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Neutral competition of stem cells is skewed by proliferative changes downstream of Hh and Hpo

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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.)

Editor: Thomas Schwarz-Romond

1st Editorial Decision 20 December 2013

Thank you very much for submitting your study on neutral competition of stem cells in Drosophila testis stem cells for consideration to The EMBO Journal editorial office.

I do enclose consistent and considerate comments from two expert scientists that should enable revisions to eventually make the study suitable for publication in The EMBO Journal.

As you will see, both scientists recognize the merits analyzing the concept of neutral competition in the studied stem cell compartment, particularly using mutations known to influence cellular proliferation. Irrespective of this principle interest, both raise significant concerns with regard to

-data quantification (estimates versus direct counting, please see ref#1 point 3)
-formal (probably direct) proof for cell proliferation as the cause of increased cellular competitiveness (ref#2, point 4)
-experimental expansion to and integration of similar analyses in GSC-clones (ref2 point 3)
-rather more careful introduction of theoretical underpinnings that should include restructuring of the first figures;

Further, I would like you to really take on board and synthesize ref#1's comments on the underlying

rational of cell competition for the reported phenotypes and at least discuss/exclude possible alternative explanations.

I am fully aware that these are rather demanding, experimental additions. I am however certain, that these would result in a dramatically improved and this significantly stronger and informative study, as already indicated by some of the encouraging remarks.

We would therefore be pleased if you would be willing to invest the necessary time and efforts and accommodate this with additional time beyond our usual three month revision period.

Please get in touch with regard to feasibility and anticipated timeline for the necessary significant revisions (due to time constrains preferably via e-mail).

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

REFEREE REPPORTS:

Referee #1:

In "Neutral competition of stem cells is skewed by proliferative changes downstream of Hh and Hpo", Amoyel et al investigate stem cell population dynamics using a well established in vivo model (the Drosophila testis stem cell niche). In the first part of their manuscript, the authors present experimental data and theoretical modelling to support the claim that stem cells in the fly testis conform to a neutral competition (or neutral drift) model. According to this model, stem cells do not invariably undergo asymmetric divisions as once thought; instead, asymmetry is achieved at the population level through stochastic (yet balanced) asymmetric and symmetric divisions, such that a steady pool of stem cells is maintained because stem cells lost to symmetric differentiating divisions are replaced by stem cells that divide symmetrically to generate two stem cell daughters (and viceversa). In the second part of their manuscript, the authors explore and describe a series of genetic pathways and mechanisms that can provide stem cells with a competitive advantage in their "neutral" competition with wild type counterparts, allowing them to eventually take over the stem cell niche. As suggested by the title, the authors conclude that it is increased proliferation what underlies such advantage (as opposed to alternative possibilities, such as killing of wild type competitors or enhanced attachment to the niche).

The work presented in this manuscript is not exceedingly novel, either from a conceptual or an experimental point of view. Neutral drift as a paradigm for in vivo stem cell homeostasis is slowly emerging as the norm, and the idea of stem cells competing with one another for niche occupancy has been explored before, particularly in flies [1-4]. However, this manuscript discusses experimental data in a context that combines both concepts, by analyzing stem cell competition from a neutral drift standpoint. While the theoretical predictions of such competitions have been appreciated since the very formulation of the neutral drift paradigm (ie, no news there), the experimental evidence to support such predictions is still somewhat sporadic and anecdotal. For that reason, this work has the potential to be a significant step forward in the field. In addition, this manuscript establishes a new modelling paradigm for a system that has been (and will surely be) repeatedly and successfully used to investigate stem cells in vivo, which contributes to its significance.

In my opinion, however, some conclusions in the present version of the manuscript could use stronger experimental support. Likewise, I do think that the proposed theoretical framework needs significant revisions and/or clarifications. I hope that the authors find the following suggestions helpful to strengthen their message and conclusions.

General comments.

1) Title: Neutral drift is emerging as a common feature among diverse stem cell populations across tissues and species. However, it wouldn't be too surprising if it's achieved and regulated through very divergent and context-dependent mechanisms (on p16 the authors recognize, for instance, that

"...within the stem cell niche, two markedly different strategies for self-renewal are in use...") For that reason, it is probably fair that the title of the manuscript be modified to better reflect the (naturally) limited scope of the work. I would respectfully suggest that it be expanded to "Neutral competition among cyst stem cells in the Drosophila testis is skewed by proliferative changes downstream of Hedgehog and Hippo". Such modification could avoid the impression that the authors are trying to oversell the reach of their work by implying that it communicates a universal mechanism (proliferative advantage) and underlying genetic pathways (Hh and Hpo), when neither of which may apply to all stem cells. In addition, I am not familiar with EMBO Journal's policy on titles, but it may be appropriate to avoid the "Hh" and "Hpo" gene name abbreviations in consideration of readers outside the field.

2) One of my major concerns relates to the claim that increased proliferation is, as stated in the abstract and discussed throughout the manuscript, "the driving force behind [niche colonization]". Intuitively, I would imagine that increased proliferation is to the process of neutral drift as enzymatic catalysts are to biochemical reactions. Enzymes drastically accelerate a reaction, but they don't change the thermodynamic equilibrium between substrates and products. Analogously, I can see how increased proliferation rates may accelerate the outcome of neutral drift, but it shouldn't per se bias the partial contributions of faster and slower proliferating clones within the population. In other words, I can see that a faster proliferating stem cell clone may beat wild type cells to replacing a missing stem cell from the niche; but I would argue that, likewise, faster proliferating clones would also be more quickly lost from the niche as well.

In the end, it should balance out, except that more quickly.

The true reason behind colonization of the niche by mutant stem cells is that the drift is not neutral anymore; instead, it is (as the authors rightly point out) skewed or biased. It is this shift from neutrality in a mutant cell's chance to replace a missing stem cell that truly leads to niche colonization. The authors argue that "altering the rate of cell division skews the stochastic process of stem cell loss and replacement at the niche in favor of the faster proliferating CySCs" (p.13). In the last paragraph of the Discussion, the authors say "Our study exemplifies how harnessing the naturally occurring process of neutral competition endows a stem cell with greater competitiveness..." But the mutant cells are not engaging in neutral competition. They are twisting it, biasing it. The phrase should probably read "... how corrupting the naturally occurring process of neutral competition..."

The question is, of course, "How?". And the authors do not provide a clear explanation. If there is a straightforward (ie. mathematically tractable) connection, then the authors need to discuss it more clearly. How is proliferation rate related to ? In fact, if I am understanding their modelling correctly, it seems like the rate of proliferation was not factored in their equations. On p.12 the authors claim that CySC dynamics "... can be resolved analytically an depends on just two parameters: the loss/replacement rate, , of CySCs from the hub, and the total number of CySCs contacting the hub". When trying to fit the data for ptc clones to a modified competition model, the authors arbitrarily split the balanced loss/replacement rate onto an increased *(1+) rate of replacement and a decreased *(1-) rate of loss. If this can be achieved by simply accelerating the proliferation rate of a clone, then this needs to be properly modeled and reported.

On the other hand, I do believe and appreciate the reported data. Therefore, I'm not arguing against a connection between proliferation rate and the propensity of cells to undergo symmetric self-renewing divisions. But if there's no straightforward connection between the two, the manuscript would at least benefit from a thorough and informed discussion about potential mechanisms linking the cell cycle and the biased choice towards self-renewal.

3) Another important issue relates with the choice of inferring clone size as the ratio of marked Zfh-1+ multiplied by a constant (N=13). Why not count CySCs directly? The Materials and Methods section states that "CySCs were scored as Zfh-1+ or Tj+ cells one cell diameter away from the hub", but the only reported data with regards to CySC numbers (clone sizes) seems to have been estimated as indicated above instead of by direct counting. What would the data look like if GFP+/Zfh-1+ one cell diameter from the hub were directly counted instead of inferring them? Would the fitting to the models still hold? Or is direct counting grossly misleading of true CySC numbers? If so, how?

In either case, the manuscript would benefit from a direct comparison of the estimates obtained by both methods (counting cells 0 to 1-cell diameters away from the hub vs. calculating the rate of marked somatic cells normalized to 13). Indeed, the latter approach is particularly misleading when dealing with mutations that affect the rate of proliferation. If ptc mutant cells divide more frequently, then even "classical" asymmetric self-renewal/commitment CySC divisions would contribute several GFP+ cells to the pool of Zfh-1+ cells, biasing the proportion of GFP+/Zfh-1+ cells upwards and causing an overestimation of true CySC numbers. This needs to be properly addressed. For these reasons, directly counting CySCs seems easier and may be less misleading. The authors should provide stronger arguments for relying on indirect estimations.

In relation to this point, what was the average number of Zhf-1+ cells one cell diameter away from the hub in the Fig. 1C experiment? Conversely, were the GFP+ cells touching the hub always one cell-diameter away from it, or could you ever find Zfh-1+ cells that were further away and still projected membrane extensions to the hub?

4) Related to point 3: why do the authors rely on GSC counts as a measure of niche colonization in all of their following experiments?

Again, why not count marked CySCs directly? Or, if there are reasons against that approach, why not maintain the (% of GFP+/Zfh-1+ * 13) estimation instead? The authors performed a series of controls to support the conclusion that ptc mutant clones displace GSCs by competition. But the possibility remains for all the other experiments that there were cell-autonomous effects that may have biased (in either direction) the ability of GSCs to remain in contact with the hub (and therefore undermine the conclusions). For instance, anytime that FRT clones were induced, there must have been a given rate of germline clones generated as well, and the resulting genetic manipulations could affect GSCs directly. (In fact, germline clones can be seen throughout the images, with Fig. S4A being an extreme example in which, in my honest and respectful opinion, all GFP+ cells are also VASA+ and have a morphology consistent with that of germ
cells). The same applies, of course, to background genetic mutations against which the clones were generated. Lastly, and as rightly emphasized by the authors, "GSC loss is only observed once the majority of CySCs are replaced by ptc mutant CySCs (Fig. S3G)". If this applies to other genetic manipulations, then partial effects on niche colonization may be missed, because GSC loss is clearly a less sensitive read out of niche occupancy by CySCs.

