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Drosophila midgut homeostasis involves neutral competition between symmetrically dividing intestinal stem cells

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(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.)

1st Editorial Decision 17 November 2011

Thank you very much for submitting your research manuscript proposing neutral competition between symmetrically dividing stem cells as component in the drosophila midgut for consideration to The EMBO Journal editorial office.
I did receive comments from three scientists that confirm timeliness of the topic and a certain interest in your findings. However, the reports also reveal significant shortcomings that relate to (i) the conclusiveness of the clonal tracing analysis as alternative interpretations of the results cannot be ruled out and (ii) absence of molecular interrogation(s) to further substantiate the symmetric component proposed. To be certain about the rather critical comments that range from potential live-imaging to long-term clonal studies and extensive pathway interrogations (and ref#3 in fact recommending rejection outright), I did involve a fourth scientists to not only assess the paper but also to comment on the referee's views.
This crystallized the possible impact of clonal age on your analyses and thus the neutral drift-model that will have to be appropriately considered and more cautiously presented. In case even longer-term data might be readily available, these should be incorporated. Rather more importantly, the role of regulatory pathway(s) on symmetric divisions has not (yet) been assessed in your work. Though a necessary condition, I would in the interest of time suggest to limit such feasible experiments to a critical set of candidate molecules. As this would significantly advance and thus also distinguish your findings from related data published in Cell, I would urge you to approach these in a timely manner. Conditioned on such modifications/expansions, we would be happy to pursue publication of your work in The EMBO Journal with no need to attempt live-imaging at this stage.
I do understand that this seems still very demanding and would involve considerable further

experimentation. I would thus be happy to discuss/clarify specific points also with regard to the potential timeline in more detail. Please do not hesitate to contact me in case of further questions, OR should you decide that under these circumstances you would prefer to take the current dataset for more rapid publication elsewhere (preferably via E-mail).

Lastly, I do have to formerly remind you that it is EMBO_J policy to allow a single round of revisions only and that the ultimate decision depends on the content and strength of your adequately modified version.

I am very much looking forward to your response and remain with best regards

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

This manuscript includes a thorough clonal analysis of Drosophila midgut stem cell proliferation, based on large number of clones studied at different time points after induction. The paper makes a few important and novel observations that revise our thinking about midgut ISC behavior; notably, the authors can show convincingly that not all ISC divisions are asymmetric and that lineage composition is highly variable. The authors propose a detailed model explaining the interplay between cell death and ISC-based cell regeneration that is compatible with the types and dynamics of clones they observe. Here are a few points that need to be addressed:
1.The descriptions of "stem cell nests" in fixed material, using esg and Su(H) markers, assumes that one can separate neighboring nests. After looking at many midgut preparations with these nests myself I would not accept that this is possible. Both ISCs and EBs are not epithelial cells; that is, they are not connected to their EC neighbors by a junctional complex (unlike EEs, which are). One has to assume that following ISC divisions, progeny move. For adult gut, that has not been directly shown (see #2); but one has to assume that individual cells, as the ones shown in Figs.2A-E, have moved away by a small amount from their parent cluster. I would not be able to say which of the labeled cells in Fig.2A, D, E form part of a given nest. Movement of the progenitors of ISCs (for example, in the larval gut) is abundant: they migrate over large distances.
2.In order to evaluate the degree to which ISCs and their progeny move, live imaging of adult gut preparations is overdue. The problem is of course that midgut will undergo muscle contractions; but is it possible to establish a culture system where muscular movement is blocked? It would be an enormous boost for the importance of this paper if it could be shown how an individual ISC clone evolves over 1-2 divisions.
3.The authors should check whether the fact that ISCs and EBs could move, even over small (1 cell diameter?) distances, affect their quantitative model.

Referee #2:

The authors performed extensive counting of ISCs and their clones in the adult Drosophila midgut. Counting of fixed cells after immunofluorescenct staining and following patterns of clones lead them to interpret the results as indications of symmetric division and neutral competition. This interpretation would be very similar to the recently proposed model of ISC in mouse small intestine.

Overall it is an interesting manuscript and the ideas help to advance the field. The experimental results themselves are well presented with large amount of well-collected data. I do think the readership, as myself, will enjoy reading this paper, but I also think it generates more arguments than answering the core question.

As in the published mouse ISC papers, the interpretation of fly midgut ISC symmetric/asymmetric division remains arguable after reading all the data presented. One problem is that the authors use quite extreme scenarios such as suggesting that previous fly midgut publications argue for "invariant asymmetry" in the strictest sense. Previous papers clearly showed that the Delta-Notch and cell fate asymmetry are detected in most ISCs after division. Most review articles in the field present a balanced view of possible symmetric division followed by some mechanism that establish asymmetric cell fates, except that the review by Hou et al 2010, defined "asymmetric division" in a very loose term of "division with asymmetry" and this review was cited as the representative view and is not that fair {(p. 3. Although an emphasis has been placed on invariant asymmetry (reviewed in Hou, 2010)....}.

The results presented in this ms. showed that 70% of the ISCs have what previously shown, asymmetric fate and Delta-Notch asymmetry at the time of detection. The new insight is about the other 30%, which the authors show that they have ISC-ISC or EB-EB properties. This I think, nonetheless, is an important observation and is highly consistent with the newly publish paper by O'Brian et al, Cell, 2011.

An important discussion is even if the midgut ISCs do not follow "invariant asymmetry", it does not mean that they divide symmetrically. There is no evidence that argues against asymmetric division, except that Dl is likely segregated symmetrically and no asymmetric component during division has been found. For example, one possible interpretation of the results presented in this ms. is that there is a yet to be defined asymmetric mechanism during ISC division that leads to quick asymmetric Dl-Notch signaling. This mechanism, as in most biological system, is not perfect and the occasional breakdown leads to equal Dl or equal N in both daughter cells. Just because we do not know this mechanism does not imply that it is symmetric division all the time. Nonetheless, I do agree that the interpretation the authors present is also a possible mechanism. I just think it is only an interpretation, not a conclusion based on the data.

The other argument the ms presented is neutral drifting and compensation of ISC loss by the symmetric duplication of neighbours. In the case of fly midgut, we cannot see that the whole gut becomes clonal, as in the intestinal crypt. Therefore, the neutral drifting theory does not apply easily here. The fact that some clones become very big and the clone density reduced to very low do suggest that many clones are lost in the process and other clones survive better and keep growing. This could mean not all ISCs are equipotent, in terms of survival. On top of that, there remains a possibility that the mitotic ISCs labeled at different time may be different, as well as the genetic technique, for example the overexpression of Gal4 or GFP can lead to different survival rate of the marked clone vs non-marked ISCs. The consideration of different division rates of ISCs, fast dividing vs. slow dividing populations, can also be a factor. Overall, while the authors' choice of interpretation is interesting, different interpretations of the data remain and perhaps equally possible.

The last point, age of the flies has very important consequence on the numbers as they counted. In the main text, the authors rarely tell the age of the flies in the experiment. Although in the material and method they stated the age of flies used for experiments, they have to reiterate the age when they make arguments. For example, the results in figure 2 will be vastly different if one uses 5 days old versus 10 or 15 days old flies. This experimental condition should be mentioned as frequent as possible in the main text.

Referee #3:

In this manuscript the authors consider the adult Drosophila intestines detailing the expression pattern of well-established marker genes and analyzing the behavior of wild type lineage clones. None of the experimental tools available in Drosophila for perturbation are utilized. The central advance claimed is that intestinal stem cells undergo symmetric division at a low frequency. The manuscript is clearly written and data is carefully documented. However, there is no perturbational analysis and most of the figures report findings that have been published previously. In this sense, essentially nothing shown here is new. Moreover, the authors present an iconoclastic view of stem cell regulation, however the issue of symmetric stem cell division has been raised many times in the

past and has been successfully addressed experimentally (e.g. Xi and Spradling, 2000). Two studies from the Jones lab and one from the Bilder lab have now documented the role of symmetric ISC division in replacement, which were left uncited (McLeod et al., 2010; Wang et al., 2011; O'Brien et al., 2011). The authors attempt to import a new scientific lexicon into a fully mature field of stem cell niche biology (e.g. Symmetric stem cell division is an established mode of stem cell replacement. Recasting the phenomenology of stem cell replacement by symmetric division as "population asymmetry leading to neutral drift dynamics in which..." tells us little that is new about stem cells or how they behave.). This type of jargon should be minimized. Ultimately the key to broad scientific appeal is clarity.

