscript clearly shows that the adult-specifying transcription factor E93 in insects also plays a major role in eliciting the death of the prothoracic glands that secrete ecdysone that causes molting. This death normally occurs after the adult molt in hemimetabolous insects and during metamorphosis in holometabolous insects. What they show here for the cockroach Blattella

germanica is that the suppression of E93 by RNAi on the last day of the final nymphal instar does not interfere with the formation of a normal adult except that the prothoracic glands do not die and consequently the adult undergoes an molt (which normally does not happen in insects). They further show that E93 normally initiates the cell death program in the prothoracic glands by interacting with the transcription factor β-FTZ-F1 that normally appears at the end of each molting cycle just before ecdysis when the ecdysteroid titer falls. β-FTZ-F1 then activates the cell death program in the glands (that β-FTZ-F1 was necessary for this activation was known from previous work of this laboratory). Here they show that this interaction between E93 and β-FTZ-F1 in the final instar seems to be specific to the prothoracic gland. Blattella is ideal for this study since it is very sensitive to RNAi and the critical time periods for E93 to determine the adult molt and to initiate prothoracic gland degeneration are sufficiently well-separated so that the latter can be disrupted without disrupting the former.
E93 and β-FTZ-F1 have previously been implicated in initiating cell death programs in the salivary glands, midgut and fat body of Drosophila and the fat body of the silkworm Bombyx mori. This is the first paper to show that E93 is involved in the cell death of the prothoracic glands in any insect.

Comments for the author

This revised version has answered all my comments and in so doing has made it a substantially better paper.

There are several minor points that need attention before publication:

1) In lines 42 and 77, they use the word “consubstantial”. I think that they mean “concomitant” in both cases.

2) line 85: USP referring to the protein should be capitalized according to Drosophila conventional usage.

3) The sentence beginning in line 111, “However, the body of evidence…” is cumbersome and needs rewriting. I suggest the following: “However, the body of evidence from both Drosophila and other insects using E93 RNAi supports a role for E93 in adult-specific development in general as well as in associated PCD processes (Lee and Baehrecke, 2001; Lee et al., 2002a; Liu et al., 2014; Liu et al., 2015; Urena et al, 2014; Uyehara et al., 2017).

4) line 168-9: better to say “…could be observed under the transparent old cuticle.”

5) line 172: better to say “could be seen under the transparent veins of the e93-depleted…”

6) line 362: It is better to say ”…can be observed under the transparent old cuticle.”

7) line 368: It is better to say “…that we suppressed E93 with RNAi in B. germanica….”

6) lines 382-5: Are those two genes Bg10435 and 10431 normally only expressed in the nymphs or are they expressed also in the adult at any age, possibly at much lower levels. Cuticle genes may be expressed in two different stages if they are specific for a particular type of cuticle (i.e., flexible or stiff, for instance) which may be in differing amounts in the two stages. Please clarify here so that the reader does not have to look up the paper which describes these in detail.

Reviewer 2

Advance summary and potential significance to field

The authors have made minor revisions according to my request. This is an exciting manuscript that is sure to be important in the field of insect developmental physiology.

Comments for the author

I have no further comments.

Reviewer 3

Advance summary and potential significance to field

As stated in the original review.

Comments for the author

With all due respect, the authors do not seem to have taken seriously the two major concerns raised by this reviewer. I will outline them here again and try to communicate why the authors' responses do not adequately address those concerns. As is, the conclusions drawn in the manuscript misrepresent the data and avoid the real issues that surround this topic in the field. This reviewer simply requests that the authors represent the data and the experimental caveats accurately. This could be achieved with a thoughtful rewrite.

Major concerns
1) As stated in the original review, based on published literature it is widely accepted that E93 does not regulate programmed cell death in Drosophila melanogaster. The paper that demonstrates this (Duncan et al 2017) not only shows that the original E93 alleles characterized were not in the E93 gene, but they also show that a complete deletion of the E93 gene does not disrupt cell death. The authors seem to disregard this latter piece of information; instead, they seem to suggest that there might be original alleles of E93 that might be in the real E93 gene. This is misleading since the original E93 alleles used in the previous studies were the ones shown not to be in E93. And returning to the earlier point, a complete loss of E93 didn't disrupt histolysis of the salivary glands. It's difficult to ignore or argue around this data.

The authors also argue against this data by using studies in other insects. These arguments are valid however, they raise an important additional question: are there differences in the role of E93 within the "ecdysone" transcriptional hierarchy between different insect species? The published data indicates that E93 disrupt "ecdysone" signaling other insects; similar studies, to my knowledge, have not been done in Drosophila melanogaster with the "new" E93 alleles. The reason the role of E93 within the hormonal hierarchy is critical is because disrupting hormonal signaling will of course disrupt cell death. There are multiple layers of evidence that the death response in salivary glands require the global hormonal pulse and its readout in this tissue. Separating this global signal from the tissue specific response is central to the second major concern.