5) There is a fundamental disconnection between the idea that mutant CySCs simply have a higher chance of replacing a vacant place at the hub left by a spontaneously differentiating CySCs (mutant or wt) and niche colonization. Given the author's argument that CySC behavior is governed by neutral competition, I can understand how mutant CySCs can take over the CySC niche in a "passive" manner, ie. by simply replacing spontaneously differentiating CySCs. But what about the GSCs? The data indicate that mutant CySCs end up outcompeting GSCs as well. Are the mutant CySCs also passively replacing GSCs that spontaneously left a vacancy at the hub, or are the mutant CySCs more actively pushing out the GSCs from the hub? The authors refer to previous work by others to mention that GSCs are also governed by neutral competition, and that GSCs can spontaneously leave the hub albeit at a slower rate. Are such rates such that they could account for outcompetition form the hub by mutant CySCs that will swiftly take their former spot?

Also, and in relation to the above, the authors say in their description of the model that "As a simplification, we do not attempt to correlate the fate behavior of CySCs with the neighboring GSCs". But if mutant CySCs are occupying spots that are left vacant by GSCs as part of a homeostatic loss of GSCs, I think this should be incorporated in a revised version of the model.

6) Related to point 1, and how different stem cells populations (in different organs and species) implement neutral competition, the manuscript would greatly benefit from acknowledging the work by Trian et al. [1] and contrasting their main conclusions with regards to stem cell competition strategies. Also, and since the use of mathematical models is beginning to emerge as a powerful approach in the field, this may be an appropriate time to, as much as possible, start comparing the the strengths and weaknesses of the mathematical approaches used.

Specific comments (in decreasing order of importance).

7) Fig. 1G,H: In these figures it might be a very useful comparison to show additional curves to show how the predictions for both parameters (average clone size and distribution of clone sizes) change when modifying the relevant parameters in the neutral drift model.

For instance, in Fig. 1G could use additional dashed curves corresponding to modified values of , N or time shift to contrast with the orange dashed line. This might give readers an idea of how sensitive the model is to variations in these parameters, and how tightly the data fit the predictions.

8) p.9 and Fig. S3G: "... the rate of loss of unmarked stem cells is higher when ptc mutant CySCs are present than when control marked CySCs are present [...] These results indicate that ptc mutant CySCs expand at the expense of their wild type neighbors" These claims need to be better supported. Fig. S3G should show average number of Zhf-1 positive cell/testis (marked or unmarked), some measure of variation and there should be a statistical analysis of the data. While it is true that the purple line has a steeper negative slope than the red line, it is hard to assess if such difference would be statistically significant. These data are another reason for seriously considering a whole re-analysis based on a direct measurement of Zfh-1+ cells (GFP+ or not) close to the hub. A visual approximation to the total number of Zfh-1+ cells (marked + unmarked) based on the lines in Fig. S3G reveals approximately the following values:

2dpci: control=45 (2+43), ptc=46 (4+42)
14dpci: control=45 (18+27), ptc=58 (36+22)

If we apply the approximation of CySCs as (fractions * 13), then we have to assume that testis harboring ptc mutant clones have more CySCs than those carrying wt clones, which goes against the author's assumption that testis homeostatically maintain a fixed number of CySCs, and that lost CySCs are "perfectly compensated" (p.12 SM) by replacing CySCs.

9) Fig. S2G: What was the statistical test used? (for GSC counts, which is a discrete variable, a non-parametric test should be used in place of a Student's t-test). Also, what are the error bars? (this should be indicated in the legend as well). If these data correspond to the values shown in Fig. S3G, I would be quite surprised that such a spread in GSC values (especially for the "ptc" sample) gives rise to such small error bars in Fig. 2G. If these are not the same datasets, the authors should explain the reason for the difference.

10) Fig. 3H,I: What are the error bars? What are the statistical tests used? Given the number of conditions in both tests (>2), and the fact that GSC counts are a discrete variable, a non-parametric ANOVA should be used. If in Fig. 3I a simple pairwise comparison (eg. Student's t-test or a non-parametric version) was used to conclude a statistically significant difference between ptc and ptc/Stat92E samples, then this should be revised to include a post-ANOVA analysis (eg. Dunn) for pairwise comparisons.

11) p.11, Fig. 3I: Without including GSC counts for wt clones in a Stat92E/+ background, the authors should refrain from talking about "enhancement" of the GSC loss phenotype following the induction of ptc mutant clones. If the wt;Stat92E/+ data looked like the green bar, then yes, it is appropriate to conclude an enhancement of the "ptc phenotype"; but if they were indistinguishable from the dark red bar, then one would have to conclude that Stat92E is epistatic to ptc. These data are necessary to conclude either way.

12) Fig. 6B: The legend should indicate the dpci corresponding to these values. Also, this experiment should include GSC counts in the corresponding genetic backgrounds in the absence of ptc clones (for instance, by looking at flies of the same genotype that are not heat shocked, or siblings lacking hs-Flp subjected to the same heat shock regime). This is important to see if the changes observed could not be attributed to differences in the baseline numbers of GSCs in each of the corresponding genetic backgrounds (like the authors did in Fig. 4I with the rhea[1] background). It may turn out that GSC counts are higher in this backgrounds regardless of the induction of ptc mutant CySCs.

13) Table S2: Here "CySC clones" means GFP+/Zfh-1+ cells one cell-diameter away from the hub? What were the percentages of testis with marked CySC clones at 2dpci? Although in some cases it is

hard to imagine, the clonal induction in a given genetic background may have been very low, partly explaining the drastic differences reported at 14dpci. Also, were there marked cyst cells at 14dpci? Data (and preferably images) showing this would support the idea that CySCs mutant for, say, Pten and Tsc1 existed at some point, but were lost to differentiation.

14) Fig. 3A: It may be informative to look at Stat92E levels in ptc mutant CySCs at later time points (8-12dpci?), because a reduced ability of ptc mutant CySCs to support neighbor GSCs may not become evident until later. If the authors claim that the GSC numbers in the presence of ptc mutant clones at 2dpci is comparable to controls, then it may help to look at time points closer to when GSC loss starts to become evident.

15) Fig. 4A: In Issigonis et al., the staining for bPS-integrin is of much higher quality than in this figure. It is very hard to see much bPS-integrin expression outside of the sheath in this image. Therefore, the author's conclusion that there are undetectable changes in bPS-integrin levels should be supported with better stainings and much higher magnification images (like those used in Issigonis et al).

16) Fig. S3C: The data do not seem to fit well the model. But if I understand correctly, the model was generated using parameters
calculated based on measurements with the FRT40A control clones. For instance, it was those data that were used to calculate a q=0.18, which was then used in modelling the FRT42D and FRT42D,ptc data. If this is correct, shouldn't the authors revise their calculation of q based on FRT42D data? If they did so, wouldn't their data in Fig. S3C fit more tightly the theoretical predictions?

17) p.14, Fig. 6A (and Fig. S1A): "... in ptc mutant CySCs PCNA-GFP is upregulated to the level observed in GSCs". I find that the PCNA:GFP expression is rather variable, with even some wt CySCs expressing as much as GSCs. Better images (definitely at higher magnification) and perhaps some sort of intensity quantification are needed to better support this claim.

18) p.7-8 (and further): The choice of "clone size" to refer to clonal CySCs may be slightly confusing. The term "clone size" is often used in closely related fields to refer to a total number of cells that includes stem (or progenitor) cells and all of their progeny. I would suggest that the authors consider revising the use of such terminology, and replace it by "number of labeled CySCs" or "clonal CySCs", or something that better reflects what is being represented. For instance, assuming that many readers may be biased to thinking of a CySC and all of its progeny when thinking of a "clone", the statement "Under the invariant asymmetry model, clone sizes and recovery rate should not change over time" may be particularly confusing at first.

19) Fig. 1C: Please indicate dpci for this figure.

20) Fig. 2A-D: There should be an indication of what the arrow means in the legend. There's one in the main text, but it's placed much later than the first reference to these images.

21) Fig. S3A legend: There should be an indication of what the dashed orange line is.

22) p.17: "Our results also show that the predominant force driving CySC maintenance is proliferation" Related to point 5 above, I think the authors should consider replacing "maintenance" with "niche colonization". While niche occupancy is a sine qua non condition for CySC maintenance, according to the authors' argument, the primary reason for a mutant (faster dividing) CySC to occupy a spot in the hub is that it was left vacant by a CySC (or GSC) that spontaneously differentiated. And THAT spontaneous loss of maintenance had nothing to do with proliferation rate (or it may have, we just don't know based on the presented data). Such revision would in my opinion better reflect the findings in this manuscript.

23) p.17: "...gain of Stat92E activity in CySCs should lead to expansion (not loss) of GSCs because JAK/STAT signaling in CySCs enables their extended niche function to support GSC self-renewal (Leatherman and Dinardo, 2008, 2010)". The authors might want to revise this statement. Notably, Leatherman and Dinardo report that restoring STAT activity in CySCs in an otherwise Stat mutant background, allowed CySCs to maintain GSCs that were no longer in contact with the hub. In other

words, if we applied the same criteria for GSC identity (ie. touching the hub) Leatherman and Dinardo actually reported GSC loss. So, maybe, one should expect GSC loss upon hyperactivation of JAK/STAT in CySCs (although it should be noted that the GSCs were mutant for Stat in Leatherman and Dinardo's experiments). In any case, this sentence should be more carefully phrased. As a side note, this comment leads to a whole different can of worms: whether or not the GSCs displaced by, say, ptc mutant or Yki overexpressing CySCs, can still function as GSCs despite their lack of attachment to the hub (much in the way reported by Leatherman and Dinardo). But that is a discussion that goes beyond the scope and conclusions from this paper and does not alter the author's conclusions about niche occupancy/colonization.

24) p.5: "We find that CySCs are lost and replaced stochastically" This is a slight overstatement. The data don't directly address this. The authors found that the behavior of the CySC clones is consistent with them being lost and replaced stochastically, as predicted by the neutral drift model. Consider revising.

25) p.5: "...patched (ptc), which encodes the Hh receptor (ref); loss of ptc causes constitutive activation of the pathway." This may sound counterintuitive for someone unfamiliar with the Hh pathway. Consider describing the pathway just a bit further (2-3 lines) for clarity.

