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Regulation of Drosophila intestinal stem cell maintenance and differentiation by the transcription factor Escargot.

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Editor: Thomas Schwarz-Romond

1st Editorial Decision 16 June 2014

Thank you very much for submitting your paper on the role/signaling interplay of escargot in intestinal stem cell maintenance vs differentiation for consideration to The EMBO Journal.

I enclose comments from three scientists that are in principle supporting publication. They suggest however a few clarifying and further reaching experiments to consolidate the conclusions that were drawn on the presented dataset.

For the Notch-pathway interactions, ref#1 suggests more stringent epistatic experiments in sensitized genetic backgrounds. This type of analysis was similarly requested for the complementary study from Bruce Edgar's lab. I was thus wondering (and communicated accordingly) whether this could be coordinated/jointly approached as to safe valuable time and resources?).

More importantly and re the functional connection to Amun, both ref#1 in point 5 as well as ref#3 in point 3 demand further results on Amun expression/its dependence on Esg in the respective cell population before being able to support publication of the study.

Lastly, I am not certain whether the mostly negative and/or not further mechanistically-developed data on Stat/Ecad-interaction add too much to the major trust to the paper. Assuming further results being readily available in the lab, I would prefer significantly expand OR dramatically shorten this paragraph as to not deviate from the major contribution of the study.

Please do not hesitate to get in touch regarding feasibility/anticipated timeline for the rather extensive revisions (due to time constrains preferably via e-mail and also to enable coordination with the back-to back submission).

Lastly, I have to formally remind you that The EMBO Journal only considers one round of revisions and look forward to hear from you/receive a suitably revised version of your study!

REFEREE REPORTS:

Referee #1:

In this manuscript by Loza-Coll et al. the authors analyze the role of the transcription factor Escargot (Esg) in intestinal stem cell (ISC) homeostasis in Drosophila. By means of loss of function experiments they uncover a dual role of Esg in the maintenance of stemness in ISC while also influencing enteroblast (EB) differentiation through regulation of Notch signaling in the latter cell type. Furthermore, through DNA binding analysis by DamID the authors identify Amun, a negative regulator of Notch signaling, as one direct target of Esg, which they go on to further characterize. They conclude that Esg activates Notch signaling in EBs by repressing Amun expression in those cells and thus drives their differentiation. This manuscript complements well with work by Korzelius et al., which has also been submitted for consideration as back-to-back publications. Overall, the data is well presented and experiments have been carefully done and controlled for. I have, however, concerns regarding the interpretation of some of the results. I also find the latest part of the manuscript, regarding the characterization of the role of Amun as a mediator of Esg function, rather weak. I suggest that the authors should address the points bellow before the manuscript is considered for publication:

Major points:

1- From the results presented in Figure 3A and E3A the authors conclude that esg knockdown results in down-regulation of Notch signaling as visualized by Su(H)LacZ reporter, which is expressed in EBs. This is a very difficult argument to make given that knockdown of esg results in premature differentiation of EBs into EE cells and ECs. Therefore, the loss of EBs will indefectibly lead to less Su(H)LacZ.

2- Figure 3B: The green channel should be shown and the proportion of EC in each condition quantified.

3- Related to Figure 3B, I think the epistasis between Esg and Notch is not properly tested since both, esg-RNAi and Notch-intra overexpression, have a phenotype of their own. Therefore, I believe that manipulating one of the components on a sensitized background for the other is a much more rigorous way to address genetic interaction. For example, knocking down and/or overexpressing esg and removing one copy of N or overexpressing Notch-intra in an esg-/+ background.

4- Data on regarding the connection between Esg-Jak-Stat-Ecad and Notch signaling is largely over interpreted. The current evidence linking Jak-Stat and Notch is only correlative and, to my knowledge, no link between these pathways has been clearly established so far. The same applies to the relationship between Ecad junctions and Notch signaling activation in the midgut. Therefore, most of the results in this section of the manuscript relay on speculations. The data presented by the authors simply suggest that esg is required to keep Jak/Stat signaling activation in ISCs. Regarding this point, the authors should assess the levels of Socs36E mRNA to rigorously and quantitatively assess Stat activity upon esg knockdown. I find the data on Ecad puzzling. If this represents an intermediate situation, the authors should knockdown esg for a longer period or using a stronger driver to see what happens to Ecad at the end.