Suggestions that could significantly improve the manuscript:

1. The analysis in Figs. 1 and 2 of the manuscript rely on widely used markers for the ISC lineage. The data show that esg+ cell nests are heterogenous and vary in their gene expression profiles. Yet the conclusion that esg+ nests vary in the content of ISCs and EBs cannot be rightly drawn from this data. While ISCs and EBs express these genes, it does not necessarily follow that these genes mark all/only ISCs and EBs. Only self-renewing, pluripotent lineages in the posterior midgut can be consider stem cells. In the end, the analysis presented does not distinguish the possibility that esg+ nests vary in ISC and EB content from the possibility that the transgeneic markers utilized simply have variable expression in midgut cells that does not correspond to stem cells and their daughters. The authors need to address this point in a convincing manner with additional experiments. In addition the data panels in Figs. 1B and 2A-E are confusing and individual channels need to be shown in order to be convincing. The authors should show Arm and Pros channels separately and provide appropriate controls to demonstrate that Arm and Pros antigens are recognizing mutually exclusive cellular domains.

2. Two problems plague the wild type lineage clone tracing analysis presented in the manuscript. First, biologically inappropriate time points have been selected. Second, the analysis was performed in the context of tissue damage and not under baseline conditions. Thus, this analysis is confounded by a significant proportion of transient clones present in the interval chosen for analysis and stem cell clones responding to environmental perturbation. The authors need to analyze later time points (10-50 days after labeling) and employ a clone marking strategy that allows lineage labeling under normal homeostasis not following heat shock (e.g. the esg F/O method, Jiang et al., 2009), since the system has been shown to be highly dynamic in response to environmental transients.

More specifically, the clonal analysis performed in Fig. 3 A-E focused on 1, 2, 3, 4, 5, 8 and 16 day post heat shock time points. The methods state that data from "whatever clones were found" were acquired. Previous studies demonstrate that multiple classes of clones can be distinguished up to 10 days following induction (Ohlstein and Spradling, 2006). Stem cell clones are distinguished from transient clones 7-10 days following induction. Therefore, data presented in Fig. 3 does not distinguish the kinetics of stem cell and non-stem cell (transient) clones in the midgut. The analysis needs to be applied to the interval between 10 and 50 days in order to be relevant to ISCs.

Moreover, these cannot rightly be considered "long term" studies of the ISC as the authors claim. The precedent in the field is 36-50 days (e.g. Margolis and Spradling, 1995, Xi and Spradling, 2000, Boyle et al., 2007, Wallengang and DiNardo, 2006). Symmetric division has been well documented to occur at later time points, so the potential effects of aging may not be a caveat until much later (e.g. Xi and Spradling, 2000; Biteau et al., 2008, 2010). In addition the effects of pyloric scarring need to be functionally linked to ISC behavior, since this is invoked as an informative measure for the midgut but the reference cited pertains to the hindgut.

In contrast to what is asserted, if we look at the measured data in Fig. 3A, B at the relevant time points to detect ISC clones (after 8 days) it appears that there is very little change in density or clone size between day 8 and 16 (Moreover, there is no comment in the deviation of the model from the actual observed 16 day time point.). Yet, the authors assert that there is a progressive expansion of clone size when the data shows that it plateaus by day 16. This disparity needs to be clearly addressed.

3. When plotted as a function of $n/\langle n(t)\rangle$, the cumulative clone size distributions converge at early time points as seen in Fig. 3D. This analysis needs to be performed specifically for stem cell clones

to exclude the early effects of transient clones in the analysis.

4. The expression of Dl in Fig. 4 appears to be highly variable and indistinguishable from background in many cases. The authors need to distinguish the possibility that clone composition varies from the possibility that their Dl staining protocol itself is variable, supplying appropriate controls where needed. Moreover the black levels seem to have been brought down in Fig. 4B and brought up in Fig. 4C. Laser intensity levels for confocal acquisition and post processing in photoshop (or equivalent) must be identical to be convincing.

5. The authors suggest that the clone in Fig. 4F is undergoing symmetric duplication. The authors need to distinguish this from the possibility that the clone indicated is simply an asymmetric division in progress with an EB cell that has yet to differentiate. The fact that one of the cells in Fig. 4F' (red clone) has a larger nucleus than the other suggest that there has been an asymmetric division. If the argument is that the red and green clones are products of symmetric division, they need to convincingly rule out that this is the product of two independent events, for the twin spot marking system. In this case, if the products of symmetric division cannot migrate far from the site of the original stem cell, this should have the effect of distorting the overall distribution of stem cells in the midgut over time, which has never been reported. Please address these points.

6. The discussion of lateral inhibition as a potential mechanism of neutral competition is completely speculative and unsupported by any of the correlative data presented. Thus, it should be removed.

Author Correspondence 21 November 2011

We are grateful for the comments of the reviewers and welcome the opportunity to respond to their questions and concerns. As we understand it, there are three central points of criticism: (1) the existence of symmetrical intestinal stem cell (ISC) division, is not new; (2) our claim that tissue homeostasis involves a neutral process is not substantiated; and (3) we do not provide data on the question of molecular regulation. Having reflected on the detailed reports, we believe that key aspects of our analysis - particularly in relation to points (1) and (2) - have been overlooked by the reviewers, while at the same time we have identified a short-list of experiments that can address other concerns and which can be delivered in a timely manner. Following your e-mail, we have set out below how we intend to respond to these central issues. We would be most grateful if you could advise us on whether you believe that the proposed experimental programme would be sufficient to warrant a further stage of the review process.

First, as concerns the novelty of the findings (issue 1), of the three papers that reviewer #3 says cover the issue of symmetrical ISC division (McLeod et al., 2010; Wang et al., 2011; O'Brien et al., 2011), only one actually provides direct evidence, as we do, for symmetrical ISC division during homeostasis (O'Brien et al. 2011 - which, incidentally, went online on the 28th of October, some 15 days after our paper went out for review!). The other two papers, both from the Leanne-Jones lab, investigate the effect of cycles of starvation and re-feeding, and they report that, under re-feeding conditions, the ISC pool has the capacity to expand alongside the whole tissue. In particular, we can find no reference in these two papers to the pattern of ISC turnover in homeostasis. Also, although O'Brien et al. documents the existence of symmetric multiplicative ISC division during homeostasis, its potential significance for homeostasis is not addressed. Indeed, these authors identify this question as fundamental. Referring to the closing discussion of their paper:
"Indeed, our data indicate that, even during non-growth periods, symmetric fates occur approximately once out of every three stem cell divisions. The regular occurrence of fate symmetry and the ability of at least some stem cells to switch between symmetric and asymmetric modes raise the possibility that stochastic competition creates a pattern of neutral drift akin to the mouse small intestine (Simons and Clevers, 2011). Whether fate regulation is intrinsic or extrinsic to the dividing stem cell and how symmetric:asymmetric ratios are modulated during homeostasis and growth are key questions that emerge from our findings."
This issue is the focus of our manuscript and we not only provide a substantial amount of evidence for symmetric divisions and their kinetics but also reveal a mechanism that reconciles them with the homeostasis of the tissue. In particular, our analysis of the clonal fate data shows that, alongside symmetrical multiplicative ISC division, there are symmetric differentiating ISC divisions occurring

with equal probability, and leading to (and, indeed, providing evidence of) neutral drift dynamics. Moreover, we are able to provide significant new insight into the "key question" concerning the pattern of ISC regulation, which implicates extrinsic factors controlling the balance between ISC proliferation and differentiation.

In respect of issue 2, the interpretation of the clonal fate data hinges on the homeostatic nature of turnover, at least over the period germane to our quantitative analysis. In this context, our measurements of tissue size, composition and density provide circumstantial evidence that the tissue is indeed in steady-state. However, by itself, this information does not establish neutrality of the self-renewal process, i.e. the equipotency of the ISC pool. Our key evidence for neutrality rests on the quantitative analysis: By supposing that the process was neutral, we obtain an excellent fit to the detailed clonal fate data - both in terms of the overall size distribution, and in relation to the respective cell types. This agreement, which spans both the long-term scaling regime as well as the short-term behaviour provides strong, albeit indirect, evidence for neutral drift dynamics - evidence that is completely overlooked by the reviewers.
In addition, there is further direct evidence in our data that we did not highlight, that stresses the fact that our experiments address a homeostatic tissue and that the process is indeed neutral: When analyzing the expression of the ISC marker, Delta, shortly after induction, when clones comprise one or two cells, an analysis of the doublets (which, at that time, could only arise from one round of division of an ISC), we observe that the ratios of ISC/ISC, ISC/non-ISC and non-ISC/non-ISC pairs coincide precisely with the ratios of asymmetric, symmetric renewal and symmetric differentiation that we inferred from the analysis of the two whole datasets. This observation provides additional and direct evidence that the process is homeostatic and neutral (symmetric renewal and symmetric differentiation are balanced). However, the number of two-cell clones that were scored is relatively low, and therefore arguably not statistically significant and this is the reason why we chose not to place emphasis on this correspondence in the paper. Since we see that this direct evidence would allay concerns about the homeostatic and neutral nature of the turnover, we propose to consolidate the existing data by further analyzing the short-term clonal evolution of a large cohort of flies. By focusing on this very early timecourse, we are confident that this programme can be achieved within six weeks.