2) The original review raises concerns about over interpreting whole-animal RNAi experiments for a tissue specific response. I can appreciate that Drosophila has the advantage of tissue specific promoters and somatic clones that are not routine in other insect models, however, those techniques have revealed the dangers of using one approach at the exclusion of the other. The authors focus on their Fig 1 data as justification for their argument, so let's dive into that data. It is true that E93 appears to be expressed in a stage specific manner in PG (Fig 1A), no arguments there. However, E93 is also expressed in CC-CA at that same stage (Fig 1B). Keep in mind that the data shown in the figure is relative expression (not absolute expression), so it is possible that the expression in CC-CA is in fact equal to or higher than that of E93 in PG
(in other words, the relative increase in PG could simply be a reflection of the extremely low levels prior to that). Regardless, the point is that E93 is present in CC-CA, a critical endocrine tissue responsible for sending global signals during development. Now, returning to the original concern, how is it possible that the authors can exclude the possibility that the injected RNAi is not reducing E93 in CC-CA? Or that this reduction of E93 in CC-CA might be disrupting CC-CA function which in turn may disrupt a subset of CC-CA functions during development. The same concern applies when interpreting the results with similar approaches in other insects.

Second revision

Author response to reviewers' comments

REVIEWER # 1.
Reviewer 1. Advance summary and potential significance to fieldThis manuscript clearly shows that the adult-specifying transcription factor E93 in insects also plays a major role in eliciting the death of the prothoracic glands that secrete ecdysone that causes molting. This death normally occurs after the adult molt in hemimetabolous insects and during metamorphosis in holometabolous insects. What they show here for the cockroach Blattella germanica is that the suppression of E93 by RNAi on the last day of the final nymphal instar does not interfere with the formation of a normal adult except that the prothoracic glands do not die and consequently the adult undergoes an molt (which normally does not happen in insects). They further show that E93 normally initiates the cell death program in the prothoracic glands by interacting with the transcription factor ß-FTZ-F1 that normally appears at the end of each molting cycle just before ecdysis when the ecdysteroid titer falls. ß-FTZ-F1 then activates the cell death program in the glands (that ß-FTZ-F1 was necessary for this activation was known from previous work of this laboratory). Here they show that this interaction between E93 and ß-FTZ-F1 in the final instar seems to be specific to the prothoracic gland. Blattella is ideal for this study since it is very sensitive to RNAi and the critical time periods for E93 to determine the adult molt and to initiate prothoracic gland degeneration are sufficiently well-separated so that the latter can be disrupted without disrupting the former.
E93 and ß-FTZ-F1 have previously been implicated in initiating cell death programs in the salivary glands, midgut and fat body of Drosophila and the fat body of the silkworm Bombyx mori. This is the first paper to show that E93 is involved in the cell death of the prothoracic glands in any insect.
Reviewer 1. Comments for the authorThis revised version has answered all my comments and in so doing has made it a substantially better paper.
There are several minor points that need attention before publication:
1) In lines 42 and 77, they use the word “consubstantial”. I think that they mean “concomitant” in both cases.
REPLY: The reviewer is right. We have replaced consubstantial by concomitant in both instances.
2) line 85: USP referring to the protein should be capitalized according to Drosophila conventional usage.
REPLY: We agree. We refer now to USP.
3) The sentence beginning in line 111, “However, the body of evidence…” is cumbersome and needs rewriting. I suggest the following: “However, the body of evidence from both Drosophila and other insects using E93 RNAi supports a role for E93 in adult-specific development in general as well as in associated PCD processes (Lee and Baehrecke, 2001; Lee et al., 2002a; Liu et al., 2014; Liu et al., 2015; Urena et al, 2014; Uyehara et al., 2017).
REPLY: We agree. The sentence has been simplified, as suggested.
4) line 168-9: better to say “…could be observed under the transparent old cuticle.”
REPLY: We agree. The sentence has been written as suggested.
5) line 172: better to say “could be seen under the transparent veins of the e93-depleted…”
REPLY: We agree. The sentence has been written as suggested.
6) line 362: It is better to say ”…can be observed under the transparent old cuticle.”
REPLY: We agree. The sentence has been written as suggested.
7) line 368: It is better to say “…that we suppressed E93 with RNAi in B. germanica….”
REPLY: We agree. But we used the word “depleted” (instead of “suppressed”, as RNAi does no suppress the transcript 100%). Thus the new sentence is “…that we depleted E93 with RNAi in B. germanica….”.
6) lines 382-5: Are those two genes Bg10435 and 10431 normally only expressed in the nymphs or are they expressed also in the adult at any age, possibly at much lower levels. Cuticle genes may be expressed in two different stages if they are specific for a particular type of cuticle (i.e., flexible or stiff, for instance) which may be in differing amounts in the two stages. Please clarify here so that the reader does not have to look up the paper which describes these in detail.
REPLY: Bg10435 and 10431 are expressed also in the adult at any age at much lower levels as guessed by the reviewer. As suggested by her/him, this has been clarified in the new version of the manuscript (lines 378-381).