26) p.7: "We reasoned that there are 3 possible outcomes..." These outcomes have been repeatedly described for other systems. The term "reasoned" seems therefore somewhat inappropriate. Consider revising.

27) p.19 (M&M): Is the anti-bGal from chicken? The most commonly used Cappel anti-bGal is from rabbit.

28) Some colons and semi-colons are misplaced in describing the genotypes in the supplementary material. Please revise carefully.

REFERENCES:

[1] Tian JP, Jin Z, Xie T. Mathematical model for two germline stem cells competing for niche occupancy. Bull Math Biol. 2012 May;74(5):1207-25.

[2] Issigonis M, Tulina N, de Cuevas M, Brawley C, Sandler L, Matunis E. JAK-STAT signal inhibition regulates competition in the Drosophila testis stem cell niche. Science. 2009 Oct 2;326(5949):153-6.

[3] Rhiner C, DÌaz B, Portela M, Poyatos JF, Fern·ndez-Ruiz I, LÛpez-Gay JM, Gerlitz O, Moreno E. Persistent competition among stem cells and their daughters in the Drosophila ovary germline niche. Development. 2009 Mar;136(6):995-1006.

[4] Jin Z, Kirilly D, Weng C, Kawase E, Song X, Smith S, Schwartz J, Xie T. Differentiation-defective stem cells outcompete normal stem cells for niche occupancy in the Drosophila ovary. Cell Stem Cell. 2008 Jan 10;2(1):39-49.

Referee #2:

In this interesting and unusual article, Amoyel, Simons, and Bach present novel results showing cell competition in the Drosophila testis. They show that the cytoplasts stem cells (somatic, CySC) are subject to neutral drift and that this can be biased by deleting the HH co-receptor Ptc, by over expressing the cell cycle regulators CycE and Stg, or by suppressing Hippo signaling. In this case the supercompetetive CySCs not only out-compete WT CySCs, but also displace germ line stem cells (GSCs). These are interesting results that should generate some excitement in the stem cell field, especially amongst those working on the Drosophila gonads. While the central story seems to be solidly supported, the manuscript has some issues with presentation as well as technique, and a

revision is in order, at least to improve the presentation and at best to improve the quality of the data presented. Details listed below:

1. The results section should be presented in the past tense, not the present tense. The experiments were done in the past, and they are over.

2. The section on neutral drift in WT CySCs and ptc mutatant CySCs (Figs 1, 2) severely neglects the theoretical treatment of the data. The theory of analysis is not discussed in any detail whatsoever, and most of the relevant figure material is relegated to the supplement (Figs S2, S3). The theoretical treatment needs to be described in much more detail the main text and the, and some of the data from figs S2 and S3 moved to the main figs. It should be much more clearly demonstrated how the data conforms to the neutral drift model, and why it does not conform to a model of invariant asymmetric stem cell division.

3. All the analysis in done for CySC clones, and none for GSC clones. The paper would be twice as good if GSC clones also showed neutral drift, cell competition, and competition with CySCs. Admittedly this would be a lot of work, but perhaps the authors would like to provide some data on the GSCs as well.

4. Nearly all the cell competition assays are indirect, in that the loss of GSCs is measured as a way of assaying super competition of mutant CySCs. This is not ideal. For some of the most important experiments, such as with CycE+Stg, and Hpo or Yki, direct measurements of cell competition should be done by clonal analysis, as it was for WT and ptc CySCs in Fig 1GH and 2EF.

5. The cell cycle analysis is not ideal (Fig 5). If the authors really wise to show that cell proliferation is sped up by Stg or CycE+Stg, then they should clonal express these genes and tally the numbers of cells per clone and calculate cell doubling times from the data. The EdU and Stg-GFP pictures provided are not so convincing (even though these genes have been shown to have strong proliferative effects in other Drosophila cell types).

6. This same criticism applies to the interpretation that ptc mutant cells outcompete their neighbors due to a more rapid cell cycle. The arguments are direct, and it would be very nice to see data that directly show an accelerated cell cycle in these ptc mutant cells. The data presented is all indirect. Given the well known effects of HH and Ptc signaling on cell identities and fates and patterning, the conclusion that ptc simply affects the cell cycle is a bit fishy. Even though the data are all consistent with this idea, and no effects on other processes such as cell growth and adhesion were observed, it seems likely that ptc could affects cell competition via something other than just the cell cycle.

Additional Author Correspondence 24 December 2013

Thank you for your considered response to the reviews of our manuscript.
We are happy to see that our manuscript was well received by the reviewers and that you would consider the study suitable for the EMBO Journal, with appropriate revisions. We thank the reviewers for their comments, which when addressed will greatly improve this study. We believe we can address many of the reviewers' concerns. However, some of the experiments required are by their nature very long, as detailed below. Thus we think that we would need more than 3 months for the revisions to the manuscript (for reference, obtaining and analysing the data for the FRT40 neutral clones presented in Figure 1 required 4-5 months of almost full-time experiments. While we realise we may not need such a complete data set with so many time points for the additional experiments, we still estimate that this time frame is what will be required). Furthermore, Reviewer 1 raises a number of good points for required controls (points 11 and 12) and requires additional data for a number of clonal analyses (point 13), which are relatively easy to address but require large numbers of experiments, and as such, are time-consuming.

Below, we answer the main points you and the reviewers raise, which we believe are valid, and answering them would improve this manuscript.
There are however reasons that may make it difficult to address all of these concerns, which we outline below.

-data quantification (estimates versus direct counting, please see ref#1 point 3)
We agree that the greatest flaws in our manuscript lie in the way clones are quantified, and in how stem cell competition is assessed (Referee 1 points 3 and 4, and Referee 2 point 4). There are two related points, one being how we count CySC clone sizes, and the other how we use GSC number as an indirect readout for CySC competitive behaviour.

For the first, we want to point out that it is extremely difficult to assess CySC number (in fact, we describe this in the very first section of the results). Position is an indicator, but does not correlate with niche contact (Fig. 1C, for instance, shows two labelled somatic cells that are positioned 1 cell diameter away from the hub, one of these cells contacts the hub (and is a CySC), the other does not). Thus we were able to infer the number of CySCs by relying on hub contact. However, it is extremely difficult to estimate which cells do contact the hub when all cell membranes are labelled (using a membrane marker for instance, or also when clones become so large that most somatic cells are labelled), as the membrane extensions that CySCs use to contact the hub are very thin and difficult to assign to one cell or another when many labelled cells are present. Thus, any measure that doesn't rely on using markers (as we do with Zfh1) becomes subjective, and not necessarily a truer reflection of CySC numbers than using Zfh1. We have however recently obtained a MARCM stock for FRT42 marked with a membrane-tagged GFP (as opposed to the nuclear GFP we used) and we will attempt to quantify clone sizes for /ptc/ and control clones using this method, or by estimating cells within 1 diameter of the hub, as suggested by Reviewer 1.

For the second, we agree that GSC number is an indirect readout, however it is well correlated with CySCs' competitive abilities (see Fig. S3G), and very robust. Considering the very large number of genotypes we examine and the fact that clone behaviour can only be sampled accurately by having very large sample sizes (>50 clones, which implies many more samples, considering clone recovery rates are not 100% and decline with time), it would require too many man-hours to quantify all our experiments using CySC numbers. We can however do so for a limited number of important genotypes (as we have already done for /ptc/ mutants), such as /hippo/ mutants (as suggested by Reviewer 2). These are very time-consuming experiments, over a long time-scale (at least 10 days to obtain adults, plus 14-28 days minimum after heat shock to obtain the desired time points), which need to be repeated as our previous experiments with /hippo/ clones used Traffic Jam and not Zfh1 as a marker, meaning we cannot use this data to estimate Zfh1-positive cell numbers.

Thus we believe we understand and we can attempt to answer these concerns, but the experiments required are not trivial, require a long time-frame, and the analysis may not be more informative/objective than our current data.

-formal (probably direct) proof for cell proliferation as the cause of increased cellular competitiveness (ref#2, point 4)

We are a bit puzzled as to what could be a more direct measurement of proliferation than S-phase index. We want to point out that /ptc/ mutant CySCs have been shown by another group to have increased M-Phase index (Michel et al Development 2012) and that Stg over-expression alone has been shown to increase proliferation of CySCs (Inaba et al Development 2011). Thus it has already been shown in the same cells as we study that both these factors regulate proliferation rates. However, we believe we can strengthen this aspect of the manuscript, in particular by demonstrating that Hippo mutant and CycE+Stg over-expressing cells do indeed have increased cell cycle. Further, we will quantify /hippo/ mutant clone growth relative to unmarked cells, to demonstrate that they do indeed outcompete wild type cells. We believe this will improve our manuscript, in particular we agree with the reviewer that the increased proliferation of Hippo mutant cells was implied and taken for granted, based on the role of Hippo in other tissues.

-experimental expansion to and integration of similar analyses in GSC-clones (ref2 point 3)
While we agree that quantitative analysis of GSC behaviour would be interesting, we believe that this lies beyond the scope of our work. In this manuscript, we attempt to understand the behaviour of CySCs through modelling and experimental manipulations. Although CySCs and GSCs share a niche, the behaviour of GSCs is quite different, as alluded to in our discussion. However, we should point out that extensive analysis of clone behaviours has been carried out by the Dinardo lab (Wallenfang et al Aging Cell 2006), and their conclusion is that "This tendency of a given unit to win out in a subpopulation while its frequency remains roughly constant over the whole population

is reminiscent of 'random drift' observed in population genetics", which suggests that neutral drift dynamics also apply to GSCs. However, the importance of symmetric division in GSCs (eg Yamashita et al Science 2003 etc), the fact that symmetric events are fairly rare (~20% of total live imaging performed by the Matunis lab (Sheng and Matunis, Development 2011)), and the lack of a requirement for the Hippo pathway (our study) suggest that proliferation is not the main driver of replacement in GSCs, the way it is in CySCs. A PhD student in my lab is currently exploring the mechanisms at work in GSCs that regulate their competitiveness for niche occupancy, however this work would form part of a separate manuscript and this student's PhD thesis, as it reveals an entirely different set of genes and mechanisms required in both populations.