Finally, in respect of point 3, the analysis of two-cell clones can also serve as a reference to probe different signalling pathways for perturbation that alter the ratios of asymmetric division/symmetric renewal/symmetric differentiation with respect to wild type. However, you will agree that an analysis of this kind would require careful genetic manipulation, in adult stages, coupled to tightly controlled lineage tracing, a programme that is not straightforward. Coming as you do from a Drosophila background, you know that this requires producing complex strains (Flp, 2xFRT, UAS, Gal4, Gal80TS - all six chromosomes involved!) and raising the experimental flies at 18∫C (one month generation time), which would likely extend the timeline beyond 90 days. Therefore, we propose to approach this issue first by using chemical inhibitors of key signalling molecules. This approach has already been employed successfully in the adult midgut with DAPT and Notch signalling (Ohlstein and Spradling, 2006), and we could extend it to Wg signaling for which there are chemical agonists and antagonists - these are the two candidate pathways that, by phenotype, are worth testing. We estimate that these experiments could be completed within two months, the time course that we are considering here. At the same time, we plan to set the Gal4-based experiments in train, but that will most likely take at least a further month.

In our view, these are the only experiments that are feasible in an early time frame and that would yield quality data germane to the focus of the present paper. In our hands, the available twin-spot methods are simply not reliable for statistical analysis (as outlined in our methods section of the paper). However, we believe that the readout of the experiments proposed above will be sufficient to provide evidence that we are dealing with a process of homeostasis through neutral competition, and to identify the key signalling pathways involved in regulating the balance between ISC differentiation and proliferation.

In our formal response to the reviewers, we will of course deal with all of the points raised in their report, and in a point-by-point manner. In the meantime, we would value your views on whether the proposed experimental programme will be sufficient from an editorial perspective.

Editorial Correspondence 22 November 2011

Thank you very much for your correspondence that highlights once more the additional impact of your findings and outlines possible experimentation to address the concerns raised from the referees. I do recognize that the desirable genetic interference seems genetically complex and would thus consume significantly more time than currently at hand, particularly if performed in a vigorous manner. I do however appreciate that you suggest consolidation of the short-term clonal evolution, very interesting experiments that might indeed support neutral drifts in a homeostatic tissue and not prominently featured in the original submission. Given the overall timeliness of the dataset and to not delay their presentation too much (also with regard to the O'Brien paper), I suggest to develop this aspect, refocus your paper accordingly, integrate the O'Brien paper in a constructive manner and try to address ALL referee comments in your rebuttal letter.
This should enable efficient presentation of these, mostly descriptive current results and warrant careful delineation of the underlying genetic control in subsequent analyses that deserve necessary time and care, without relying on still questionable chemical interference.

I hope that this letter clarifies and offers a constructive way forward and would be happy to receive a thoroughly revised paper within a few weeks.

Yours sincerely,

Editor
The EMBO Journal

1st Revision - Authors' Response 05 March 2012

In response to the reports of the referees, we have undertaken some further experiments and implemented a number of changes in the text which we believe address their concerns in full. Below we discuss these concerns in detail, indicating how we have tried to improve the paper with regard to each particular issue.

REFEREE #1

This manuscript includes a thorough clonal analysis of Drosophila midgut stem cell proliferation, based on large number of clones studied at different time points after induction. The paper makes a few important and novel observations that revise our thinking about midgut ISC behavior; notably, the authors can show convincingly that not all ISC divisions are asymmetric and that lineage composition is highly variable. The authors propose a detailed model explaining the interplay between cell death and ISC-based cell regeneration that is compatible with the types and dynamics of clones they observe.

We thank Referee #1 for their views on the significance of our work and its impact on the current understanding of *Drosophila* ISCs behaviour.

Here are a few points that need to be addressed:

1.The descriptions of "stem cell nests" in fixed material, using esg and Su(H) markers, assumes that one can separate neighboring nests. After looking at many midgut preparations with these nests myself I would not accept that this is possible. Both ISCs and EBs are not epithelial cells; that is, they are not connected to their EC neighbors by a junctional complex (unlike EEs, which are). One has to assume that following ISC divisions, progeny move. For adult gut, that has not been directly shown (see #2); but one has to assume that individual cells, as the ones shown in Figs.2A-E, have moved away by a small amount from their parent cluster. I would not be able to say which of the

labeled cells in Fig.2A, D, E form part of a given nest. Movement of the progenitors of ISCs (for example, in the larval gut) is abundant: they migrate over large distances.

We regret that this section on nest composition may have caused some confusion with the reviewer. In our modelling scheme, we propose that ISCs undergo routine loss and replacement by neighbours. Such behaviour would necessitate the constant reorganization of tissue at the cellular scale, precisely the point made by the reviewer. We wanted to use the analysis of clusters of esg+ cells – the nests – to provide indirect evidence for this changing environment.

In our assay, we were careful to design a rigorous criterion to associate esg+ cells in closest proximity, and score them on the basis of Su(H)GBE expression. Indeed, we are confused by the affirmation of the Referee that it is not possible to separate "neighbouring nests". Our working definition of a nest is unambiguous: the group of esg+ cells that are in direct contact with at least one other esg+ cell of the group, as judged by Armadillo/b-catenin (Arm) membrane staining (laid down in shorter wording in page 5, paragraph 3, and page 16, paragraph 2 of the original manuscript; in page 12, paragraph 2, and page 18, paragraph 4 of the revised manuscript). This definition is based on, and motivated by, previous work. Arm was identified as general membrane marker in the midgut by Ohlstein and Spradling in their seminal paper in 2006:

> Staining for […] Armadillo outlines nests of 2–3 basally located diploid cells that lack Prospero […] as well as polyploid enterocytes […] and Prospero-positive enteroendocrine cells.

Moreover, adherens junctions (AJs) between undifferentiated cells have been claimed to play a fundamental role during Notch/Delta interactions (Maeda et al., 2008) and it has been recently suggested that the downregulation of AJs contacts between ISCs and ECs have a functional role in regulating ISC turnover (Choi et al., 2011). Though it might be questionable whether ISCs are connected with the EC neighbours by AJs, the relevant connections here are those between esg+ cells. Therefore it seems reasonable to use Arm at the membrane to define the esg+ nests, as it reveals their cell-cell contacts and these might be functionally relevant for cell fate.

In summary, we believe that the results are useful in providing further support to the modelling scheme. Equally, we accept that in the original version of the manuscript the relevance of this component of the study was less than clear. We have now revised down and relocated this section to a position where its significance for the modelling scheme is more clear (page 12 of the revised manuscript).

2. In order to evaluate the degree to which ISCs and their progeny move, live imaging of adult gut preparations is overdue. The problem is of course that midgut will undergo muscle contractions; but is it possible to establish a culture system where muscular movement is blocked? It would be an enormous boost for the importance of this paper if it could be shown how an individual ISC clone evolves over 1-2 divisions.

There is no question that live imaging of the adult gut would be a boost not only for this paper but the for whole field. Unfortunately long-term live imaging protocols are not routine techniques in the field at this point, and we think that only the setting up of such methods would fall beyond the scope of this work.

3.The authors should check whether the fact that ISCs and EBs could move, even over small (1 cell diameter?) distances, affect their quantitative model.

Since cell rearrangement (i.e. movement) lies at the heart of the current modelling scheme (viz. ongoing ISC loss and replacement by neighbours), the question does not seem to arise. The only situation that could impact the model is the presence of long-range movements (over several cell diameters), as one of its predictions is that clones remain relatively contiguous. However, this assumption is widely justified, as we have checked for the cohesiveness of the clones (discussed in page 18, paragraph 3 of the oritinal manuscript; in page 19, paragraph 1 of the revised manuscript

and page 4, paragraph 2 of the revised Supplementary Methods), and this has been explicitly recognized by others as well (Buchon et al., 2009; O'Brien et al., 2011; Ohlstein and Spradling, 2006). This assumption is in fact implicit throughout the literature on the adult midgut, which makes extensive use of lineage labelling techniques.