5- Characterization of the role of Amun in the system:

a-The data regarding the in situ expression of Amun is poor. I acknowledge that ISHs in the midgut are hard and I guess there are no good antibodies for Amun? In such case, I suggest the authors that they assess Amun expression domain by using EC and ISC/EB specific drivers to drive Amun RNAi and then assess presence or absence of gene knockdown by RT-PCR. This would provide a good test for the efficiency of the Amun RNAi and also confirm the expression domain of the gene in control and esg-RNAi midguts. For example; one would expect to see a knockdown of Amun in control midguts when using the EC driver but not the ISC/EB driver. On the contrary, using the latter driver with esg- and Amun- RNAis should show Amun knockdown when compared to esg-RNAi only.
b- I wonder whether using a stronger driver to overexpress Amun (Figure 4B, C) would result in a more robust/stronger phenotype.
c- Figures 5 and E5 show the same control and esg-RNAi guts. Different examples should be presented for different experiments.

Minor point:

I find that most of the discussion is a repetition of the results. I suggest the authors try to modify it to minimize this.

Referee #2:

In this paper, Loza-Coll and colleagues investigate the role of the transcription factor esg in controlling the physiology of Intestinal Stem Cells in Drosophila. The authors first show that clones mutant for esg display a decrease in progenitor cells, and that differentiated cells are enriched for one cell type, enteroendocrine cells. They further confirm these results by using in vivo RNAi in all progenitors(esgts) and in enteroblasts only. Lack of esg in progenitors is associated with a decrease in Notch signaling from ISC to EBs, and this increase is responsible for the increase in EE cells in the gut. The authors then suggest that JAK-STAT signaling is not involved in esg mediated regulation of Notch signaling. In addition, reduction in esg leads to an increase in E-cadhering expression in progenitor cells. In order to identify esg targets in progenitor cells, the authors performed DAM-ID on esg. This approach led to the identification of Amun, which is both down-regulated by esg and a known negative regulator of Notch signaling. The authors convincingly show that Amun is required for the effect of esg on Notch signaling induced. Altogether, the authors convincingly show that esg is required for ISC maintenance and affect lineage differentiation through modulation of Notch signaling. This paper is clearly written and brings interesting data to the understanding of the role of snail family transcription factors on Stem cell function. I recommend this paper for publication but advise to take into account few suggestions down below:

Major concerns

1- The authors claim a loss of ISC in clones mutants for esg. It would be useful to see in the main figure a direct quantification of ISCs per clone by staining for delta, a marker of ISC.

2- The authors show that the effect of esg RNAi is stronger in EBs. However we do not have the results of experiments done with an ISC specific driver. The authors should look at the effect of knocking down esg only in ISCs (delta-Gal4 driver).

3- The authors present negative data to explain that the JAK-STAT pathway is not responsible for the effect of esg on Notch. However we lack here crucial information to understand the results and conclude. First the authors should show the levels of cytokines, and JAK-STAT target genes by RT-qPCR, second the authors should look at the epsitasis of esg and STAT modulation.

Minor points:

P9: restricted should be restrict.

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P10: ISC/SB should be ISC/EB

Referee #3:

The manuscript by Loza-Coll et al. describes the functions of the conserved Snail family gene escargot in Drosophila intestinal stem cells (ISCs). They found that Esg maintains ISC stemness and activates Notch signaling in the enteroblast (EB) to promote EB cell fate. They then used DamID to identify the loci downstream of Esg that regulate Notch activity, and found that Amun mediates such regulation. The concept that the continuous expression of Snail family gene maintains the stem cell stemness is not new (as has been shown in the Drosophila neural stem cells; Lai et al 2012). However, the work does provide a link between two well-established signaling pathways, esg and Notch.