-rather more careful introduction of theoretical underpinnings that should include restructuring of the first figures;

We agree that the theoretical model was introduced slightly rapidly.
This was done mainly as the novelty in our manuscript does not rely on the idea of neutral competition (as noticed by Reviewer 1), but on the exploitations of the predictions of this model. Thus, although we had to establish the neutral drift dynamics, this is simply as a foundation for the rest of the work. We understand however that we should rewrite and reorganise this section, and we agree that the manuscript would benefit from better description of the neutral and biased drift models.

If you are in agreement with our assessment of the work to be done, would it be possible to have 6 month's time to complete the experiments?

Thank you for your consideration of our manuscript.

Additional Editorial Correspondence

I am pleased that you are prepared to take the referee points to your heart and develop the most critical aspects much further. I also much appreciate that you aim for this relatively labor intensive and time consuming task and are thus happy to grant necessary time for execution (please see below).

I also realize that the complementary work on GSCs is well underway in your lab though the outcome possibly dedicated to a different paper. Although I can see the rationale of this from an author perspective, I am wondering whether in light of the anticipated timeframe for the CySC revisions, one may have to rethink presentation of these distinct, though still complementary projects? In other words, and depending on how these data unfold, direct comparison/cross-reference of some aspects of CySCs versus GSCs may at the end benefit from integrated presentation in a single study and I would be very much prepared to discuss this further down the line...

Irrespective of these considerations, you may also be pleased to learn that The EMBO Journal offers 'scooping protection' for papers formally under revision in a way that potential competing work published while you are working on the expansion of your dataset will not impinge/undermine the novelty of your study.

However, and I am sure you fully understand the logic here, this has to be a case-by case decision as we cannot afford to maintain such a policy to simply warrant endless revisions. I therefore like to encourage efficient proceedings of the proposed experimental routes and their timely pursuit to resubmit the paper possibly within the next 3-5 month please.

1st Revision - authors' response 06 June 2014

We are very grateful to both Reviewers for their insightful comments. In response to the issues they raised, we have substantially altered the revised manuscript. Specifically,

1) we have added three entire time courses (2,7,14,28 dpci) for control, *ptc* and *hpo* in which we directly count labeled CySCs (instead of relying on the previous indirect method, which is now in Supplementary Figures).
2) we have reduced our reliance on GSC counts as a readout of niche competition by CySCs in at least 2 instances.
3) we have provided many more details and better explanations for the biophysical modeling, including the assumptions and limitations of the model.
4) we have improved our statistical analyses and added controls for a number of figures.

In sum, in responding to the Reviewers' comments, we have greatly improved the manuscript and we hope that the Reviewers find the changes satisfactory.

Referee #1:

In "Neutral competition of stem cells is skewed by proliferative changes downstream of Hh and Hpo", Amoyel et al investigate stem cell population dynamics using a well established in vivo model (the Drosophila testis stem cell niche...Neutral drift as a paradigm for */in vivo/* stem cell homeostasis is slowly emerging as the norm, and the idea of stem cells competing with one another for niche occupancy has been explored before, particularly in flies [1-4]. However, this manuscript discusses experimental data in a context that combines both concepts, by analyzing stem cell competition from a neutral drift standpoint. While the theoretical predictions of such competitions have been appreciated since the very formulation of the neutral drift paradigm (ie, no news there), the experimental evidence to support such predictions is still somewhat sporadic and anecdotal. For that reason, this work has the potential to be a significant step forward in the field. In addition, this manuscript establishes a new modelling paradigm for a system that has been (and will surely be) repeatedly and successfully used to investigate stem cells */in vivo/*, which contributes to its significance.

1) Title: Neutral drift is emerging as a common feature among diverse stem cell populations across tissues and species. However, it wouldn't be too surprising if it's achieved and regulated through very divergent and context-dependent mechanisms (on p16 the authors recognize, for instance, that "...within the stem cell niche, two markedly different strategies for self-renewal are in use...") For that reason, it is probably fair that the title of the manuscript be modified to better reflect the (naturally) limited scope of the work. I would respectfully suggest that it be expanded to "Neutral competition among cyst stem cells in the */Drosophila/* testis is skewed by proliferative changes downstream of Hedgehog and Hippo". Such modification could avoid the impression that the authors are trying to oversell the reach of their work by implying that it communicates a universal mechanism (proliferative advantage) and underlying genetic pathways (Hh and Hpo), when neither of which may apply to all stem cells. In addition, I am not familiar with EMBO Journal's policy on titles, but it may be appropriate to avoid the "Hh" and "Hpo" gene name abbreviations in consideration of readers outside the field.

We agree that it would be nice to have a longer title that includes "*Drosophila* testis" and "Hedgehog" and Hippo" in the title. However, per *EMBO J* instructions to authors "the total length of the title should not exceed 100 characters (including spaces)." Our title is 93 characters with spaces, so unfortunately we cannot even write out Hedgehog and Hippo in the title (which would bring the length to 101 characters including spaces). If allowed by the Editor, we would be very happy to change the title to "Neutral competition among Cyst stem cells in the *Drosophila* testis is skewed by proliferative changes downstream of Hedgehog and Hippo", which is 135 characters including spaces.

2) One of my major concerns relates to the claim that increased proliferation is, as stated in the abstract and discussed throughout the manuscript, "the driving force behind [niche colonization]". Intuitively, I would imagine that increased proliferation is to the process of neutral drift as enzymatic catalysts are to biochemical reactions. Enzymes drastically accelerate a reaction, but they don't change the thermodynamic equilibrium between substrates and products. Analogously, I can see how increased proliferation rates may accelerate the outcome of neutral drift, but it shouldn't ***per se*** bias the partial contributions of faster and slower proliferating clones within the population. In other words, I can see that a faster proliferating stem cell clone may beat wild type cells to replacing a missing stem cell from the niche; but I would argue that, likewise, faster proliferating clones would also be more quickly lost from the niche as well.

In the end, it should balance out, except that more quickly.

We assume that proliferation and loss are not accelerated in the same way but rather that they are separable (i.e., cells are lost (displaced or otherwise) at the same rate, but replaced at a higher rate by clonally marked cells, giving them an advantage.) We think that instead of faster proliferating clones being lost more quickly, they produce more offspring. Having more offspring increases the chance of the clone replacing an unmarked neighboring cell. Under the analogy described by the reviewer, one would predict that slower growing clones would be retained longer than wild type or faster proliferating clones. Conversely, our model predicts the opposite, that slow proliferating stem cells are more likely to be lost from the niche. We show here that this is true for *yki* mutant CySC clones (which are lost from the niche (Fig. 8A,E,F)). The Yamashita lab has shown that *stg* mutant clones are also lost (Inaba et al, 2011). In a different but comparable system, the Kalderon lab has shown that in the ovary, follicle stem cells with lower CycE or Yki activity are also lost (Huang & Kalderon, 2014; Wang & Kalderon, 2009).

In summary, following this question raised by the reviewer, we have revised the text to explain more carefully the basis of the non-neutral model behavior, noting where the symmetry between wild type and *ptc* mutant CySCs is broken.

The true reason behind colonization of the niche by mutant stem cells is that the drift is not neutral anymore; instead, it is (as the authors rightly point out) skewed or biased. It is this shift from neutrality in a mutant cell's chance to replace a missing stem cell that truly leads to niche colonization. The authors argue that "altering the rate of cell division skews the stochastic process of stem cell loss and replacement at the niche in favor of the faster proliferating CySCs" (p.13). In the last paragraph of the Discussion, the authors say "Our study exemplifies how harnessing the naturally occurring process of neutral competition endows a stem cell with greater competitiveness..." But the mutant cells are not engaging in neutral competition. They are twisting it, biasing it. The phrase should probably read "... how /***corrupting***/ the naturally occurring process of neutral competition..."

We thank the reviewer for the suggestion. We have used the word "corrupting" in the Discussion of the revised manuscript on p. 23.

The question is, of course, "How?". And the authors do not provide a clear explanation. If there is a straightforward (ie. mathematically tractable) connection, then the authors need to discuss it more clearly. How is proliferation rate related to λ? In fact, if I am understanding their modelling correctly, it seems like the rate of proliferation was not factored in their equations. On p.12 the authors claim that CySC dynamics "... can be resolved analytically an depends on just two parameters: the loss/replacement rate, λ, of CySCs from the hub, and the total number of CySCs contacting the hub". When trying to fit the data for ptc clones to a modified competition model, the authors arbitrarily split the balanced loss/replacement rate λ onto an increased λ *(1+δ) rate of replacement and a decreased λ *(1-δ) rate of loss. If this can be achieved by simply accelerating the proliferation rate of a clone, then this needs to be properly modeled and reported.

On the other hand, I do believe and appreciate the reported data. Therefore, I'm not arguing against a connection between proliferation rate and the propensity of cells to undergo symmetric self-renewing divisions. But if there's no straightforward connection between the two, the manuscript would at least benefit from a thorough and informed discussion about potential mechanisms linking the cell cycle and the biased choice towards self-renewal.

In responding to this point, it is worth again recapitulating the basis of the modeling scheme, its limitations, and what it offers to the current study. In developing the biophysical model, our aim is to introduce the simplest model that is consistent with the experimental clonal fate data. In particular, our goal is to understand (a) whether the wild type clonal behavior is consistent with population asymmetric behavior of the CySC compartment, and (b) whether the *ptc* mutant experiences a survival bias over their wild type neighbors. Although the model, as defined, can address these questions, it cannot provide direct insight into the factors that effect loss and replacement, or the mechanism of bias. These are left to the subsequent biological assays.

To answer the points raised by the reviewer in turn, indeed, the clonal dynamics of the CySCs are insensitive to divisions that lead to asymmetric fate outcome, as such divisions will not change CySC number. Instead, clonal dynamics depends on the CySC loss/replacement rate and not the rate of CySC proliferation *per se*. Since the target of our study is to resolve evidence for neutral and non-neutral stem cell competition, this insensitivity is not in itself a limitation.