REFEREE #2

The authors performed extensive counting of ISCs and their clones in the adult Drosophila midgut. Counting of fixed cells after immunofluorescenct staining and following patterns of clones lead them to interpret the results as indications of symmetric division and neutral competition. This interpretation would be very similar to the recently proposed model of ISC in mouse small intestine. Overall it is an interesting manuscript and the ideas help to advance the field. The experimental results themselves are well presented with large amount of well-collected data. I do think the readership, as myself, will enjoy reading this paper, but I also think it generates more arguments than answering the core question.

We are grateful for Referee #2 comments on the interest and quality of our work and its contribution to advancing the field. We agree that our manuscript raises important questions with respect to the molecular mechanisms of homeostasis in the *Drosophila* midgut, questions that we now begin to address experimentally in the revised manuscript. However, to resolve those mechanisms, it is first necessary to identify the cellular strategy of tissue homeostasis, and we maintain that this key question (do adult *Drosophila* ISCs balance differentiation and division at the lineage level, or at the population level?), is answered in full, for the first time, in this work.

As in the published mouse ISC papers, the interpretation of fly midgut ISC symmetric/asymmetric division remains arguable after reading all the data presented.

We assume that Referee #2 is referring here to the specific issues that follow, which we discuss below on a point-by-point basis.

One problem is that the authors use quite extreme scenarios such as suggesting that previous fly midgut publications argue for "invariant asymmetry" in the strictest sense. Previous papers clearly showed that the Delta-Notch and cell fate asymmetry are detected in most ISCs after division. Most review articles in the field present a balanced view of possible symmetric division followed by some mechanism that establish asymmetric cell fates, except that the review by Hou et al 2010, defined "asymmetric division" in a very loose term of "division with asymmetry" and this review was cited as the representative view and is not that fair {(p. 3. Although an emphasis has been placed on invariant asymmetry (reviewed in Hou, 2010)....}.

The scientific motivation of our study is, as stated above, the elucidation of the cellular strategy of tissue maintenance in the fly intestine (lineage- vs. population-based), which is fundamental to interpret the molecular regulation. When presenting the background of the field, it is obvious that this question has been overlooked. Our reading of the literature suggests that the zeitgeist of the field clearly favours a lineage-based regulatory strategy (see Appendix), and that the attempts to resolve the molecular mechanisms of homeostasis have been largely guided by this paradigm. However we think that many authors realize the importance of this question, and we have presented the scientific question with a softer line (see pages 3-4 of the revised manuscript). We did not seek to reinvent a whole new model for the adult midgut, but simply solve a well-defined problem.

The results presented in this ms. showed that 70% of the ISCs have what previously shown, asymmetric fate and Delta-Notch asymmetry at the time of detection. The new insight is about the other 30%, which the authors show that they have ISC-ISC or EB-EB properties. This I think, nonetheless, is an important observation and is highly consistent with the newly publish paper by O'Brian et al, Cell, 2011.

We appreciate the comments of Referee #2 on the importance of our observation. We would however like to point out that the new insight of our manuscript is not only reporting the existence of symmetric divisions, but the identification of the cellular mechanism of tissue homeostasis as one of population asymmetry, with the consequences that this has on the possible signalling mechanisms regulating the homeostatic process as a whole. Population asymmetry requires the presence of a unified mechanism whose output is the three possible fate outcomes (2ISC, 2EB, 1ISC+1EB) in homeostatic proportions. Therefore, our findings do not concern only a particular, separate class of ISC divisions, but the behaviour of the whole system.

An important discussion is even if the midgut ISCs do not follow "invariant asymmetry", it does not mean that they divide symmetrically. There is no evidence that argues against asymmetric division, except that Dl is likely segregated symmetrically and no asymmetric component during division has been found. For example, one possible interpretation of the results presented in this ms. is that there is a yet to be defined asymmetric mechanism during ISC division that leads to quick asymmetric Dl-Notch signaling. This mechanism, as in most biological system, is not perfect and the occasional breakdown leads to equal Dl or equal N in both daughter cells. Just because we do not know this mechanism does not imply that it is symmetric division all the time. Nonetheless, I do agree that the interpretation the authors present is also a possible mechanism. I just think it is only an interpretation, not a conclusion based on the data.

The alternative explanation that Referee #2 proposes seems reasonable, but on close examination, it cannot easily account for the observed phenomenology. In this scenario, an intrinsically asymmetric mechanism would lead to a unidirectional inducing Dl signal from the ISC towards the EB. In the absence of the signal, as has been observed for null *N* or *Dl* clones, EB differentiation is blocked and the ISC offspring remain in the stem compartment. However, to produce the observed lineage dynamics, the frequent (20% of the divisions) breakdown of such a signal would need to result in ISCs dividing into either two ISCs or two EBs, and in *precisely balanced proportions*. It is easy to imagine that insufficient signal may result in two ISC daughters. But how could the breakdown of a unidirectional signal produce two EBs? Only if *exactly half* of the "breakdown" events consisted in the cell-autonomous activation of N in both ISC daughters. It is difficult to imagine how accidents of different molecular nature could be balanced.

Moreover, frequent signalling breakdowns could arguably lead the resulting cells into an irreversible chain of signalling errors, translated in chained symmetric multiplications. However neutral competition requires the offspring of each division to have the same chances to divide leading to any of the three possible fate outcomes.

By contrast, if one accepts that the ISC offspring is equivalent at birth (and we provide ample evidence that this is the case), one has to assume that the divisions are *initially* symmetric with respect to fate. Therefore, the Dl signal would naturally occur in both directions (and involve non-siblings), which is physically possible as the ISC offspring has both Dl and N. We provide now evidence in the paper, from the analysis of some gain of function alleles of *N*, that this seems to be the case (pages 13-14 and Figure 8A of the revised manuscript). Bi-directional signal is only a half-step away from lateral inhibition, which could explain all three possible fate outcomes occurring in homeostatic balance. Moreover, the constancy of processes such as the development of sensory bristles in *Drosophila* or that of the vulva in *C. elegans* witness the robustness of lateral inhibition as a mechanism for defining the fraction of competent cells allowed to take a particular differentiation program – which is the essence of homeostatic regulation.

Referee #2 writes that "there is no evidence that argues against asymmetric division". Indeed, it is true that many divisions result in asymmetric fate outcome. However, if Referee #2 refers to evidence against *intrinsically asymmetric* division, it is noteworthy that so far there is not much evidence in favour of it, either. The only strong datum supporting such asymmetry is the partition of

the esg+ population in Dl+ and Su(H)GBE+, which could be *as easily* explained by lateral inhibition. By contrast, the symmetric presence of both Dl and N in newborn ISC offspring is suggestive of symmetry breaking *after* division, also easily compatible with lateral inhibition.

With the information available and the data we present, we believe that our proposal of lateral inhibition is by far the most plausible scenario. However, in the original manuscript, we also discussed how the alternation between symmetric and intrinsically asymmetric divisions could give rise to the observed lineage dynamics – a discussion that has been clarified in the revised text (in page 14, paragraph 2 of the original manuscript; in page 16, paragraph 2 of the revised manuscript).

The other argument the ms presented is neutral drifting and compensation of ISC loss by the symmetric duplication of neighbours. In the case of fly midgut, we cannot see that the whole gut becomes clonal, as in the intestinal crypt. Therefore, the neutral drifting theory does not apply easily here. The fact that some clones become very big and the clone density reduced to very low do suggest that many clones are lost in the process and other clones survive better and keep growing. This could mean not all ISCs are equipotent, in terms of survival.

Of course, as Reviewer #2 points out, it is not possible to observe monoclonality in an extended tissue such as the midgut. Indeed, to reach fixation in a stem compartment of about 900 ISCs (vs.14-16 in the mammalian crypt) would take much longer than the age span of the fly. But we are mystified by the affirmation that, in the absence of fixation, "neutral drifting theory does not apply easily". In the numerous applications of neutral drift dynamics to population genetics, genealogy, mathematics and ecology, fixation is rarely observed. Rather, it is the characteristics of the drift process itself that provides a signature of the underlying dynamics. Indeed, it is in these characteristics that we can find the evidence for equipotency. In particular, the clonal fate data show hallmark scaling behaviour over time thereby ruling out the dominance of a lineage primed population over a weaker population (Klein and Simons, 2011) a key point emphasized in the original manuscript (in pages 8-9; in pages 7-8 of the revised manuscript).

