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escargot maintains stemness and suppresses differentiation in Drosophila intestinal stem cells

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Corresponding author: Bruce Edgar, DKFZ-ZMBH Alliance -Member

Review timeline:

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Editorial Decision:	16 June 2014
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Transaction Report:

(Note: With the exception of the correction of typographical or spelling errors that could be a source of ambiguity, letters and reports are not edited. The original formatting of letters and referee reports may not be reflected in this compilation.)

Editor: Thomas Schwarz-Romond

Editorial Decision 16 June 2014

Thank you very much for submitting your manuscript on the functional role of escargot in (intestinal) stem cell maintenance for consideration to The EMBO Journal.

Having received three referee comments, I am pleased to invite formal revisions based on the very explicit and constructive referee remarks (attached for your info below).

Specifically, I deem the statistical clarifications/extensions and presentational issues as outlined from refs#2 and #3 essential. I would however not insist (though very much encourage) the epistatic expansions proposed by ref#1. As the latter have been similarly requested for the complementary study by the Jones-lab, I wonder whether it would make sense to coordinate/join forces on particularly this aspect of the revisions?

Please do not hesitate to get in touch with regard to feasibility and anticipated timeline for the necessary revisions (due to time constrains preferably via e-mail).

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 Korzelius et al. the authors analyze the role of the transcription factor Escargot (Esg) in intestinal stem cell (ISC) homeostasis in Drosophila. By means of gain and loss of function experiments they uncover a dual role of Esg in the maintenance of stemness in ISC and suppression of ISC differentiation. Furthermore, through extensive transcriptomic profiling and DNA binding analysis by DamID the authors present a comprehensive molecular characterization of the role of Esg in the midgut. They find the transcription factor and EC marker, Pdm1 as one direct target of Esg, which they go on to further characterize. They conclude that Esg maintains stemness in the midgut by repressing differentiation factors, such as Pdm1. This manuscript complements well with work by Loza-Coll et al., which has also been submitted for consideration as back-to-back publications.
This work presents a very thorough analysis of the molecular events regulated by Esg in the midgut. It also provides an impressive data-set of genes associated with stemness and cell differentiation in the midgut, which are likely to be useful for the field. The data is well presented and experiments have been carefully done and controlled for. I have a few comments, which I suggest that the authors should address before the manuscript is considered for publication:

Major points:

1- Representative images of midguts as in Figure 1D with and without Pe and at the different time points should be shown.

2- Figure 7: I think the epistasis between Esg and Notch is not properly tested since both, esg-/-; esg overexpression; Notch-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 or overexpressing esg and removing one copy of N; overexpressing Notch-intra or Notch-RNAi in an esg-/+ background.

Referee #2:

In this article, Korzelius et al. tackle the role of the transcription factor esg, in the activity of intestinal stem cells (ISCs) in Drosophila. To note, esg is the classical marker for progenitor cells in Drosophila, but its function is still surprisingly remaining mysterious. First, the authors show by clonal analysis, and in vivo RNAi that esg is required for ISC maintenance, as seen by the disappearance of esg>GFP cells and the stem cell marker delta. Upon removal of esg, progenitor cells differentiate in all the postmitotic cell types of the gut (EEs and ECs). Interestingly, esg is required in enteroblasts, committed progenitors on their way to differentiation, to maintain the EB fate, indicating that esg is a major negative regulation of differentiation in progenitors. Overexpression of esg in progenitors prevents them for differentiating, generating accumulating progenitors that retain their proliferating ability. The authors then proceed to transcriptomics and DAM-ID to identify genes both bound and regulated transcriptionally by esg in progenitor cells. In addition, the authors show that esg expression in differentiated ECs both repressed the expression of differentiation markers of the EC, and induced the expression of genes associated with stem cells or cell cycle, altogether inducing stress and cell death in the intestine. The authors further show that the overexpression of one putative target of esg, pdm1 is enough to trigger premature differentiation of

progenitor cells. Interestingly, activation of Notch pathway along with activation of esg can partially rescue the lack of differentiation. Covertly, the authors show that in absence of escargot, tumors induced by lack of Notch signaling in progenitors are less frequent, due to increased differentiation of progeny. Altogether the authors present a very well constructed paper that both clarifies the role of the esg transcription factor in the maintenance of progenitors in the gut, and informs on the mechanisms that lead to differentiation. This paper is well written, clear, and the mix of genomics and functional genetics very informative. I believe this paper deserves publication. I only have few major comments that could improve the paper:

Major points:

1- the authors suggest that all ISCs differentiate and there is no cell loss. However it is hard to be sure that there is no cell loss and replacement after loss of esg in ISCs. The authors could perform an immunostaining (for instance anti casp3) to be sure no apoptotic cell death occurs.