In refining the neutral drift model to accommodate a potential survival advantage, we allow for a disparity between the rate at which a *ptc* mutant CySC can replace a neighboring wild type CySC and the reverse. Without any loss of generality, we can parameterize these two rates by two independent parameters, the net loss/replacement rate, λ, and a relative bias, δ. This is quite generic and separate to the question of how these parameters may be related to a defined mechanism.

Then, if the loss rate of CySCs does not depend differentially on whether *ptc* is expressed, an increase of proliferation in the *ptc* mutant can confer a survival advantage. The argument is straightforward: in the race to replace the loss of a neighboring CySC (wild type or *ptc* mutant), a CySC that divides faster will have a greater chance of succession. (Note that the same argument would hold if cell division drove the displacement and differentiation of neighbors, regardless as to their expression of *ptc*.) Crucially, the theoretical analysis cannot provide insight into whether this is the correct mechanism. It can only record whether biased drift is consistent with the observed clonal dynamics. Other mechanisms could give rise to the same model behavior. It is therefore a question for the subsequent experimental analysis to determine

whether increased proliferation is implicated as a mechanism.

We acknowledge that these arguments were not marshaled clearly in the original version of the manuscript. We have therefore made revisions that we hope detail more clearly both the basis of the modeling scheme and its limitations.

3) Another important issue relates with the choice of inferring clone size as the ratio of marked Zfh-1+ multiplied by a constant (N=13). Why not count CySCs directly? The Materials and Methods section states that "CySCs were scored as Zfh-1+ or Tj+ cells one cell diameter away from the hub", but the only reported data with regards to CySC numbers (clone sizes) seems to have been estimated as indicated above instead of by direct counting. What would the data look like if GFP+/Zfh-1+ one cell diameter from the hub were directly counted instead of inferring them? Would the fitting to the models still hold? Or is direct counting grossly misleading of true CySC numbers? If so, how?

In either case, the manuscript would benefit from a direct comparison of the estimates obtained by both methods (counting cells 0 to 1-cell diameters away from the hub vs. calculating the rate of marked somatic cells normalized to 13). Indeed, the latter approach is particularly misleading when dealing with mutations that affect the rate of proliferation. If ptc mutant cells divide more frequently, then even "classical" asymmetric self-renewal/commitment CySC divisions would contribute several GFP+ cells to the pool of Zfh-1+ cells, biasing the proportion of GFP+/Zfh-1+ cells upwards and causing an overestimation of true CySC numbers. This needs to be properly addressed. For these reasons, directly counting CySCs seems easier and may be less misleading. The authors should provide stronger arguments for relying on indirect estimations.

In relation to this point, what was the average number of Zhf-1+ cells one cell diameter away from the hub in the Fig. 1C experiment? Conversely, were the GFP+ cells touching the hub always one cell-diameter away from it, or could you ever find Zfh-1+ cells that were further away and still projected membrane extensions to the hub?

We thank the reviewer for raising these important issues. In addressing them, we have made significant experimental and textual additions, and their incorporation has dramatically improved the study and our confidence in the results.

By way of background, we tried to use proteins expressed in CySCs as a means to directly count them. Ptc and Stat92E are not reliable as we show in Fig. 1B. Zfh1 labels 43±7 cells in the testis, but only 30% of them are in direct contact with the hub (Fig. 1C). When we randomly label the membranes of Zfh1-expressing cells using FRT^{82B} CD8-GFP MARCM for the data in Fig. 1C, we find that some of them residing 1-cell diameter away from the hub do not in fact contact the niche. [However, to address the last of the reviewer's points, all clones that do contact the niche reside immediately adjacent or 1-cell diameter away from the hub.] Therefore, having exhausted these easier approaches to directly counting CySCs in clones, we decided ultimately to use the proportion of Zfh1-positive cells as a less biased (albeit less direct) approximation of the true CySC pool.

Nevertheless, in response to these insightful comments, we repeated the **entire** clonal analysis for control, *ptc* and *hpo* clones at 2, 7, 14, 28 dpci using a membrane targeted CD8-GFP MARCM stock for FRT^{42D} (on which both *ptc* and *hpo* reside). This new stock allowed us to count directly the number of cells within the clone that contact the hub. These new results with FRT^{42D} CD8-GFP MARCM clones are now

presented in the main body text and in Figures 1, 2 and 7 with those obtained with the original FRT^{40A} and FRT^{42D} nls GFP MARCM stocks being moved to Figures S2 and S3. We then subjected these new control CD8-GFP MARCM data to quantitative analysis and found that they fit the model even better than the original control FRT^{40A} and FRT^{42D} nls GFP MARCM stocks. However, even though the new data have greatly improved our study, two caveats remain: (1) the number of CySCs that are not within the CD8-GFP clone has to be estimated by position since their membranes are not labeled and (2) once many cells around the niche are labeled like in *ptc* and *hpo* samples and at later time points in control, it becomes difficult to distinguish the membranes of individual cells. One consequence of this new, improved methodology is that the number of "CySCs" in the CD8-GFP analysis is greater than 13 and ranges from 16-21 in controls, which for the reasons just given is an over-estimation. Since we were aware of the caveats of the membrane MARCM labeling at the outset of this study, we had chosen to use the less biased (albeit less direct) method of counting Zfh1-positive cells and normalizing to 13 CySCs. However, this latter method has caveats, too, as the reviewer has correctly pointed out. Regardless, we now find very good agreement between the two methods and taken together, they allow us to draw conclusions about the behavior of CySCs.

As far as the modeling is concerned, we have chosen to use 13 as the number N of CySCs. By the nature of the dynamics, different values of N would be acceptable from the perspective of the clonal analysis (meaning that the fit to a model with a different N would generate quite reasonable agreement with the data). Indeed, according to the neutral drift dynamics, the effective control parameter is the ratio N^2/λ where λ is the CySC loss/replacement rate. It means that, for a larger N, we would require a proportionately larger λ. For example, if we take N=20 (see Figs. R1 and R2 below in response to point 7), the data would be consistent with a loss/replacement rate some $(20/13)^2 = 2.37$ times larger, and potentially in excess of the CySC division rate. Conversely, if not all of the cells anchored to the hub function as stem cells, or there was variable self-renewal potential according to particular location relative to the hub, then the number of "effective" CySCs may be smaller than 13 and the loss/replacement rate proportionately reduced (as an example, see a recent study on this issue (Ritsma et al, 2014)).

However, in summary, we emphasize that the goal of the modeling scheme is not to reach a definitive estimate for the stem cell number or their loss/replacement rate, but rather to find rigorous evidence for population asymmetry and neutral dynamics of the wild type system, and to use this as a platform to detect and study survival bias and niche competition in the *ptc* mutant.

Per the reviewer's suggestion, we now point out the advantages and drawbacks of each counting method in the results section of the revised manuscript (pp. 7-10).

4) Related to point 3: why do the authors rely on GSC counts as a measure of niche colonization in all of their following experiments?

Again, why not count marked CySCs directly? Or, if there are reasons against that approach, why not maintain the (% of GFP+/Zfh-1+ * 13) estimation instead? The authors performed a series of controls to support the conclusion that ptc mutant clones displace GSCs by competition. But the possibility remains for all the other experiments that there were cell-autonomous effects that may have biased (in either direction) the ability of GSCs to remain in contact with the hub (and therefore undermine the conclusions). For instance, anytime that FRT clones were induced, there must have been a given rate of germline clones generated as well, and the resulting genetic manipulations could affect GSCs directly. (In fact, germline clones can be seen throughout the images, with Fig. S4A being an extreme example in which, in my honest and respectful opinion, all GFP+ cells are also VASA+ and have a morphology consistent with that of germcells). The same applies, of course, to

background genetic mutations against which the clones were generated. Lastly, and as rightly emphasized by the authors, "GSC loss is only observed once the majority of CySCs are replaced by ptc mutant CySCs (Fig. S3G)". If this applies to other genetic manipulations, then partial effects on niche colonization may be missed, because GSC loss is clearly a less sensitive read out of niche occupancy by CySCs.

We thank the reviewer for this thoughtful comment. In addressing this issue, we have again strengthened the results as well as our confidence in the conclusions. In the revision, we have tried to reduce our reliance on indirect counting methods, in particular GSC numbers. We now report CySC numbers directly for *hpo* clones (Fig. 7D,E) and show through quantitative analysis that their behavior is consistent with a biasing of neutral drift dynamics.

As described in the preceding answer, obtaining CySC counts is not a trivial task. Moreover, even in cases of bias, clone size are always observed over a wide range, meaning that one needs large n values for every genotype, which is not feasible. For example, for a single time point (like 14 dpci), we need to scan and analyze at least 40 clones. Since clone recovery rate decreases as a function of time (Fig. S2G), we need to scan and analyze 2-3 times as many samples. Obviously, it would be very cumbersome to perform such analyses for all of the many genotypes we use (see the list of genotypes in the Supplementary Materials and Methods). By contrast, we have found counting GSCs to be a very robust and substantially less cumbersome readout of CySC competition. In fact, we stress that GSC number is a highly sensitive readout, as evidenced by the dominant suppression of niche colonization by *ptc* or *hpo* mutant CySCs when the genetic dose of *stg* is reduced (Figs. 6B,7F). This method of counting GSCs as a proxy for competition by CySCs has allowed us to screen through a relatively large number of candidate genes and potential cellular mechanisms, and we would not have been able to do so otherwise. However, using the improved FRT^{42D} CD8-GFP MARCM technology, we have found that heterozygosity for *stg* suppresses the number of *ptc* mutant CySCs (Fig. 6A) as well as the loss of GSCs (Fig. 6B) that we reported in the original submission. These new data give us confidence that we can use GSC number as a faithful readout of CySC competition. Thus, due to the perceptive comments from this reviewer, we have reinforced the central conclusion of this study, namely that accelerated proliferation is required to give CySCs a competitive advantage.