However, to provide further evidence that the labelled cell population undergoes homeostatic turnover involving neutral competition, we have performed a new clonal induction experiment, where we focus on the population of clones chased for only 28h (pages 11-12 and Figure 6 of the revised manuscript). If this is a neutral process, we would expect these early clones (mostly originating from the first one or two divisions of the founder ISC) to be equally partitioned in ISCs and EBs (or differentiated cells), and therefore in Dl+ and Dl- cells. Moreover, they should reflect +/+, +/- and -/- divisions in homeostatic proportions. Indeed, we find that this is the case. Altogether, we believe that the case for neutrality and equipotency is strongly supported by the wide range of experimental data.

On top of that, there remains a possibility that the mitotic ISCs labeled at different time may be different, as well as the genetic technique, for example the overexpression of Gal4 or GFP can lead to different survival rate of the marked clone vs non-marked ISCs. The consideration of different division rates of ISCs, fast dividing vs. slow dividing populations, can also be a factor. Overall, while the authors' choice of interpretation is interesting, different interpretations of the data remain and perhaps equally possible.

The concerns raised by Referee #2 with respect to the use of markers are potentially relevant, we do not think that they apply to our study.

First, the labelling technique we used is known to be very reliable and we have provided specific experimental evidence that induction only occurs at significant levels when inducing FLP expression by heat treatment (see page 3, paragraph 2 of the revised Supplementary Methods, and Supplementary Figure S2A). This has also been noted by others (Fox et al., 2009). Therefore we are certain that above 99% of the observed clones were born in the first 48h after the FLP induction. This temporal offset alone is by and large insufficient to provide the *increasing* divergence in size that we observe later on (effectively spanning two orders of magnitude from day 5).

Second, there is the problem of the biological effect of the molecular marker that we use for lineage tracing, b-galactosidase/LacZ. There are two reasons to assume this marker is orthogonal to our experiment. (1) LacZ has been used for decades as both lineage and cell type marker in *Drosophila*, and in particular in *Drosophila* adult stem cell biology (Fox et al., 2009), and to our knowledge it has never been reported to cause damage nor elicit a specific response in the expressing cells. (2) In the particular context of the adult midgut, it is clear that the *esg-lacZ* marker that we and others use in the midgut (esg^{k00606}) expresses LacZ in all ISCs at levels much higher than the ones found in ISCs expressing the *tub-FRT-lacZ* lineage marker that we use in our tracing experiments. This strain is perfectly healthy and its tissue composition and structure is undistinguishable from tissue described in the literature as healthy and homeostatically normal. Therefore, it is very unlikely that the expression of the LacZ marker in the labelled lineages could induce a specific response in the ISCs at all, and much less so that it would lead precisely to clone size distributions with the characteristics of exponential shape, convergence and scaling.

Third, Referee #2 raises the possibility that the heterogeneity in clone size could reflect a heterogeneity in potency, now due to division rate, rather than fitness. To the clonal size distributions, it is the same: the observation of scaling behaviour rules out engrained heterogeneity and provides positive evidence for equipotency. Moreover, the composition of the clonal population offers additional evidence against invariant asymmetry at different rates. If that was the case, the presence of clones larger than the "proliferative unit" of the tissue (5-6 cells) would result in a reduction of the ISC fraction within the labelled population. By contrast, our data show that the labelled clones at day 5, when ~70% (!) of the clones are larger than 6 cells (and many comprising tens of cells), the whole of the labelled tissue is representative of the composition of the surrounding tissue (with 19% of cells being Delta+). This representativeness with clones larger than the proliferative unit is a strong indication that tissue homeostasis is achieved not by invariant asymmetry but by population asymmetry/neutral drift.

Finally, based on all the remarks made above, we believe that the only possible interpretation of the data, taken in full but in particular in relation to the observation of scaling, is what we defend in the paper: that homeostasis in the *Drosophila* adult midgut results from neutral competition, externally regulated, between symmetrically dividing ISCs. In particular, we believe that the alternatives offered by Referee #2, although potentially explanatory, must be ruled out once confronted with the data. Although the precise molecular mechanism that we propose (lateral inhibition) is not proven beyond the shadow of a doubt, it is the one most consistent with our data and the literature, and we provide additional evidence in support of this hypothesis in the revised manuscript (pages 13-14 and Figure 8A of the revised manuscript).

The last point, age of the flies has very important consequence on the numbers as they counted. In the main text, the authors rarely tell the age of the flies in the experiment. Although in the material and method they stated the age of flies used for experiments, they have to reiterate the age when they make arguments. For example, the results in figure 2 will be vastly different if one uses 5 days old versus 10 or 15 days old flies. This experimental condition should be mentioned as frequent as possible in the main text.

We agree with Referee #2 that the readership will have an interest in the precise age of the experimental flies and thank them for raising this point. We have provided a more explicit discussion regarding the selection of the time window for the sake of the non-expert reader (in page 5 paragraph 2 of the revised manuscript; in pages 2-3 of the revised Supplementary Methods), and the age of the flies is provided explicitly in the revised main text (wherever an experiment is presented).

REFEREE #3

In this manuscript the authors consider the adult Drosophila intestines detailing the expression pattern of well-established marker genes and analyzing the behavior of wild type lineage clones. None of the experimental tools available in Drosophila for perturbation are utilized. The central

advance claimed is that intestinal stem cells undergo symmetric division at a low frequency. The manuscript is clearly written and data is carefully documented. However, there is no perturbational analysis and most of the figures report findings that have been published previously. In this sense, essentially nothing shown here is new.

We thank Referee #3 for the compliments on the clarity of the manuscript and documentation of the data. Naturally, we completely disagree with the statement that "nothing shown here is new": we actually address for the first time whether, during homeostasis of the *Drosophila* posterior midgut, the balance between proliferation and differentiation is achieved on a lineage basis or on a populational basis, and find the latter to be the case (and in particular through interactions between proximate ISCs). Not only do we provide direct evidence for the necessary symmetric ISC divisions, we provide the appropriate phenomenological context to understand their significance. These findings alone are of great interest to the field and so it is acknowledged in recent studies. Moreover, we propose a molecular mechanism that might be the cause of the observed phenomenology, not only in the light of previous work in the field but, in the revised version, with additional evidence in support of it, based on genetic perturbations of Notch signalling.

Moreover, the authors present an iconoclastic view of stem cell regulation, however the issue of symmetric stem cell division has been raised many times in the past and has been successfully addressed experimentally (e.g. Xi and Spradling, 2000). Two studies from the Jones lab and one from the Bilder lab have now documented the role of symmetric ISC division in replacement, which were left uncited (McLeod et al., 2010; Wang et al., 2011; O'Brien et al., 2011).

We agree with Referee #3 in that the significance of symmetric stem cell division has been addressed successfully in the past *in other tissues and model organisms*. In the case of the *Drosophila* adult posterior midgut, we are not aware of any report addressing the issue. Of the three papers that Reviewer #3 says cover the issue of symmetrical ISC division (McLeod et al., 2010; Wang et al., 2011; O'Brien et al., 2011), only one actually provides direct evidence, as we do, for symmetrical ISC division during homeostasis (O'Brien et al., 2011 – which, incidentally, went online on the 28th of October, some 15 days after our paper went out on review!). The other two papers, both from the Jones lab, investigate the effect of cycles of starvation and re-feeding, and they report that, under re-feeding conditions, the ISC pool has the capacity to *expand* alongside the whole tissue. In particular, we find no reference in these two papers to the pattern of ISC turnover in *homeostasis*, nor to ISC *symmetric divisions* for that matter. Also, although O'Brien et al. (2011) document the existence of symmetric *multiplicative* ISC division during homeostasis, its potential significance for homeostasis is not addressed in their paper. Actually, O'Brien and colleagues identify this question as fundamental. Referring to the closing discussion of their paper:

> "The regular occurrence of fate symmetry and the ability of at least some stem cells to switch between symmetric and asymmetric modes raise the possibility that stochastic competition creates a pattern of neutral drift akin to the mouse small intestine (Simons and Clevers, 2011). Whether fate regulation is intrinsic or extrinsic to the dividing stem cell and how symmetric:asymmetric ratios are modulated during homeostasis and growth are key questions that emerge from our findings."

This issue is precisely the focus of our manuscript and we not only provide a substantial amount of evidence for symmetric divisions and their kinetics but also reveal a mechanism that reconciles them with the homeostasis of the tissue. In particular, our analysis of the clone fate data shows that, alongside symmetrical multiplicative ISC division, there are symmetric differentiating ISC divisions (of which we provide direct evidence as well) occurring with equal probability, and leading to (and, indeed, providing evidence of) neutral drift dynamics. Moreover, we are able to provide significant new insight into the "key question" concerning the pattern of ISC regulation, which implicates extrinsic factors controlling the balance between ISC proliferation and differentiation. This goes well beyond merely reporting the existence of symmetric divisions.