2- The authors indicate that progenitors differentiate upon loss of esg. A quantitative estimation of the number of EE and EC cells should be estimated.

3- The authors suggest that esg does not control polyploidy in the gut because they detect prospero positive cells. This does not indicate that these EE cells are indeed diploid. Could the author show some measurement to quantify/estimate that the ploidy of EEs and ECs remains constant when progenitors differentiate upon loss of esg.

4- overexpression of esg in progenitors affects differentiation. One could legitimately ask whether the transition between ISC and EB is altered too. The authors should present quantifications of delta and Su(H) positive cells upon overexpression of esg. Does the number of ISCs remain constant?

5- I believe that panel 4B is not mentioned in the text.

Minor point
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1. The authors need to provide a brief explanation or scheme of esg-F/O or MARCM system for readers who are not familiar with the fruit fly genetics.
2. The authors have to define ROI in the main text.
3. The authors need to explain the approaches to conclude that "a highly significant overlap between Esg-Dam-bound genes and genes upregulated upon est[RNAi]" in page 11. I don't understand the conclusion from the Venn diagrams in the Figure 4E. The same issue with Figure 5H.
4. Figure legend of 4A or the labels of X/Y axis are swapped.
5. No explanation of the purpose and methodologies of Figure 4B.
6. Figure 4C is very confusing. I had a hard time to figure out what I should focus on. The authors have to revise this figure.
7. In the figure 7J, NotchRNAi knock-down shows bimodal distribution; therefore I don't think the statistics present here are meaningful. Furthermore, the conclusion in the figure is contradictory to what they claim in page 17 "midguts from estG66B NotchRNAi animals still had much lower

mitotic indices than seen in NotchRNAi control." I would suggest the authors to show the percentage of clones that have no Ph3+ cells in wildtype versus NotchRNAi, or use a different approach to present the results.
8. Please outline the clone in Figure 2 to help readers to see the difference.
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10. Figure 2F-2I, is the labeling supposed to be 0"."17%, not 0","17%?
11. Figure E3. What are merged in E3A', E3B', E3C', and E3D'?

1st Revision - authors' response 25 August 2014

We would like to thank the reviewers for their constructive responses and for their support of our manuscript. We have addressed the remaining issues in our manuscript as follows from the point-by-point list below:

Referee #1:

In this manuscript by Korzelius et al. the authors analyze the role of the transcription factor Escargot (Esg) in intestinal stem cell (ISC) homeostasis in Drosophila. By means of gain and loss of function experiments they uncover a dual role of Esg in the maintenance of stemness in ISC and suppression of ISC differentiation. Furthermore, through extensive transcriptomic profiling and DNA binding analysis by DamID the authors present a comprehensive molecular characterization of the role of Esg in the midgut. They find the transcription factor and EC marker, Pdm1 as one direct target of Esg, which they go on to further characterize. They conclude that Esg maintains stemness in the midgut by repressing differentiation factors, such as Pdm1. This manuscript complements well with work by Loza-Coll et al., which has also been submitted for consideration as back-to-back publications.

This work presents a very thorough analysis of the molecular events regulated by Esg in the midgut. It also provides an impressive data-set of genes associated with stemness and cell differentiation in the midgut, which are likely to be useful for the field. The data is well presented and experiments have been carefully done and controlled for. I have a few comments, which I suggest that the authors should address before the manuscript is considered for publication:

Major points:

1- Representative images of midguts as in Figure 1D with and without P.e. and at the different time points should be shown.

Response: We added representative images to our revised Figure 1 that show midguts 2 days after *P.e*. infection for both control *esg-F/O* and *esg-F/O* > esg^{RNAi} midguts. These illustrate that upon *P.e*. infection, midguts shrink dramatically and are disorganized in esg^{RNAi} animals due lack of the ability to regenerate the damaged epithelium. We have also counterstained for the mitosis marker pH3S10 to demonstrate a lack of actively dividing ISCs in these midguts. Furthermore, we quantified the total number of pH3S10-positive cells/midgut in control and esg^{RNAi} animals upon P.e. infection as well. We also discussed these findings in the Main text (p7).