I have two major concerns.

(1) The first is the organization of the manuscript. I found myself constantly trapped in the text trying to understand the context of the experiment. Is this experiment about ISC, EB, ISC/EB or EE/EC? For example, Notch signaling is not activated in ISC, but the second half of the text about Notch signaling combines ISC/EB as a general study subject; I was deeply confused. Is Notch also activated in the ISC? Why is the ISC included in this part? What is the importance of Notch signaling in the ISC? Does Delta trigger cis-activation of Notch in ISC? I would suggest the authors to reorganize the manuscript and clarify the context of each experiment to help readers to understand the functions of esg in ISC stemness, EB fate, and EE/EC fate specification, respectively. The second section about Notch signaling should also be more precise (EB or EE/EC), rather than combining ISC/EB as a general subject in the text.
(2) The second major concern is the conclusion of "incoherent feed forward loop" from the study. I found the authors used the term differently from the conventional definition. I don't think it is appropriate for the authors to join two linear pathways in two different cells to create a "loop". I would suggest the authors to revise this part of conclusion.
(3) My final major concern is that I don't (yet) believe the expression of Amun is restricted to EC cells in wild type and upregulated in ISC/EE cells following loss of Esg. The position of the EC cell is not identified in the figure, and no EC marker is mentioned. The quantification is barely significant. The n's are not given, and perhaps are low. Perhaps a larger sample size would increase the significance of the result (or show it is not significant).
The minor concerns are:
a) Figure 1A needs to be recolored. I got confused with the exact same color of Su(H), Pros and Delta. Besides, Gfp in EB is supposed to be ubiquitous, but the presentation shows that Gfp is nuclear and does not make sense at all. The production of EC and EE is from EB or ISC? The authors have to revise this figure to help readers understand the model system in this manuscript.
b) I don't understand why the authors wanted to show the results of esgshof in the first paragraph. The allele is not used further in the manuscript and what is the importance and advantage of this specific allele compared to the null allele esgG66? The authors will have to explain why esgshof has to be characterized here.
c) Figure 1F. Where is the morphological change?
d) Since esgts is considered a stronger driver, why do the authors switch back and forth between esgts and drug-induced driver (5961GS)? I think a simple explanation of the advantage and disadvantage of two different drivers will be appreciated.
e) The intro says that previous work has shown Amun is a Notch target. Please give the citation.
f) Please explain how the Esg-DamID was expressed in the text or methods (it is too important to bury in the supplemental methods).

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1st Revision - authors' response 20 October 2014

We thank the Referees for their constructive criticism and insightful suggestions. We think that our revisions have significantly strengthened the manuscript. Below, please find a point-by point response to the critiques.

Referee #1:

Major points:

1- From the results presented in Figure 3A and E3A the authors conclude that esg knockdown results in down-regulation of Notch signaling as visualized by Su(H)LacZ reporter, which is expressed in EBs. This is a very difficult argument to make given that knockdown of esg results in premature differentiation of EBs into EE cells and ECs. Therefore, the loss of EBs will indefectibly lead to less Su(H)LacZ.

We are aware of this caveat and have alluded to it in the Discussion. More importantly, we now include data that demonstrate that Esg and Notch interact genetically (new Figure 3B). Moreover, we have also included new data where Esg *depletion is restricted specifically to ISCs. We did not use the Delta-Gal4 driver, which has reportedly worked with variable efficacy for colleagues in the field, as in our hands, crosses with this driver were all very sick or lethal, and the few escapers that we obtained showed a spatially restricted pattern of expression that excludes the posterior midgut (see figure at the end of this document). Therefore, we also used an esg-Gal4 driver combined with Su(H)-Gal80, which suppresses Gal4 activity in EBs (new Fig. 2A and Fig. E2A). However, as we acknowledge above, this strategy is also susceptible to the caveat that Gal80 expression may be partial due to any effects on Notch signaling as a consequence of* esg *depletion in ISCs.*

2- Figure 3B: The green channel should be shown and the proportion of EC in each condition quantified.