Regarding "the possibility remains for all the other experiments that there were cell-autonomous effects that may have biased (in either direction) the ability of GSCs to remain in contact with the hub (and therefore undermine the conclusions)", we and the Bökel lab have shown that loss and gain of Hh signaling has no discernible autonomous effect on GSCs (Amoyel et al, 2013; Michel et al, 2012). Similarly, *yki* gain and loss does not appear to perturb the male or female germ line cell-autonomously ((Huang & Kalderon, 2014; Sun et al, 2008) and this study). Additionally, we now control for all of the potential effects of the genetic background on GSCs (see also responses to points 11 and 12 below). Finally, we are aware that we are making clones in the germ line which unfortunately cannot be avoided by the heat-shock methodology we employed. However, we have re-analyzed the over-expression clones and counted clonal GSC number. When GSC loss occurs in competitive situations, we observed a loss of 35% of GSCs compared to controls. If clonal over-expression positively affects GSC retention [and therefore prevents them from being outcompeted by CySCs], more marked GSCs should be recovered. Conversely if clonal over-expression causes GSC autonomous displacement from the niche, we would expect to recover fewer labeled GSCs. We do not observe an increase in marked GSCs for DE-Cadherin or βPS-integrin compared to control. There is a slight (9%) but not statistically significant increase in GSCs expressing Hop but this would not compensate for the predicted loss of GSCs if Hop-expressing CySCs were competing with them. With regards to the latter prediction, over-expression of CycE+Stg in CySCs leads to loss of GSCs. However, within these samples we find the same proportion of labeled GSCs compared to control. This suggests that our manipulations are not affecting GSCs directly.

In Fig. S4A (now Fig. S5A), which shows dMyc-expressing MARCM clones, CySC clones are rarely recovered (see also Table S2). One of the few examples of a CySC clone over-expressing dMyc happened to be in a testis containing several marked GSCs.

5) There is a fundamental disconnection between the idea that mutant CySCs simply have a higher chance of replacing a vacant place at the hub left by a spontaneously differentiating CySCs (mutant or wt) and niche colonization. Given the author's argument that CySC behavior is governed by neutral competition, I can understand how mutant CySCs can take over the CySC niche in a "passive" manner, ie. by simply replacing spontaneously differentiating CySCs. But what about the GSCs? The data indicate that mutant CySCs end up outcompeting GSCs as well. Are the mutant CySCs also passively replacing GSCs that spontaneously left a vacancy at the hub, or are the mutant CySCs more actively pushing out the GSCs from the hub? The authors refer to previous work by others to mention that GSCs are also governed by neutral competition, and that GSCs can spontaneously leave the hub albeit at a slower rate. Are such rates such that they could account for outcompetition form the hub by mutant CySCs that will swiftly take their former spot?

Also, and in relation to the above, the authors say in their description of the model that "As a simplification, we do not attempt to correlate the fate behavior of CySCs with the neighboring GSCs". But if mutant CySCs are occupying spots that are left vacant by GSCs as part of a homeostatic loss of GSCs, I think this should be incorporated in a revised version of the model.

We are grateful to the reviewer for pointing these out. In the discussion of the revised manuscript, we have addressed the questions (pp. 22-23). Briefly we have speculated that increased proliferation leads to increased offspring, which because of their increased number have a higher probability of replacing a CySC that is lost to differentiation. We acknowledge that our results do not permit us to resolve whether the outcompetition of CySCs is due to passive replacement or active displacement but speculate that live imaging (which is beyond the scope of this study) might be able to distinguish these scenarios. Finally, regarding the correlation of behavior between CySCs and neighboring GSCs, we agree that this is a very interesting question that unfortunately we cannot answer at this time. We emphasize that the model is not designed to reveal mechanism, and if we were to pursue this question experimentally, we would need to observe stem cell replacement *in vivo* using live imaging, something we plan to do in the future but is beyond the scope of this paper.

6) Related to point 1, and how different stem cells populations (in different organs and species) implement neutral competition, the manuscript would greatly benefit from acknowledging the work by Trian et al. [1] and contrasting their main conclusions with regards to stem cell competition strategies. Also, and since the use of mathematical models is beginning to emerge as a powerful approach in the field, this may be an appropriate time to, as much as possible, start comparing the the strengths and weaknesses of the mathematical approaches used.

In the discussion on p. 22 of the revised manuscript, we have added text about the Tian et al paper. There is a major conceptual difference between the approach in the Tian study and the one implemented here. In our analysis, we are not trying to use the dynamics to resolve mechanism. Rather, we are asking what is the simplest model that is capable of capturing the clonal dynamics. We can infer that in wild type there is stochastic stem cell loss and replacement effected by neighboring cells, and that this process becomes biased in CySCs mutant for *ptc* or *hpo*. The model cannot tell us whether loss drives

replacement, or *vice versa*; nor can it reveal the biological or mechanistic origin of bias. Rather, these issues are left to the experimental methodology to resolve. By contrast the paper by Tian et al. sets out to introduce a more "physical" or "biological" model that attempts to synthesize some of the possible mechanistic constraints, viz. cell interactions with the niche and with each other, etc. This is a complementary approach. However, we note that any model that is regulated by local extrinsic cues and that respects homeostasis will converge onto the model implemented in this work. Therefore the question of which is "correct" is a redundant one. Rather, we are using our model as a platform to revisit mechanism experimentally.

Specific comments (in decreasing order of importance).

7) Fig. 1G,H: In these figures it might be a very useful comparison to show additional curves to show how the predictions for both parameters (average clone size and distribution of clone sizes) change when modifying the relevant parameters in the neutral drift model.

For instance, in Fig. 1G could use additional dashed curves corresponding to modified values of λ, N or time shift to contrast with the orange dashed line. This might give readers an idea of how sensitive the model is to variations in these parameters, and how tightly the data fit the predictions.

With respect to the neutral model, there are as discussed above two adjustable parameters, the total number of CySCs, which we take from experiment to be N=13, and the CySC loss/replacement rate, λ. In addition, to apply the model to the experimental data, we also have to set the induction frequency, q, of the CySC following heat-shock. Operationally, the induction frequency is determined for the primary clonal assay (the membrane marker) by measurement of the frequency of unlabeled testes, and then corroborated by comparison with measurements of the frequency of GFP-positive clusters at 2 dpci (Fig. 1H). Moreover, as shown in the figures for the mean fractions (orange dashed curves in Figs. 1G, S2E,F), the longer time clonal evolution 7 dpci and onwards, is rather insensitive to the initial labeling efficiency.

Although clonal evolution is predicted to depend sensitively on the individual parameters, N and λ, previous studies (Lopez-Garcia et al. 2010) have shown that the effective control parameter is the ratio N^2/λ, i.e. any adjustment of N or λ that keeps this ratio fixed will lead to a similar quantitative clone size dependence. Therefore, for given N, we can achieve a high degree of accuracy in estimating the loss/replacement rate. Unfortunately, as a corollary, the clonal data cannot provide an independent means to estimate N itself. Instead, we have to rely on detailed measurements of processes contacting the hub to estimate the CySC number.

To illustrate this point, we have included as a guide for the eye an auxiliary figure showing a fit to the control data for N=20, similar to the values found from the membrane-GFP assay. Taking a loss/replacement of $(20/13)^2 \times 0.84=1.99$ per day, one may see that the model provides a good agreement with the data (See Figs. R1, R2 below). (Here we have shown the predictions of the model with revised parameters as dashed lines.) We would be happy to include this plot in the manuscript if the reviewer thought it helpful.

In the light of the questions and comments on this point by the reviewer, we have sought to revise the main and supplementary text to emphasize more clearly the validity of the modeling scheme and the strategy used to fit the data.

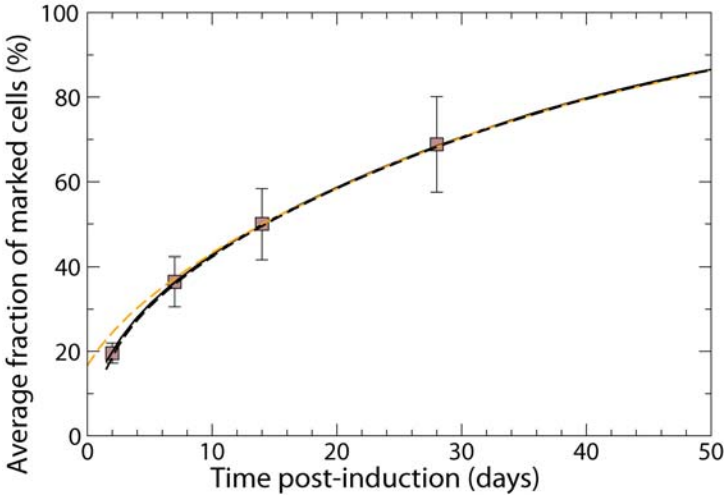

Fig. R1. Hypothetical modeling of variation of average size of control clones as a function of time using N=20. The dashed curve shows the model prediction with N=20 and a loss/replacement rate of 1.99 per day while the remaining curves and data are reproduced from Fig. 1G.

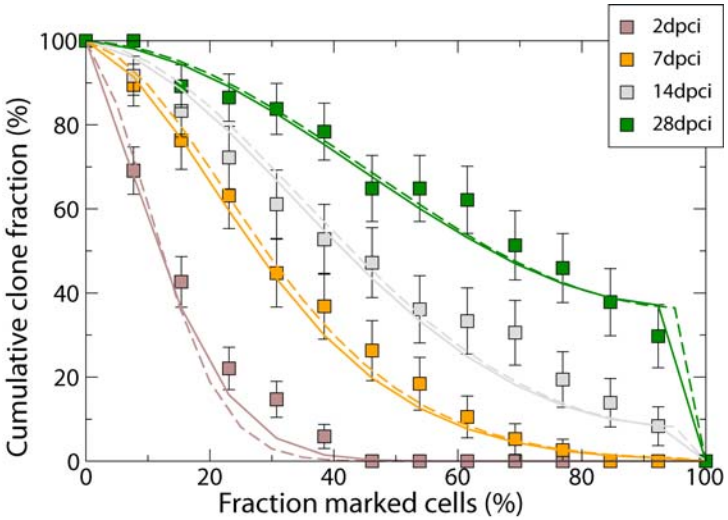

Fig. R2. Predicted clone size distributions using N=20. The dashed curves show the respective model predictions with N=20 and a loss/replacement rate of 1.99 per day while the remaining curves and data are reproduced from Fig. 1I.