2- Figure 7: I think the epistasis between Esg and Notch is not properly tested since both, esg-/-; esg overexpression; Notch-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 or overexpressing esg and removing one copy of N; overexpressing Notch-intra or Notch-RNAi in an esg-/+ background.

Response: Our epistatis experiments showed that Notch activation can override Esg in stem/progenitor cells, but also that Esg is required for the propagation of Notch mutant tumors. Admittedly, these tests, while useful do not fully explain the relationship between Notch signaling and Esg in ISC differentiation. However, since the single phenotypes from Notch and Esg LOF and overexpression are so strong, we doubt that the dosage-dependent genetic interactions as proposed by the reviewer would resolve the issue. Also consider that we have no evidence that Notch and Esg directly control each other, so a strong genetic interaction is not necessarily expected. As depicted in our model figure (revised Fig 8), we propose that the downregulation of Esg by Notch signaling is indirect, via Pdm1, and also requires an accessory factor, Stat, that is independently activated. Given this, we don't expect a strong genetic interaction between Esg and Notch. But even if such an interaction were seen, we don't believe it would bring us closer to understanding the mechanism of their interaction. (Experiments testing Stat might be more fruitful). Furthermore, the genetics required to perform the genetic interaction tests proposed by the reviewer would take a rather long time. Hence, assuming a large time-investment and little pay off, we've decided not to perform these experiments for this revision. We hope the reviewer can be persuaded that this is the most practical course.

Referee #2:

In this article, Korzelius et al. tackle the role of the transcription factor esg, in the activity of intestinal stem cells (ISCs) in Drosophila. To note, esg is the classical marker for progenitor cells in Drosophila, but its function is still surprisingly remaining mysterious. First, the authors show by clonal analysis, and in vivo RNAi that esg is required for ISC maintenance, as seen by the disappearance of esg>GFP cells and the stem cell marker delta. Upon removal of esg, progenitor cells differentiate in all the postmitotic cell types of the gut (EEs and ECs). Interestingly, esg is required in enteroblasts, committed progenitors on their way to differentiation, to maintain the EB fate, indicating that esg is a major negative regulation of differentiation in progenitors. Overexpression of esg in progenitors prevents them for differentiating, generating accumulating progenitors that retain their proliferating ability. The authors then proceed to transcriptomics and DAM-ID to identify genes both bound and regulated transcriptionally by esg in progenitor cells. In addition, the authors show that esg expression in differentiated ECs both repressed the expression of differentiation markers of the EC, and induced the expression of genes associated with stem cells or cell cycle, altogether inducing stress and cell death in the intestine. The authors further show that the overexpression of one putative target of esg, pdm1 is enough to trigger premature differentiation of progenitor cells. Interestingly, activation of Notch pathway along with activation of esg can partially rescue the lack of differentiation. Covertly, the authors show that in absence of escargot, tumors induced by lack of Notch signaling in progenitors are less frequent, due to increased differentiation of progeny. Altogether the authors present a very well constructed paper that both clarifies the role of the esg transcription factor in the maintenance of progenitors in the gut, and informs on the mechanisms that lead to differentiation. This paper is well written, clear, and the mix of genomics and functional genetics very informative. I believe this paper deserves publication. I only have few major comments that could improve the paper:

Major points:

1- the authors suggest that all ISCs differentiate and there is no cell loss. However it is hard to be sure that there is no cell loss and replacement after loss of esg in ISCs. The authors could perform an immunostaining (for instance anti-casp3) to be sure no apoptotic cell death occurs.

Response: Our data argue against an induction of apoptosis due to loss of Esg for these reasons: 1) We present cell counts in our revised Fig 2J for *esg-F/O > esg^{RNAi}* midguts 1 day after RNAi induction. This analysis shows that the majority of the affected ISCs/EBs differentiate into larger Pdm1-positive EC cells (revised Fig 2I). During this differentiation process however, the total number of cells/region of interest measured does not significantly change, which strongly argues against cell loss (revised Fig. 2J). 2) Furthermore, differentiated *esg^{G66B}* null mutant cell clones are maintained over time and can still be found at least 10 days after clonal induction. This is also supported by the accompanying manuscript from Loza-Coll et al., which shows that *esg^{G66B}* clones

are maintained, but do not grow between 4 and 10 days after clonal induction. Hence, based on our cell counts and clonal analysis data we do not think that there is significant apoptosis taking place upon loss of Esg.