These data (new Fig. 3C) and all the measurements (new Fig. 3D), including specific counts of ECs (Fig. E3E), have now been included.

3- Related to Figure 3B, I think the epistasis between Esg and Notch is not properly tested since both, esg-RNAi and Notch-intra overexpression, have a phenotype of their own. Therefore, I believe that manipulating one of the components on a sensitized background for the other is a much more rigorous way to address genetic interaction. For example, knocking down and/or overexpressing esg and removing one copy of N or overexpressing Notch-intra in an esg-/+ background.

We agree and tried a series of approaches to address the Referee's concerns. Although it was difficult to find viable, allelic combinations that showed no phenotype on their own, ultimately, we used a combination of a Notch heterozygous background and a more modest Esg knockdown achieved by using a lower concentration of the drug RU486. The new data (Fig. 3B) shows a striking enhancement in the Notch loss of function phenotype by simultaneous dowregulation of esg.

4- Data on regarding the connection between Esg-Jak-Stat-Ecad and Notch signaling is largely over interpreted. The current evidence linking Jak-Stat and Notch is only correlative and, to my knowledge, no link between these pathways has been clearly established so far. The same applies to the relationship between Ecad junctions and Notch signaling activation in the midgut. Therefore, most of the results in this section of the manuscript relay on speculations. The data presented by the authors simply suggest that esg is required to keep Jak/Stat signaling activation in ISCs. Regarding this point, the authors should assess the levels of Socs36E mRNA to rigorously and quantitatively assess Stat activity upon esg knockdown. I find the data on Ecad puzzling. If this represents an intermediate situation, the authors should knockdown esg for a longer period or using a stronger driver to see what happens to Ecad at the end.

These data have been omitted from the revised manuscript.

5- Characterization of the role of Amun in the system:

a-The data regarding the in situ expression of Amun is poor. I acknowledge that ISHs in the midgut are hard and I guess there are no good antibodies for Amun? In such case, I suggest the authors that they assess Amun expression domain by using EC and ISC/EB specific drivers to drive Amun RNAi and then assess presence or absence of gene knockdown by RT-PCR. This would provide a good test for the efficiency of the Amun RNAi and also confirm the expression domain of the gene in control and esg-RNAi midguts. For example; one would expect to see a knockdown of Amun in control midguts when using the EC driver but not the ISC/EB driver. On the contrary, using the latter driver with esg- and Amun- RNAis should show Amun knockdown when compared to esg-RNAi only.

To address the Referees' concern regarding the data indicating that Aumn is a target of Esg, we used FACS to sort ISC/EBs from intestines in which esgRNAi was expressed in those cells (new Fig. 4D, Fig. E4B). Importantly, the new data support our previous conclusions that Amun *expression is upregulated upon* esg *depletion (Fig. E4A, C).*

b- I wonder whether using a stronger driver to overexpress Amun (Figure 4B, C) would result in a more robust/stronger phenotype.

We originally chose to use the 5961 driver to avoid any issues arising due to depleting esg *with it's own promoter (*esgGal4). *However, we've now repeated our experiments using the* esgGal4 driver, which indeed gives rise to a stronger phenotype *(new Fig. 4B,C).*

c- Figures 5 and E5 show the same control and esg-RNAi guts. Different examples should be presented for different experiments.

Different images for the control and esgRNAi images have now been included in the new Fig. E5A, as suggested by the Referee. However, we explicitly indicate in the figure legend that they are different examples from the same experiment shown in Fig. 5B.

Minor point:

I find that most of the discussion is a repetition of the results. I suggest the authors try to modify it to minimize this.

We have now revised the Discussion and think it is more streamlined.

Referee #2:

Major concerns

1- The authors claim a loss of ISC in clones mutants for esg. It would be useful to see in the main figure a direct quantification of ISCs per clone by staining for delta, a marker of ISC.