8) p.9 and Fig. S3G: "... the rate of loss of unmarked stem cells is higher when ptc mutant CySCs are present than when control marked CySCs are present [...] These results indicate that ptc mutant CySCs expand at the expense of their wild type neighbors" These claims need to be better supported. Fig. S3G should show average number of Zhf-1 positive cell/testis (marked or unmarked), some measure of variation and there should be a statistical analysis of the data. While it is true that the purple line has a steeper negative slope than the red line, it is hard to assess if such difference would be statistically significant. These data are another reason for seriously considering a whole re-analysis based on a direct measurement of Zfh-1+ cells (GFP+ or not) close to the hub. A visual approximation to the total number of Zfh-1+ cells (marked + unmarked) based on the lines in Fig. S3G reveals approximately the following values:

2dpci: control=45 (2+43), ptc=46 (4+42)
14dpci: control=45 (18+27), ptc=58 (36+22)

If we apply the approximation of CySCs as (fractions * 13), then we have to assume that testis harboring ptc mutant clones have more CySCs than those carrying wt clones, which goes against the author's assumption that testis homeostatically maintain a fixed number of CySCs, and that lost CySCs are "perfectly compensated" (p.12 SM) by replacing CySCs.

We are grateful to the reviewer for pointing this out as we believe that using the method suggested by the reviewer has substantially increased the quality of our paper. Per the reviewer's suggestion and as noted above, we have repeated the *ptc* MARCM analysis (and appropriate control) with a CD8-GFP MARCM stock, which allows us to directly measure CySCs. We have replaced Fig. S3G with these new data, now presented in Fig. 2E, which show that there is a significant decrease in wild type (unmarked) CySCs in these samples. Furthermore, using this method, we find that although there is an apparent increase in total CySC number (marked+unmarked) in testes containing *ptc* mutant clones, this increase is fairly small (from ~17 in control to ~20 in *ptc* mutant). It would be possible to adapt a model that responds to this increase by accommodating a small degree of CySC replacement uncompensated by CySC loss, i.e. symmetrical division. However, since this adjustment is small (compare around 20 loss/replacement events per CySC over the time course with a fractional increase in CySC number of 10%), we do not believe that the incorporation of such an additional parameter into the model to capture this effect will provide additional insight. More importantly, with one further adjustable parameter, we do not believe that agreement of a revised model with the experimental data will prove compelling.

9) Fig. S2G: What was the statistical test used? (for GSC counts, which is a discrete variable, a non-parametric test should be used in place of a Student's t-test). Also, what are the error bars? (this should be indicated in the legend as well). If these data correspond to the values shown in Fig. S3G, I would be quite surprised that such a spread in GSC values (especially for the "ptc" sample) gives rise to such small error bars in Fig. 2G. If these are not the same datasets, the authors should explain the reason for the difference.

There was no Fig. S2G in the original manuscript and so we assume that the reviewer was referring to Fig. 2G. The data in Fig. 2G corresponded to the values in Fig. S3G. In Fig. 2G in the original manuscript we used the Student's t-test and the error bars were standard error of the mean (SEM). However, based on the insightful comment by the reviewer, we have changed the statistical test we used. In the revised manuscript, these data are now presented in Fig. 2F. We now use a non-parametric comparison test (*i.e.*, Mann-Whitney) and obtain similar trends and the differences between control and *ptc* samples are still significant. The error bars remain SEM, and this information has been updated in the Figure legend. Therefore, our conclusions remain unchanged.

10) Fig. 3H,I: What are the error bars? What are the statistical tests used? Given the number of conditions in both tests (>2), and the fact that GSC counts are a discrete variable, a non-parametric ANOVA should be used. If in Fig. 3I a simple pairwise comparison (eg. Student's t-test or a non-parametric version) was used to conclude a statistically significant difference between ptc and ptc/Stat92E samples, then this should be revised to include a post-ANOVA analysis (eg. Dunn) for pairwise comparisons.

11) p.11, Fig. 3I: Without including GSC counts for wt clones in a Stat92E/+ background, the authors should refrain from talking about "enhancement" of the GSC loss phenotype following the induction of ptc mutant clones. If the

wt;Stat92E/+ data looked like the green bar, then yes, it is appropriate to conclude an enhancement of the "ptc phenotype"; but if they were indistinguishable from the dark red bar, then one would have to conclude that Stat92E is epistatic to ptc. These data are necessary to conclude either way.

We answer #10 and #11 here. In Figs. 3H,I the error bars were SEM. We had used the Student's t-test, but based on the comment of the reviewer, we use a non-parametric ANOVA followed by pair-wise comparison with Sidak's multiple comparisons test. We now use these tests throughout the revised manuscript, except when we compare only two conditions [when we use the Mann-Whitney test as stated in the response to point #9]. This is now clearly stated in the Materials and Methods. We also now include GSC counts for control clones generated in a *Stat92E*/+ heterozygous background (control; *Stat92E*/+). Using the new statistical analysis, we find that (1) GSCs in control; *Stat92E*/+ samples is not significantly different from control, (2) the difference between *ptc* and controls are still significant but (3) GSC number in the *ptc*;*Stat92E*/+ samples is significantly different from *ptc* ($P<0.008$). These data are presented again in Figs. 3H,I in the revised manuscript.

12) Fig. 6B: The legend should indicate the dpci corresponding to these values. Also, this experiment should include GSC counts in the corresponding genetic backgrounds in the absence of ptc clones (for instance, by looking at flies of the same genotype that are not heat shocked, or siblings lacking hs-Flp subjected to the same heat shock regime). This is important to see if the changes observed could not be attributed to differences in the baseline numbers of GSCs in each of the corresponding genetic backgrounds (like the authors did in Fig. 4I with the rhea[1] background). It may turn out that GSC counts are higher in this backgrounds regardless of the induction of ptc mutant CySCs.

We are very grateful to the reviewer for pointing out the need to have control clones in the relevant heterozygous backgrounds, as this has resulted in the identification of a false positive (which was predicted by the reviewer). To address this important point, we generated control clones in *Akt1*/+, *InR*/+, *S6k*/+, *CycA*/+, *E2f*/+ and *cdk2*/+ backgrounds and counted GSC numbers in each cohort. In the case of $CycA^{H170}$/+, we observed an increase in GSC number in testes with control clones (from 14.1 to 16.8). This result prevents us from drawing any conclusions about the interaction between *ptc* and *CycA*, so we have removed this from the revised manuscript. The other new data have been added to Fig. 6C. Even though *CycA* was not informative when the appropriate control was performed, we think that the main conclusion - that genes involved in cell cycle control affect the ability of *ptc* mutant CySCs to outcompete GSCs - remain valid.

13) Table S2: Here "CySC clones" means GFP+/Zfh-1+ cells one cell-diameter away from the hub? What were the percentages of testis with marked CySC clones at 2dpci? Although in some cases it is hard to imagine, the clonal induction in a given genetic background may have been very low, partly explaining the drastic differences reported at 14dpci. Also, were there marked cyst cells at 14dpci? Data (and preferably images) showing this would support the idea that CySCs mutant for, say, Pten and Tsc1 existed at some point, but were lost to differentiation.

We have added clone recovery rates for 2 and 7 dpci to Table S2 for each of the six genotypes. Indeed, we recover CySC clones at 2 dpci in all genotypes at roughly comparable rates. At 7 dpci, we recover few differentiating cyst clones and at 14 dpci there were very few or no differentiating labeled cyst cells. We have added a new supplementary figure (Fig. S6) which shows representative images of control, *Pten* and *Tsc1* clones at 2 and 7 dpci as well as a graphical representation of Table S2.

14) Fig. 3A: It may be informative to look at Stat92E levels in ptc mutant

CySCs at later time points (8-12dpci?), because a reduced ability of ptc mutant CySCs to support neighbor GSCs may not become evident until later. If the authors claim that the GSC numbers in the presence of ptc mutant clones at 2dpci is comparable to controls, then it may help to look at time points closer to when GSC loss starts to become evident.

Stat92E levels in *ptc* mutant CySCs is unchanged at 7 and 14 dpci. In the revised manuscript we have added a new supplementary figure (Fig. S4) with representative images to show this.

15) Fig. 4A: In Issigonis et al., the staining for bPS-integrin is of much higher quality than in this figure. It is very hard to see much bPS-integrin expression outside of the sheath in this image. Therefore, the author's conclusion that there are undetectable changes in bPS-integrin levels should be supported with better stainings and much higher magnification images (like those used in Issigonis et al).

We have repeated the βPS integrin staining and obtained better quality results, which have been added to the revised manuscript in place of the original ones (Fig. 4A,A'). We still do not see an increase in βPS integrin at the hub-CySC interface in *ptc* mutant clones compared to control.

16) Fig. S3C: The data do not seem to fit well the model. But if I understand correctly, the model was generated using parameters calculated based on measurements with the FRT40A control clones. For instance, it was those data that were used to calculate a q=0.18, which was then used in modelling the FRT42D and FRT42D,ptc data. If this is correct, shouldn't the authors revise their calculation of q based on FRT42D data? If they did so, wouldn't their data in Fig. S3C fit more tightly the theoretical predictions?

In analyzing the data, we did in fact make use of the FRT^{42D} control to set the induction frequency of the FRT^{42D} *ptc* mutant. This translated to a figure of around q=0.3. However, as discussed in the text, the long-term behavior of the clone size distribution is rather insensitive to the particular value of q. Rather, the variability of the fits are more likely to reflect the statistical noise in the measurements. Indeed, we note that the new membrane-marker based clonal assay does indeed provide a very good agreement between theory and experiment for both the wild type testis and the *ptc* mutant.

17) p.14, Fig. 6A (and Fig. S1A): "... in ptc mutant CySCs PCNA-GFP is upregulated to the level observed in GSCs". I find that the PCNA:GFP expression is rather variable, with even some wt CySCs expressing as much as GSCs. Better images (definitely at higher magnification) and perhaps some sort of intensity quantification are needed to better support this claim.

We repeated the experiment and added new data scanned at higher magnification to the revised manuscript (in Fig. 5D-D'"). The new data show a wild type CySC and a *ptc* mutant CySC in the same plane and the single channel reveals that *PCNA-GFP* is more highly expressed in the *ptc* mutant clone than in control. We also quantified fluorescence intensity in control or *ptc* clones (Fig. 5F) and find a significant increase in *ptc* clones (using the Mann-Whitney test).