REVIEWER # 2
Reviewer 2 Advance summary and potential significance to field
The authors have made minor revisions according to my request. This is an exciting manuscript that is sure to be important in the field of insect developmental physiology.
Reviewer 2 Comments for the author
I have no further comments.
REPLY: No reply required.

REVIEWER #3
Reviewer 3 Advance summary and potential significance to field
As stated in the original review.
Reviewer 3 Comments for the author
With all due respect, the authors do not seem to have taken seriously the two major concerns raised by this reviewer. I will outline them here again and try to communicate why the authors' responses do not adequately address those concerns. As is, the conclusions drawn in the manuscript misrepresent the data and avoid the real issues that surround this topic in the field. This reviewer simply requests that the authors represent the data and the experimental caveats accurately. This could be achieved with a thoughtful rewrite.
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1) As stated in the original review, based on published literature it is widely accepted that E93 does not regulate programmed cell death in Drosophila melanogaster. The paper that demonstrates this (Duncan et al 2017) not only shows that the original E93 alleles characterized were not in the E93 gene, but they also show that a complete deletion of the E93 gene does not disrupt cell death. The authors seem to disregard this latter piece of information; instead, they seem to suggest that there might be original alleles of E93 that might be in the real E93 gene. This is misleading since the original E93 alleles used in the previous studies were the ones shown not to be in E93. And returning to the earlier point, a complete loss of E93 didn't disrupt histolysis of the salivary glands. It's difficult to ignore or argue around this data.
The authors also argue against this data by using studies in other insects. These arguments are valid, however, they raise an important additional question: are there differences in the role of E93 within the "ecdysone" transcriptional hierarchy between different insect species? The published data indicates that E93 disrupt "ecdysone" signaling other insects; similar studies, to my knowledge, have not been done in Drosophila melanogaster with the "new" E93 alleles. The reason the role of E93 within the hormonal hierarchy is critical is because disrupting hormonal signaling will of course disrupt cell death. There are multiple layers of evidence that the death response in salivary glands require the global hormonal pulse and its readout in this tissue. Separating this global signal from the tissue specific response is central to the second major concern.
REPLY: We admit that our review on this point was very limited and can be improved. Indeed, what we intend to show is that E93 is important in the death of the prothoracic gland in Blattella. Thus, for the purposes of our work, it is not key to argue the possible effects (or no effects) of E93 on the death of salivary glands in Drosophila. Now, these antecedents (the results of the Thummel group and those of the Duncan group), have been objectively mentioned in the new Introduction (lines 102-112). In accordance with the suggestions of the reviewer, we now state that “Duncan et al. (2017) have reported that mutant alleles of E93 used in earlier studies in D. melanogaster were in fact alleles of a nearby gene isocitrate dehydrogenase 3b (idh3b), which encodes a key enzyme of the citric acid cycle in the mitochondria. Moreover, a complete deletion of the E93 gene did not disrupt cell death, which challenges the previous observations on E93 and salivary gland PCD in D. melanogaster”. Afterwards, we mention the published data on the function of E93 in midgut and fat body death in Drosophila and Bombyx, which are more comparable with our results, as they were obtained with RNAi. Later, in the discussion, we have based the comparison of our data (obtained through RNAi) with those of Drosophila and Bombyx (also obtained with RNAi) (lines 339-344).
2) The original review raises concerns about over interpreting whole-animal RNAi experiments for a tissue specific response. I can appreciate that Drosophila has the advantage of tissue specific promoters and somatic clones that are not routine in other insect models, however, those techniques have revealed the dangers of using one approach at the exclusion of the other. The authors focus on their Fig 1 data as justification for their argument, so let's dive into that data. It is true that E93 appears to be expressed in a stage specific manner in PG (Fig 1A), no arguments there. However, E93 is also expressed in CC-CA at that same stage (Fig 1B). Keep in mind that the

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REPLY: We agree. Although the strategy of injecting the dsE93 towards the end of the last nymphal instar maximizes the effect on the prothoracic gland, the treatment has a systemic action, thus affecting (although to a much lesser extent) other tissues, notably CC-CA, which could influence the aspects studied. So we have added a new comprehensive paragraph in this sense at the end of the discussion (lines 411-417).

Third decision letter

MS ID#: DEVELOP/2020/190066

MS TITLE: E93-depleted adult insects preserve the prothoracic gland and molt again

AUTHORS: Orathai Kamsoi and Xavier Belles
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.