2- The authors indicate that progenitors differentiate upon loss of esg. A quantitative estimation of the number of EE and EC cells should be estimated.

Response: In our revised Fig 2 we show the quantification of the number of differentiated EC cells. This is shown in revised Fig. 2I, in which the % of Pdm1-positive ECs is plotted for control and esg^{RNAi} *esg*-F/O animals. We changed the text accordingly to describe this. Figure 2A-B show that EE's are produced as well. The quantification of EE cells/clone is done extensively in the accompanying manuscript from Loza-Coll et al., to which we also refer in our manuscript, so we haven't done that here.

3- The authors suggest that esg does not control polyploidy in the gut because they detect prospero positive cells. This does not indicate that these EE cells are indeed diploid. Could the author show some measurement to quantify/estimate that the ploidy of EEs and ECs remains constant when progenitors differentiate upon loss of esg.

Response: Our previous work has shown that wild-type EE cells have a 2C DNA-content (Zielke et al., Cell Reports 2014). Since it is known that Esg affects polyploidization, the Referee raises a valid point by asking if the ploidy of these EE cells changes upon loss of Esg. To determine if EE cells in esg^{G66B} null mutant clones would differ from EEs in wild-type cells, we quantified the nuclear DAPI intensity from Pros-labeled EE cells inside control (FRT40A) and esg^{G66B} *FRT40A* clones for multiple clones (revised Figure 2E). This analysis indicates that esg^{G66B} *FRT40A* Pros-positive cells have on average a doubled amount of DNA, and are thus in G2. However, they appear not to be polyploid. We have added this new data to Figure 2 and include a description in the Main text (page 8-9). We cannot say why these EEs arrest in G2 rather than G1, but our general conclusion that Esg is not critical for maintain diploidy remains unchanged (G2 cells are diploid).

4- Overexpression of esg in progenitors affects differentiation. One could legitimately ask whether the transition between ISC and EB is altered too. The authors should present quantifications of delta and Su(H) positive cells upon overexpression of esg. Does the number of ISCs remain constant?

Response: We now show in our revised Fig E3 (Extended Data) that *esg-F/O* clones that constitutively express UAS-esg have both *Su(H)GBE-lacZ*-positive EBs and *Dl-lacZ*-positive ISCs. We quantified the cell numbers for both *Su(H)GBE-lacZ*-positive EBs and *Dl-lacZ* ISCs and see only a small (but significant) shift in ISC/EB ratio between control and *UAS-esg*. Hence, the overexpression of Esg does not seem to cause a large shift in ISC/EB ratio in the progenitor compartment. We have added these data to our revised Figure E3 and discuss these data in page 10 of the Main text.

5- I believe that panel 4B is not mentioned in the text.

Response: We apologize for this. In the revised manuscript, we have added a section that briefly describes the methodology and discusses our results from the PCA-analysis presented in Fig 4B.

Minor point

Typo p11: this should be "transcriptional changes affected"

Response: We corrected this typo in the Main text

Referee #3:

The manuscript by Korzelius et al. is to study functions of the Snail family gene escargot (esg) in the intestinal stem cell (ISC) and the transit amplifying progenitor enteroblast (EB). They found that Esg is required to maintain the stemness of ISC/EB. By using whole genome DamID and RNA sequencing, they further identified that Pdm1 is a direct target of Esg. Continuous expression of Esg in ISC/EB is required to suppress Pdm1 and to prevent Pdm1-induced premature differentiation. The study is comprehensive, and the data are solid. The work will greatly facilitate delineation of the genetic network that balances ISCs between self-renewal and differentiation. I recommend the publication of this manuscript in EMBO.

Several minor points, however, need to be revised to assist readers to fully understand the paper.

1. The authors need to provide a brief explanation or scheme of esg-F/O or MARCM system for readers who are not familiar with the fruit fly genetics.

Response: We adapted the Main text to incorporate a more elaborate description of both systems (pages 6 and 7), together with the appropriate references in the literature that outline these methods in even greater detail. The general idea of the *esg*-F/O system is now also diagrammed in our revised Fig 2F and discussed in the figure legend to Fig 2F.