Our colleagues and collaborators performed this experiment in their accompanying manuscript (Korzelius et al.; Fig. 1C). In order to avoid too much redundancy between the manuscripts, we wanted to present our data in such as way as to highlight both the loss of ISCs and the increase in EE cells. Therefore, in Figs.E1D, E and Table E1, we have provided quantification of the number and detailed analysis of clones that do not contain ISCs/EBs.

2- The authors show that the effect of esg RNAi is stronger in EBs. However we do not have the results of experiments done with an ISC specific driver. The authors should look at the effect of knocking down esg only in ISCs (delta-Gal4 driver).

Please see new Figures 2A, E2A. We did not use the Delta-Gal4 driver, as in our hands, progeny from this cross were all very sick or died, and the few escapers that we obtained showed a spatially restricted pattern of expression that excluded the posterior midgut (see figure at the end of this document). Therefore, as an alternative approach, we used an esg-Gal4 driver combined with Su(H)-Gal80, which suppresses Gal4 activity in EBs (new Fig. 2A, C and Fig. E2A). However, as we acknowledge above, this strategy is also susceptible to the caveat that Gal80 expression, and therefore transgene suppression in EBs, may be partial due to any effects on Notch signaling as a consequence of esg *depletion in ISCs.*

3- The authors present negative data to explain that the JAK-STAT pathway is not responsible for the effect of esg on Notch. However we lack here crucial information to understand the results and conclude. First the authors should show the levels of cytokines, and JAK-STAT target genes by RT-qPCR, second the authors should look at the epsitasis of esg and STAT modulation.

These data have been omitted from the revised manuscript.

Minor points:

P9: restricted should be restrict.

P10: ISC/SB should be ISC/EB.

Both have been corrected.

Referee #3:

I have two major concerns.

(1) The first is the organization of the manuscript. I found myself constantly trapped in the text trying to understand the context of the experiment. Is this experiment about ISC, EB, ISC/EB or EE/EC? For example, Notch signaling is not activated in ISC, but the second half of the text about Notch signaling combines ISC/EB as a general study subject; I was deeply confused. Is Notch also activated in the ISC? Why is the ISC included in this part? What is the importance of Notch signaling in the ISC? Does Delta trigger cis-activation of Notch in ISC? I would suggest the authors to reorganize the manuscript and clarify the context of each experiment to help readers to understand the functions of esg in ISC stemness, EB fate, and EE/EC fate specification, respectively. The second section about Notch signaling should also be more precise (EB or EE/EC), rather than combining ISC/EB as a general subject in the text.

Perhaps one issue is that most of the tools that we have available to us manipulate gene expression in both the ISC and EB. Therefore, we include both in our descriptions and interpretations to avoid overinterpretation of the data. In this revised manuscript, we have tried to be more clear about the rationale and technical reach of each experiment.

(2) The second major concern is the conclusion of "incoherent feed forward loop" from the study. I found the authors used the term differently from the conventional definition. I don't think it is appropriate for the authors to join two linear pathways in two different cells to create a "loop". I would suggest the authors to revise this part of conclusion.

We have revised the Discussion to avoid using this terminology. In addition, the schematic describing the loop as been omitted in the new Figure 6.

(3) My final major concern is that I don't (yet) believe the expression of Amun is restricted to EC cells in wild type and upregulated in ISC/EE cells following loss of Esg. The position of the EC cell is not identified in the figure, and no EC marker is mentioned. The quantification is barely significant. The n's are not given, and perhaps are low. Perhaps a larger sample size would increase the significance of the result (or show it is not significant).

In response to a similar comment by Referee 1 (see above, 5a), we have taken a new approach to address the effects on Amun *expression upon reduction of* esg*. Specifically, we used FACS to sort ISC/EBs from intestines in which esgRNAi was expressed in those cells (new Fig. 4D, Fig. E4B). Importantly, the new data support our previous conclusions that* Amun *expression is upregulated upon* esg *depletion (Fig. E4A, C). However, the qRT-PCR from sorted cells revealed that Amun expression is not restricted to ECs. Therefore, we have refrained from making conclusions about where* Amun *is normally expressed and have focused on what happens within ISC/EBs in response to Esg downregulation.*

The minor concerns are:

a) Figure 1A needs to be recolored. I got confused with the exact same color of Su(H), Pros and Delta. Besides, Gfp in EB is supposed to be ubiquitous, but the presentation shows that Gfp is nuclear and does not make sense at all. The production of EC and EE is from EB or ISC? The authors have to revise this figure to help readers understand the model system in this manuscript.