The authors attempt to import a new scientific lexicon into a fully mature field of stem cell niche biology (e.g. Symmetric stem cell division is an established mode of stem cell replacement. Recasting the phenomenology of stem cell replacement by symmetric division as "population asymmetry leading to neutral drift dynamics in which..." tells us little that is new about stem cells or how they behave.). This type of jargon should be minimized. Ultimately the key to broad scientific appeal is clarity.

We agree with Referee #3's appeal for clarity. The criticism, however, leaves us confused. It is not our intention to introduce either new, or obscure, terminology in the field, but we need a term for the objects of our study: whether the strategy of homeostatic maintenance in the *Drosophila* midgut is lineage-based or population-based. We adopt terminology that has been introduced well in the past (Watt and Hogan, 2000), widely used in population genetics and increasingly in stem cell biology (Clayton et al., 2007; Doupe et al., 2010; Fre et al., 2011b; Klein et al., 2010; López-García et al., 2010; Snippert et al., 2010), and discussed specifically in the context of stem cell biology (Klein and Simons, 2011), referring to the relevant publications in the manuscript. Moreover, "population asymmetry" and "neutral drift" refer to the *global population* behaviour during homeostasis. Such phenomenology cannot be recast only in terms of "stem cell replacement", as this refers to an individual *event* that can occur in various biological contexts (regeneration, aging, growth or normal homeostasis). Therefore, we maintain that the overall terminology used in the manuscript is appropriate. However, for the sake of the readership, we have re-examined the initial passages where these terms are introduced in the manuscript, in order to improve clarity (page 4, paragraph 2; page 5 paragraph 3; and page 7, paragraphs 1 and 2, of the revised manuscript).

Suggestions that could significantly improve the manuscript:

1. The analysis in Figs. 1 and 2 of the manuscript rely on widely used markers for the ISC lineage. The data show that esg+ cell nests are heterogenous and vary in their gene expression profiles. Yet the conclusion that esg+ nests vary in the content of ISCs and EBs cannot be rightly drawn from this data. While ISCs and EBs express these genes, it does not necessarily follow that these genes mark all/only ISCs and EBs. Only self-renewing, pluripotent lineages in the posterior midgut can be consider stem cells. In the end, the analysis presented does not distinguish the possibility that esg+ nests vary in ISC and EB content from the possibility that the transgeneic markers utilized simply have variable expression in midgut cells that does not correspond to stem cells and their daughters. The authors need to address this point in a convincing manner with additional experiments.

As Referee #3 points out above, we have made use of markers that have been widely used since their description by (Micchelli and Perrimon, 2006). As such, they have been used previously by others to estimate the census of ISCs and EBs (Buchon et al., 2010; Choi et al., 2011; Xu et al., 2011), and in particular they have been instrumental, in two of the papers that Referee #3 mentions above, for monitoring the size of the stem cell pool in non-homeostatic conditions (O'Brien et al., 2011; Wang et al., 2011). In particular, (Choi et al., 2011) use the *esg* and *Su(H)GBE* reporters to calculate the total amount of ISCs in the adult posterior midgut, finding numbers very similar to the ones we estimate (page 4, paragraph 4 of the revised Supplementary Methods).

Although in theory these markers could reflect the past activity of their promoters due to a prolonged half-life of the lacZ or GFP markers, this precaution does not seem to apply here for the following reasons. First, known markers of the differentiated EE and EC populations, such as Pros, Nub/Pdm1 or myoIA, are not found in the esg+ cells (which also comprises the Su(H)GBE+ population) in homeostatic conditions (Jiang et al., 2009; Lee et al., 2009; Micchelli and Perrimon, 2006). Second, in conditions of fast division such as Notch null clones, where the problem of lacZ perdurance would exacerbated, the partition of the mutant cells between esg-Z+ and Pros+ is strikingly mutually exclusive (see Supplementary Figure S4A-B in Lee et al., 2009). Therefore we think that it is perfectly valid to make a straightforward interpretation of the expression of these markers.

In addition the data panels in Figs. 1B and 2A-E are confusing and individual channels need to be shown in order to be convincing. The authors should show Arm and Pros channels separately and provide appropriate controls to demonstrate that Arm and Pros antigens are recognizing mutually exclusive cellular domains.

This is a very legitimate concern, and certainly showing the channels separately would improve the clarity of the figure. We have followed this advice and provided separate channels for all of them (for Figure 2A-E within the same figure, for Figure 1B in Supplementary Figure S1).

The controls that Referee #3 requests, all very sensible, are already provided in the literature, so additional experimentation is not necessary. Both Pros and Arm have been extensively used in the midgut literature, and it is clear from the published data that Arm localizes exclusively to the membrane and Pros exclusively to the nucleus in all contexts examined so far:

Nuclear localization of Pros, probed alone: (Amcheslavsky et al., 2011; Apidianakis et al., 2009; Bardin et al., 2010; Beebe et al., 2010; Biteau and Jasper, 2011; Lee et al., 2009; Lin et al., 2009; Micchelli and Perrimon, 2006; Ohlstein and Spradling, 2006; Shaw et al., 2010; Staley and Irvine, 2010).

Junctional localization of Arm, probed alone: (Amcheslavsky et al., 2009; Apidianakis et al., 2009; Buchon et al., 2010; Maeda et al., 2008).

In fact, it is not uncommon practice in the field to detect with the same fluorophore both Pros and Arm, and in all cases clear-cut results in the subcellular localization of both antigens are obtained: (Amcheslavsky et al., 2011; Biteau et al., 2008; Biteau and Jasper, 2011; Biteau et al., 2010; Ohlstein and Spradling, 2006).

2. Two problems plague the wild type lineage clone tracing analysis presented in the manuscript.

First, biologically inappropriate time points have been selected. Second, the analysis was performed in the context of tissue damage and not under baseline conditions. Thus, this analysis is confounded by a significant proportion of transient clones present in the interval chosen for analysis and stem cell clones responding to environmental perturbation. The authors need to analyze later time points (10-50 days after labeling) and employ a clone marking strategy that allows lineage labeling under normal homeostasis not following heat shock (e.g. the esg F/O method, Jiang et al., 2009), since the system has been shown to be highly dynamic in response to environmental transients.

More specifically, the clonal analysis performed in Fig. 3 A-E focused on 1, 2, 3, 4, 5, 8 and 16 day post heat shock time points. The methods state that data from "whatever clones were found" were acquired. Previous studies demonstrate that multiple classes of clones can be distinguished up to 10days following induction (Ohlstein and Spradling, 2006). Stem cell clones are distinguished from transient clones 7-10 days following induction. Therefore, data presented in Fig. 3 does not distinguish the kinetics of stem cell and non-stem cell (transient) clones in the midgut.

The analysis needs to be applied to the interval between 10 and 50 days in order to be relevant to ISCs. Moreover, these cannot rightly be considered "long term" studies of the ISC as the authors claim. The precedent in the field is 36-50 days (e.g. Margolis and Spradling, 1995, Xi and Spradling, 2000, Boyle et al., 2007, Wallengang and DiNardo, 2006). Symmetric division has been well documented to occur at later time points, so the potential effects of aging may not be a caveat until much later (e.g. Xi and Spradling, 2000; Biteau et al., 2008, 2010).

In addition the effects of pyloric scarring need to be functionally linked to ISC behavior, since this is invoked as an informative measure for the midgut but the reference cited pertains to the hindgut. In contrast to what is asserted, if we look at the measured data in Fig. 3A, B at the relevant time points to detect ISC clones (after 8 days) it appears that there is very little change in density or clone size between day 8 and 16 (Moreover, there is no comment in the deviation of the model from the actual observed 16 day time point.). Yet, the authors assert that there is a progressive expansion of clone size when the data shows that it plateaus by day 16. This disparity needs to be clearly addressed.

Under point no. 2, Referee #3 raises issues regarding three main themes: the appropriateness of the time window, the damage to the tissue resulting from the heat shock-based induction of the FLP,

and the pooling of all clones without regard of their origin. We cover the first two topics immediately below and the third one after point no. 3, which is related to that topic.

Time window

The age time window of our long-term lineage tracing starts at days 5-7 after eclosion and lasts until days 21-23 after emergence from the puparium. Recent work (O'Brien et al., 2011) indicates that there is a post-emergence maturation process of the midgut, whereby the naïve organ increases its size in response to the initial food intake. This process seems to last 3-4 days and is left out of our time window. Besides, work describing the aging of the *Drosophila* posterior midgut (mostly from the Jasper lab, and cited above by Referee #3) shows clearly that flies 30-60 days old consistently display clear phenotypes associated with aging, in particular a dysplastic, disorganized epithelium, excess of esg+ cells and misdifferentiation. However a milder increase in esg+ cells can be observed as early as 15 days after emergence (Biteau et al., 2008). Therefore, there are good reasons to believe that our timepoints 1, 2, 3, 4, 5 and 8 days after induction (corresponding, in experimental time window from induction to dissection, to 5-7 to 13-15 days after emergence), are exactly within the period when the tissue is expected to be strictly homeostatic in composition and size. It is however expected that some of the organs analyzed at time point 16 (age 21-23) are outside homeostasis.