2. The authors have to define ROI in the main text.

Response: We incorporated the definition (for ROI/Region of Interest in the Main text of our revised manuscript.

3. The authors need to explain the approaches to conclude that "a highly significant overlap between Esg-Dam-bound genes and genes upregulated upon esgRNAi" in page 11. I don't understand the conclusion from the Venn diagrams in the Figure 4D. The same issue with Figure 5H.

Response: We apologize for the confusion raised by our Venn-diagram analysis in Fig 4D and 5H. We have clarified our analysis in more detail and added a reference for our methodology on page 12-13 of the Main text of our revised manuscript. This provides a more elaborate explanation of the statistical method used to determine the significance of overlap in the respective Venn diagrams in Fig 4D and Fig 5H.

4. Figure legend of 4A or the labels of X/Y axis are swapped.

Response: We corrected this mistake in the Figure legend of Figure 4.

5. No explanation of the purpose and methodologies of Figure 4B.

Response: We corrected this mistake and have now added a description and discussion of the PCA-analysis shown in Fig 4B on page 11 of the revised manuscript.

6. Figure 4C is very confusing. I had a hard time to figure out what I should focus on. The authors have to revise this figure.

Response: Figure 4C shows the binding peak profiles from the Esg-DamID experiment for 5 different gene loci. The goal of this panel was to show that the Esg-Dam fusion protein directly binds close to the transcription start site for these particular genes. Hence, Esg is likely to directly act at the promoters of these genes to regulate their transcription. To clarify this further, we altered the Main text and the figure legend and reformatted panel 4C, where we now have boxes to outline the tracks of the separate genes, as well as a separate legend within the revised Figure 4 that

highlights all the features of the Esg-DamID binding profiles.

7. In Figure 7J, NotchRNAi knock-down shows bimodal distribution; therefore I don't think the statistics present here are meaningful. Furthermore, the conclusion in the figure is contradictory to what they claim in page 17 "midguts from esg^{G66B} NotchRNAi animals still had much lower mitotic indices than seen in NotchRNAi control." I would suggest the authors to show the percentage of clones that have no Ph3+ cells in wildtype versus NotchRNAi, or use a different approach to present the results.

Response: The Referee points out an aspect of the *esg^{G66B} NotchRNAi* phenotype that we did not present clearly, namely that the majority of the *esg^{G66B} NotchRNAi* clones are similar to *esg^{G66B}* single mutant clones and consist solely of differentiated cells (that cannot undergo mitosis). The suggestion of counting the # of mitosis/clone between control (in this case: Notch RNAi alone) and experiment (*esg^{G66B}+Notch^{RNAi}*) that referee #3 makes would be a great idea to illustrate this. However, this is experimentally very difficult, since *Notch^{RNAi}* clones proliferate at such a rate that individual clones easily merge, even after shorter periods of time. Hence, one is no longer able to distinguish whether these overgrowths arise from single or multiple clones. Therefore, any reliable quantification of the number of clones/cells per clone is impossible for this genotype. However, we have added representative images of whole midguts of these genotypes (*esg^{G66B}+Notch^{RNAi}* and *Notch^{RNAi}* alone) to illustrate the difference between the overgrowths in these animals in our revised Figure E5. We hope that this added data and the revised discussion of the phenotypes in the main text (pages 18 and 19) will sufficiently clarify this issue for Referee #3.

8. Please outline the clone in Figure 2 to help readers to see the difference.

Response: We outlined the clones in panels 2A-D.

9. The authors have to explain why the GFP intensity in E2B (esgRNAi 7d) is stronger than that in E2E (esgRNAi 2d).

Response: These images were taken from separate experiments and different settings for the green and red channels were used in both cases. Both times, we used GFP-antibody to detect the GFP-signal in these immunostainings. Therefore, the difference in GFP-intensity between the 2-day and the 7-day timepoint should not be compared. Since we did not make any statements about comparing GFP-intensities between these experiments, we did not feel the need to mention the observed difference in intensity between these experiments.

10. Figure E2F-E2I, is the labeling supposed to be 0"."17%, not 0","17%?

Response: We corrected this mistake in Figure E2F-E2I.

11. Figure E3. What are merged in E3A', E3B', E3C', and E3D'?

Response: We replaced the “MERGE” label in these panels with the individual channel names (DAPI/GFP/beta-Gal) in Fig E3.