We have recolored this figure. GFP is ubiquitous in some cases, nuclear in others (depending on the UAS-Gfp used). Because there is such inherent variation in the observed staining patterns depending on the constructs used, we have revised the legend for Fig. 1A and removed any references to staining patterns.

b) I don't understand why the authors wanted to show the results of esgshof in the first paragraph. The allele is not used further in the manuscript and what is the importance and advantage of this specific allele compared to the null allele esgG66? The authors will have to explain why esgshof has to be characterized here.

We have now emphasized that shof *is the only viable allele of* esg *available; therefore, we thought we could use shof mutants to begin investigating any intestinal phenotypes. However, as we describe here, there is no loss of expression and no obvious phenotypes.*

c) Figure 1F. Where is the morphological change?

We used arrows to point at three different examples of cells that are noticeably larger and rounder than the typical triangular shape of ISC/EBs. We have stressed in the figure legend that these changes are apparent in some of the cells, and have specifically pointed out to an example that illustrates our observation.

d) Since esgts is considered a stronger driver, why do the authors switch back and forth between esgts and drug-induced driver (5961GS)? I think a simple explanation of the advantage and disadvantage of two different drivers will be appreciated.

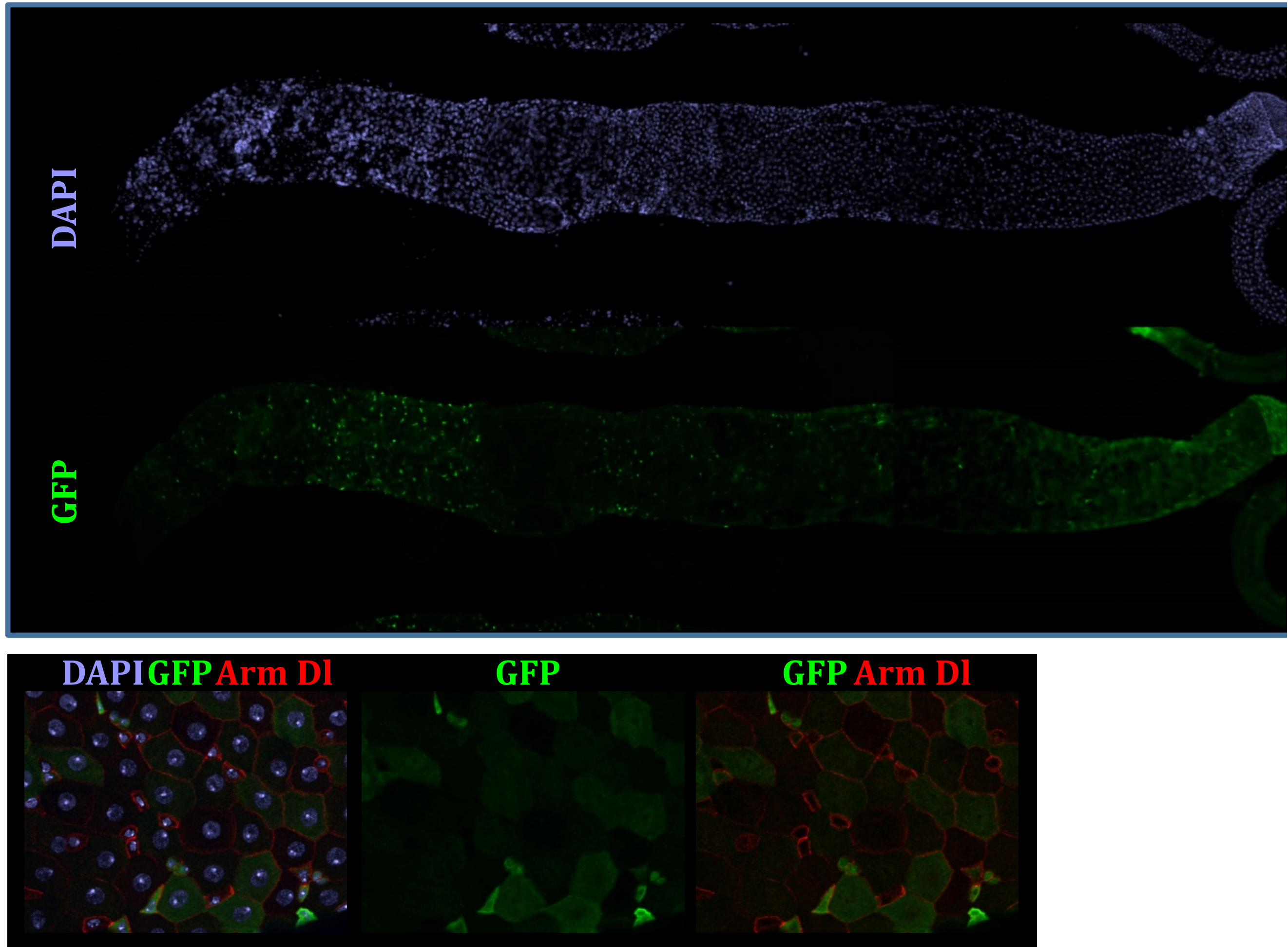
Although esgts *is stronger, we wanted to avoid any caveats from expressing* esgRNAi *with the* esg *promoter. In addition,] it is not yet possible to have a version of the esg[ts] driver that permits visualization of ISC/EB cells in uninduced controls (the GFP marker is dependent upon temperature shifts). 5961 is weaker, but much easier to control, as demonstrated by our new experiments in a Notch heterozygous background (new Fig. 3B), which could only be achieved by titrating the amount of RU486 to achieve a modest reduction in* esg *levels.*