For these reasons, it is clear that the time window proposed by Referee #3, up to 50 days of age, would not provide any information germane to the period of *homeostasis* in the posterior midgut. Even though that might be the standard for germ line stem cells as Referee #3 notes in the references provided.

As for the disagreement between some theoretical predictions and the data at time point 16, it is indeed explicitly mentioned in the main text of the original manuscript (page 11, paragraph 2), where the reader is referred to experimental procedures (page 17, paragraph 2) for more details, and also in the legend of Figure 3. Now we have also given to this matter a more prominent place in the discussion (pages 16-17 of the revised manuscript). We attribute the disagreement to the tissue not being homeostatic at that point, for the reasons given above and emanating from the literature. It was not possible, after the experiment, to re-stain those samples for probes that could reveal the dysplasia explicitly. But certainly the physical arrangement of nuclei in some areas of the experimental sample at day 16 looked dysplastic, and the degree of polyploidy in the tissue was certainly altered. However, in absence of specific staining, we could only rely on obvious histological marks to evaluate this possibility. Pyloric scars were simply the only available clue, and while they pertain to the hindgut they provide a measure of the progression of age in our experimental cohort and conditions, and are informative of the *general* intestinal homeostatic condition of the cohort. After all, it is difficult to imagine that a damaging agent could affect a tissue that is largely quiescent (hindgut) without eliciting any response in an upstream part (midgut) of the same tract – a part that by contrast, requires continuous renewal.

Tissue damage

We agree with Referee #3 that heat-shock treatments are potentially damaging and that this has not been thoroughly addressed in the literature, even though a vast deal of what is known about the posterior midgut is based on heat shock-induced wild-type and mutant lineages.

However, we did monitor the evolution of the size of the organs during the lineage tracing, and found that (a) the size corresponds to what is expected from the literature and (b) the size remains rather constant during the chase. We have now extended this observation by obtaining the cell density as well, during the first four days of the time course (enough to monitor the loss and recovery of up to 90% of the cells of the tissue after infection; Buchon et al., 2010) (see Supplementary Figure S6C). Together, the data indicate that the cellular complement of the tissue does not experience appreciable changes. This contrasts starkly with the expected behaviour of the tissue if the observed clonal growth was due to a regenerative response to damage. Given that the average size of the clones grows to 15-20 cells (three times the proliferative unit of the tissue), if such growth was a regenerative response to an increase in cell loss due to the heat shock, one would expect the midgut to have experienced a corresponding reduction in size, and then a recovery to its original size/cell density. This is not observed. Moreover, the growth of the clones seem to ramp up

only after two days after induction, with a rather slow growth before that moment. This does not seem to be behaviour associated with a regenerative crisis.

Finally, if the heat treatment elicited a regenerative crisis, one would expect the divisions of the ISCs shortly after the heat-shock to follow a non-neutral pattern (i.e. an excess of symmetric multiplicative divisions to amplify the pool of ISCs). We have performed such an experiment, and what we find is that very early clones, just 28h after induction, reveal a mixture of ISC divisions resulting in 2ISCs, 2EBs, ISC+EB in balanced combination and in proportions showing a good agreement with our theoretical prediction (pages 11-12 and Figure 6 of the revised manuscript).

Referee #3 suggests using a flip-out transgene together with the TARGET system (esg-Gal4, tub-Ga80ts, UAS-Flp). Unfortunately these techniques do not allow for induction at less than saturation density. If all ISCs and EBs were labelled, it would be simply impossible to discriminate the lineage potential of single, sparse ISCs, a condition fundamental to our analysis. It would be certainly interesting to be able to induce individual ISCs sparsely without a heat treatment, but presently there are no tools available for this purpose. Leaving aside the fact that there is no evidence for tissue damage with heat-shock, it therefore seems inappropriate to perform an experiment that, by construction, cannot give the required experimental output.

3. When plotted as a function of n/<n(t)>, the cumulative clone size distributions converge at early time points as seen in Fig. 3D. This analysis needs to be performed specifically for stem cell clones to exclude the early effects of transient clones in the analysis.

This is indeed an important point, which we discussed in the methods section (page 19, paragraph 1 of the original manuscript) and mentioned in the legend of Figure 3, but perhaps required a more prominent position. We thank Referee #3 for mentioning it and we have now made reference to this in the main text (page 8, paragraph 2 of the revised manuscript). The fact is that we had already taken that precaution specifically, simply by removing from consideration the single-cell clones. With this filter, the population of clones that we consider in our work is exactly equivalent to that identified as “stem cell clones” in the paper mentioned by Referee #3 (Ohlstein and Spradling, 2006).

4. The expression of Dl in Fig. 4 appears to be highly variable and indistinguishable from background in many cases. The authors need to distinguish the possibility that clone composition varies from the possibility that their Dl staining protocol itself is variable, supplying appropriate controls where needed. Moreover the black levels seem to have been brought down in Fig. 4B and brought up in Fig. 4C. Laser intensity levels for confocal acquisition and post processing in photoshop (or equivalent) must be identical to be convincing.

The expression of Delta is *intrinsically* variable, as described in the literature (Ohlstein and Spradling, 2007). However our acquisition conditions are indeed the same for all images. Appropriate controls are (1) the presence of Dl+ cells outside the clones, which highlights that there is no artifactual exclusion of anti-Dl antibody in the clones and (2) the fact that in our overall analysis the proportion of Dl+ ramps up to 19%, in perfect agreement with the fraction of ISCs described in the literature.

We have selected a new example for panel 4B and re-done the panels A-C from the raw data in *exactly* the following manner using ImageJ: raw confocal stacks were despeckled, z-projected using maximum intensity, and then normalized *linearly* to occupy the whole bit-depth of the image:

Ipost = (Ipre-min(Ipre))(max(Ipre)/max(Ipre)-min(Ipre))*.

As this transformation is linear this is perfectly admissible and it is common practice in image analysis. Despeckling is necessary for making the z-projection meaningful, given that the Dl vesicles are found only in particular planes in the z-series. We have also provided as Supplementary Movies S1-3 the animated z-series for the three clones, in the original *raw data*, save for showing the Dl channel also separate and the annotation of the Dl+ cells.

5. The authors suggest that the clone in Fig. 4F is undergoing symmetric duplication. The authors need to distinguish this from the possibility that the clone indicated is simply an asymmetric division in progress with an EB cell that has yet to differentiate. The fact that one of the cells in Fig. 4F' (red clone) has a larger nucleus than the other suggest that there has been an asymmetric division. If the argument is that the red and green clones are products of symmetric division, they need to convincingly rule out that this is the product of two independent events, for the twin spot marking system. In this case, if the products of symmetric division cannot migrate far from the site of the original stem cell, this should have the effect of distorting the overall distribution of stem cells in the midgut over time, which has never been reported. Please address these points.

We are surprised by this criticism, since Referee #3 states further above that ISC symmetric divisions have been documented by O'Brien et al. (2011), who use *exactly* the same technique displayed in Figure 4F.

Indeed, figure 4F show three contiguous cells labelled with CD8:GFP and two contiguous cells labelled with CD2:RFP, the two groups of cells abutting each other. A Supplementary Movie S4 displaying the animated z-stack is provided to clarify this point. The marker expression depends on the "twinspot MARCM" technique (Yu et al., 2009). Therefore, there are only two possible straightforward interpretations of this result. One, that they are twin clones (2R+3G) originated by the symmetric duplication of an ISC and the subsequent divisions of the resulting ISCs. Alternatively, two adjacent stem cells could have originated independently a pair of two-colour twinspots (1R+1G and 1R+2G) next to each other, in such a disposition that the non-sibling red cells and the non-sibling green cells appear together. Given the low density of induction with which we performed the experiment (documented in the new Supplementary Figure S3A, where a wider field of view for the same clone is provided; but also in general for this experiment in the original Figure 4D – now revised Figure 3D), the first one is the only plausible possibility and provides evidence for symmetric ISC multiplication, in agreement with the symmetric divisions reported by O'Brien et al. (2011) and corroborated by other two twinspot techniques in our study.

Referee #3 also asks about the potential distortion of ISC spatial distribution due to symmetric divisions. It is important to realize that, as pointed out in the original manuscript (in page 14, paragraph 2; in page 16 paragraph 2 of the revised manuscript), the equivalency of the ISC population means that also in subsequent divisions, all three fate outcomes are possible, and therefore one symmetric ISC duplication does not imply that there will be an inexorable, local increase in ISC density. Our analysis of the spatial localization of esg+/Su(H)GBE– cells clearly indicates that two ISCs can be found together (~19% of the nearest esg+ pairs are 2ISCs), but very rarely three (Figure 2F), and this is in agreement with Jiang et al. (2009), who report that ~20% of the Dl+ cells are found in clusters of two, while the others are isolated from other Dl+ cells.

6. The discussion of lateral inhibition as a potential mechanism of neutral competition is completely speculative and unsupported by any of the correlative data presented. Thus, it should be removed.

We disagree. Our discussion is based not only on what is known about Dl/N in the literature, but also in our finding of externally-driven neutral competition. This implies that the ISC population is equipotent and that fate allocation is correlated with the neighbours (therefore non-autonomously and therefore *after* division). Thus, our work provides *for the first time* evidence of the equivalence of the ISC offspring at birth (which conforms to the observed distribution of Notch in ISCs/EBs and Delta in newborn ISC daughters), a condition germane to the process of lateral inhibition. Moreover, in the revised manuscript we present additional evidence in support for our proposal, with the analysis of mild mutant conditions for *N* that reveal a bi-directionality of the signalling process (pages 13-14 and Figure 8A of the revised manuscript).

This is a matter of high interest in the field as it is not yet clear the mode of N/Dl interaction in this tissue, and a mechanism for symmetry breaking has not been identified. Therefore it is not pure speculation but, rather, very relevant.

APPENDIX

Relative weight of the invariant asymmetry model in the Drosophila literature addressing or mentioning the ISC/EB relationships

1. List of references *with a focus* on the posterior midgut (including reviews) that mention the ISC lineage

	Suggesting invariant asymmetry		*Suggesting* population asymmetry	
Reference	In cartoon	In writing	In cartoon	In writing
(Ohlstein and Spradling, 2006)	✓			
(Micchelli and Perrimon, 2006)				
(Ohlstein and Spradling, 2007)	✓			
(Lin and Xi, 2008)				
(Lin et al., 2008)				
(Lee et al., 2009)				
(Park et al., 2009)				
(Jiang et al., 2009)	✓			
(Amcheslavsky et al., 2009)	✓			
(Pitsouli et al., 2009)	✓			
(Apidianakis et al., 2009)				
(Karpowicz et al., 2010)	✓			
(Shaw et al., 2010)		✓ (1)		
(Ren et al., 2010)	✓	✓		
(Biteau et al., 2010)		✓ (2)		
(Zeng et al., 2010)	✓			
(Staley and Irvine, 2010)	✓	✓		
(Bardin et al., 2010)		✓ (1)		
(Beebe et al., 2010)	✓			
(Park et al., 2010)				
(Lin et al., 2010)				
(Hou, 2010)		✓ (2)		
(Wang and Hou, 2010)		✓		
(Karpowicz and Perrimon, 2010)		✓		
(McLeod et al., 2010)	✓			
(Xu et al., 2011)				
(Choi et al., 2011)	✓			
(Amcheslavsky et al., 2011)	✓			
(Biteau and Jasper, 2011)		✓ (2)		
(Jiang et al., 2011)	✓			
(O'Brien et al., 2011)				✓(3)
(Apidianakis and Rahme, 2011)		✓		
(Cordero and Sansom, 2011)		✓		

(Jiang and Edgar, 2011)	✓	✓	
(Fre et al., 2011a)	✓		
(Wang et al., 2011)			
	25 out of 36		1 out of 36

(1) using the formula "each division" to refer to an outcome of 1ISC + 1EB
(2) using explicitly the term "asymmetric division"
(3) discussed as a *possibility*

2. All statements in reviews mentioning the *Drosophila* ISC lineage

(Hou, 2010)

> During stem-cell division, ISCs divide asymmetrically to produce one new ISC (self-renewal) and one immature daughter cell, an enteroblast (EB), which further differentiates into an EC or a secretory ee cell.
>
> […]
>
> Possible Mechanisms for the Regulation of Asymmetric ISC Division: The Notch signal-transduction pathway plays a key role in regulating the asymmetrical division of ISCs
>
> […]
>
> Because the asymmetric division of ISCs is in some ways akin to the asymmetric divisions that occur in *Drosophila* sensory organ lineages, we considered whether the two systems might use similar regulatory mechanisms. […] Despite the similarity in the asymmetry of the Notch signal activation, however, there is no evidence for the asymmetric segregation of Neuralized and Numb in dividing ISCs […]
>
> […]
>
> Another possibility is that the asymmetric expression of Delta is responsible for the unidirectional Notch signaling and asymmetric ISC division
>
> […]
>
> Da is the furthest downstream of any molecule known to regulate ISC asymmetric division. […] In addition, other signal-transduction pathways may affect the asymmetric ISC division by regulating Da-interacting proteins or downstream genes.

(Wang and Hou, 2010)

> "During the stem cell division, ISCs divide asymmetrically to produce a new ISC and an immature daughter cell, an enteroblast (EB), which further differentiates into an EC or a secretory ee cell."

(Karpowicz and Perrimon, 2010)

> Lineage-tracing experiments in *Drosophila* have similarly revealed the presence of clones arising from ISCs [3] (Figure 1b). As in mice, such clones contain all of the different cells of the intestine; however, Drosophila clones are more discrete; only one stem cell is associated with each clone, and these appear to remain more or less separated from one another.
>
> […]

> Thus, ISC clones in *Drosophila* are thought to contain only one ISC and a mixture of other gut cell types. It is not yet clear whether the division of ISCs is asymmetric, as in dividing neuroblasts [10], or whether it is initially symmetric but then one daughter differentiates according to its position, as occurs in germline stem cells (GSCs) [11].

(Apidianakis and Rahme, 2011)

> Although asymmetric ISC divisions in the midgut produce transient cells called enteroblasts, these cells do not undergo further cell division and remain close to the ISC before maturation.
>
> […]
>
> For example, *Drosophila* homologs of mammalian disease-associated genes [e.g. Wnt/Wg and K- Ras/Ras1, which act as a driving force in intestinal tumorigenesis (Markowitz and Bertagnolli, 2009)] can be studied in terms of ISC symmetric divisions.

(Note the mention of symmetric divisions is in the context of cancerous mutations, therefore outside homeostasis)

(Cordero and Sansom, 2011)

> Recently the 'dogma' of intestinal stem cell biology, which assumes invariant asymmetric stem cell divisions, was challenged by two independent studies. Those reports showed that ISCs always divide symmetrically (Snippert et al. 2010; Lopez-Garcia et al. 2010). Such division will result in either two stem cells or two differentiated cells in a stochastic manner. Both events compensate for each other so that the number of stem cells is maintained over time. Thus, ISCs are maintained in a process of neutral drift between symmetrically dividing ISCs.

(Note the mention of symmetric divisions is in the context of mammalian ISCs)

(Jiang and Edgar, 2011)

> In the *Drosophila* midgut, differential Notch activation between the two daughter cells of an ISC division is generated by asymmetric Dl expression [18,22]. During mitosis, Dl is expressed in both daughter cells. However, just after the completion of the division, only one daughter cell retains punctuated Dl expression while the other daughter cell loses Dl expression but activates Notch target genes and reporters. It has been proposed that the more basally located cell retains Delta expression and self-renewing ISC identity, whereas the more apically localized daughter cell will lose Delta expression and become the committed progenitor EB [18]. The asymmetry of Dl expression between ISC and EB is further reinforced by the repression of Dl transcription in EBs. The precise mechanism for establishing asymmetric Dl expression in the midgut progenitors is still unclear and multiple models have been proposed [18,22].

(Fre et al., 2011a)

> Delta sends a signal from one daughter cell to activate the Notch transcriptional response in its adjacent sister cell, thereby promoting its commitment to differentiation, while the signal sending cell does not activate Notch and retains stem cell identity. The exact mechanisms allowing activation of Notch in one cell and preventing activation in the other cell are not fully understood.

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Editorial Decision 23 March 2012

Thank you very much for the revised study.
One of the original referees assessed the rather significant revisions and, on face value, offers now the necessary support for publication of your data.

Please allow me to congratulate to the study.

The editorial office will soon be in touch with necessary paperwork for official acceptance.

Yours sincerely,

Editor
The EMBO Journal