e) The intro says that previous work has shown Amun is a Notch target. Please give the citation.

Omission of these references was a mistake. We have now added references that conclude Amun is a negative regulator of Notch.

f) Please explain how the Esg-DamID was expressed in the text or methods (it is too important to bury in the supplemental methods).

Done.



<u>Figure R1: Expression patter of Delta-Gal4 driver line</u>. *Top*: GFP staining of one of the few escapers from a Dl-Gal4 > UAS-2xGfp cross. Please note that GFP was detected only in the most anterior region of the posterior midgut (just posterior to the gastric region). *Bottom*: Some guts did show some GFP expression in the posterior region of the posterior midgut (where we have focused all of our other observations). However, a more careful inspection of these cases showed that GFP expression does not seem to be restricted to diploid cells, but extends to a noticeable number of ECs as well.

2nd Acceptance 27 October 2014

One of the original referees commented on your revised manuscript. S/he essentially endorses publication and we are thus happy to formally accept your paper for publication in The EMBO Journal.

For efficient production, we would be grateful if you were to provide a 2-up to 4 bullet point SYNOPSIS that emphasizes the major advance provided by your study. Short and concise terms would be appreciated.

In an effort to ensure good reporting standards and to improve data reproducibility (consistent with the 'Principles and Guidelines for Reporting (Preclinical) Research' issued by the NIH in 2014), we now require the submission of a completed author checklist. This covers in a systematic manner your practices regarding animal welfare, human subjects, data deposition and research ethics. It needs to be filled (not all fields may apply to your study in particular) and returned to the editorial office, either via the online submission system as a supplementary file or simply by email (contact@embojournal.org).

I hope that this message will be received as good news and would be grateful for your timely attention and response as to facilitate rapid production/publication.

REFEREE REPORT:

This new version of the manuscript has been streamlined, and is technically acurate. Ambiguous sections of the manuscript have been removed, and the role of Amun and its interaction with esg seem robust. Thus the global message of the role of esg, and the interaction with the Notch pathways are clear. I believe this paper should be published.

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