18) p.7-8 (and further): The choice of "clone size" to refer to clonal CySCs may be slightly confusing. The term "clone size" is often used in closely related fields to refer to a total number of cells that includes stem (or progenitor) cells and all of their progeny. I would suggest that the authors consider revising the use of such terminology, and replace it by "number of labeled CySCs" or "clonal CySCs", or something that better reflects what is being represented. For instance, assuming that many readers may be biased to

thinking of a CySC and all of its progeny when thinking of a "clone", the statement "Under the invariant asymmetry model, clone sizes and recovery rate should not change over time" may be particularly confusing at first.

We thank the reviewer for pointing this out. We agree that this is potentially confusing to readers and have changed the terminology to "the number of labeled CySC" in the revised manuscript.

19) Fig. 1C: Please indicate dpci for this figure.

We have added the dpci to the figure.

20) Fig. 2A-D: There should be an indication of what the arrow means in the legend. There's one in the main text, but it's placed much later than the first reference to these images.

We have corrected this in the revised manuscript.

21) Fig. S3A legend: There should be an indication of what the dashed orange line is.

The dashed orange line represents the predicted clonal evolution if only a single clone were induced at some earlier time with the same set of parameters. By adjusting the time-shift appropriately (as recorded in the figure captions), one may note that the clone sizes observed from multiple independent induction events and from a single induction event would converge rapidly. We believe that this is a useful point of reference as the overall clonal behavior can then be compared with the analytical results that can only be obtained for single clone induction, and are presented in the supplementary materials. We have clarified this in the revised manuscript in the legend of Figures 1, 2, 7, S2, S3.

22) p.17: "Our results also show that the predominant force driving CySC maintenance is proliferation" Related to point 5 above, I think the authors should consider replacing "maintenance" with "niche colonization". While niche occupancy is a sine qua non condition for CySC maintenance, according to the authors' argument, the primary reason for a mutant (faster dividing) CySC to occupy a spot in the hub is that it was left vacant by a CySC (or GSC) that spontaneously differentiated. And THAT spontaneous loss of maintenance had nothing to do with proliferation rate (or it may have, we just don't know based on the presented data). Such revision would in my opinion better reflect the findings in this manuscript.

In the revised manuscript, we have replaced "maintenance" with "niche colonization" as per the reviewer's suggestion.

23) p.17: "...gain of Stat92E activity in CySCs should lead to expansion (not loss) of GSCs because JAK/STAT signaling in CySCs enables their extended niche function to support GSC self-renewal (Leatherman and Dinardo, 2008, 2010)". The authors might want to revise this statement. Notably, Leatherman and Dinardo report that restoring STAT activity in CySCs in an otherwise Stat mutant background, allowed CySCs to maintain GSCs that were no longer in contact with the hub. In other words, if we applied the same criteria for GSC identity (ie. touching the hub) Leatherman and Dinardo actually reported GSC loss. So, maybe, one should expect GSC loss upon hyperactivation of JAK/STAT in CySCs (although it should be noted that the GSCs were mutant for Stat in Leatherman and Dinardo's experiments). In any case, this sentence should be more carefully phrased. As a side note, this comment leads to a whole different can of worms: whether or not the GSCs displaced by, say, ptc mutant

or Ykioverexpressing CySCs, can still function as GSCs despite their lack of attachment to the hub (much in the way reported by Leatherman and Dinardo). But that is a discussion that goes beyond the scope and conclusions from this paper and does not alter the author's conclusions about niche occupancy/colonization.

As the reviewer points out, CySCs with sustained JAK/STAT signaling can support *Stat92E* deficient GSCs that lose adhesion to the niche (Leatherman and Dinardo 2010). However, they earlier showed that sustained JAK/STAT pathway activation in CySCs in an otherwise wild type background resulted in expansion of both CySCs and GSCs (Leatherman and Dinardo 2008), which is different from what we report in the current study. In order to avoid any confusion, we have rephrased the highlighted sentence on p. 23.

24) p.5: "We find that CySCs are lost and replaced stochastically" This is a slight overstatement. The data don't directly address this. The authors found that the behavior of the CySC clones is consistent with them being lost and replaced stochastically, as predicted by the neutral drift model. Consider revising.

We have changed the sentence according to the reviewer's suggestion.

25) p.5: "...patched (ptc), which encodes the Hh receptor (ref); loss of ptc causes constitutive activation of the pathway." This may sound counterintuitive for someone unfamiliar with the Hh pathway. Consider describing the pathway just a bit further (2-3 lines) for clarity.

We have explained in more detail how Hh signaling is inhibited by Ptc, on p.11 of the revised manuscript.

26) p.7: "We reasoned that there are 3 possible outcomes..." These outcomes have been repeatedly described for other systems. The term "reasoned" seems therefore somewhat inappropriate. Consider revising.

We have changed this sentence.

27) p.19 (M&M): Is the anti-bGal from chicken? The most commonly used Cappel anti-bGal is from rabbit.

The antibody we used in these studies is Chicken anti-Beta gal polyclonal from Immunology Consultants lab (Catalog number CGAL-45A-Z). We erroneously reported this reagent was from Cappel and have corrected it in the revised manuscript.

28) Some colons and semi-colons are misplaced in describing the genotypes in the supplementary material. Please revise carefully.

We have updated the list of genotypes in the Supplementary Materials and Methods to reflect the changes we have made to the manuscript. We have also corrected any errors we have found in the original manuscript.

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Referee #2:

In this interesting and unusual article, Amoyel, Simmons, and Bach present novel results showing cell competition in the Drosophila testis. They show that the cytoplasts stem cells (somatic, CySC) are subject to neutral drift and that this can be biased by deleting the HH co-receptor Ptc, by over expressing the cell cycle regulators CycE and Stg, or by suppressing Hippo signaling. In this case the supercompetetive CySCs not only out-compete WT CySCs, but also displace germ line stem cells (GSCs). These are interesting results that should generate some excitement in the stem cell field, especially amongst those working on the Drosophila gonads. While the central story seems to be solidly supported, the manuscript has some issues with presentation as well as technique, and a revision is in order, at least to improve the presentation and at best to improve the quality of the data presented. Details listed below:

1. The results section should be presented in the past tense, not the present tense. The experiments were done in the past, and they are over.

We double checked the *EMBO J* Instructions to Authors, and we could find nothing about the tense that should be used in the results section. In previous papers, we have used the past tense to describe the experiment that was performed and the present tense for the conclusions, for instance "We generated X mutant clones." "These clones colonize the niche at the expense of wild type cells." As such, we have maintained this style in the revised manuscript.

2. The section on neutral drift in WT CySCs and ptc mutatant CySCs (Figs 1, 2) severely neglects the theoretical treatment of the data. The theory of analysis is not discussed in any detail whatsoever, and most of the relevant figure material is relegated to the supplement (Figs S2, S3). The theoretical treatment needs to be described in much more detail the main text and the, and some of the data from figs S2 and S3 moved to the main figs. It should be much more clearly demonstrated how the data conforms to the neutral drift model, and why it does not conform to a model of invariant asymmetric stem cell division.

We thank the reviewer for raising the issue of the clarity of presentation of the model and the fit of our data to neutral drift (as opposed to invariant asymmetry). In the revised manuscript, we explain the neutral drift model in more detail in the results section on pp. 8-9. In addition, we now clearly state on p. 8 why our results are not compatible with the invariant asymmetry model. Per the reviewer's suggestion to move supplemental items to the main body, in the revised manuscript, we have moved the schematic of CySC outcomes from Fig. S2A to Fig. 1A. Since we have added a substantial amount of data for control and *ptc* clones using a CD8-GFP MARCM stock to the revised manuscript (see answer to Reviewer 1 #3) as a figure comprised of entirely new data, we have decided not to move any more data from Fig. S2 and S3 to

the main body. Through the presentation of the new MARCM data, we now explain in much greater detail how our data conform to neutral drift and not to invariant asymmetry (see pp. 7-10).

3. All the analysis in done for CySC clones, and none for GSC clones. The paper would be twice as good if GSC clones also showed neutral drift, cell competition, and competition with CySCs. Admittedly this would be a lot of work, but perhaps the authors would like to provide some data on the GSCs as well.

While we agree that quantitative analysis of GSC behavior would be interesting, we believe that this lies beyond the scope of the current paper. In this manuscript, we attempt to understand the behavior of CySCs through experimental manipulations and modeling. Although CySCs and GSCs share a niche, the behavior of GSCs is quite different, as alluded to in our discussion. In fact, the two pathways that control CySC self-renewal and niche competition - Hippo and Hedgehog - do not have any perceptible role in GSCs in the testis (Sun, 2008; Michel, 2012; Amoyel, 2013; and this study). As such, we used these two pathways as tools to dissect the competitive mechanisms in CySCs. A similar undertaking for GSCs would require an entirely new repertoire of genes and would dilute the message we are trying to make in our study of somatic stem cells. It is worth pointing out that extensive analysis of clone behaviors has been carried out by the Dinardo lab (Wallenfang et al, 2006), and their conclusion is that "This tendency of a given unit to win out in a subpopulation while its frequency remains roughly constant over the whole population is reminiscent of 'random drift' observed in population genetics", which suggests that neutral drift dynamics also apply to GSCs. Therefore, the Dinardo lab has already proven that GSCs exhibit behaviors consistent with neutral drift.

4. Nearly all the cell competition assays are indirect, in that the loss of GSCs is measured as a way of assaying super competition of mutant CySCs. This is not ideal. For some of the most important experiments, such as with CycE+Stg, and Hpo or Yki, direct measurements of cell competition should be done by clonal analysis, as it was for WT and ptc CySCs in Fig 1GH and 2EF.

We thank the reviewer for pointing this out, as we think that answering this has substantially improved our study. We have addressed this point in our answer to Reviewer 1 #4. To summarize, we have added a full time course of *hpo* mutant clones and counted the number of CySCs in CD8-GFP MARCM clones at 2, 7, 14 and 28 dpci. We report these results and show the fit to the biased drift model in Figs. 7D,E. Moreover, we have shown that removing a copy of *stg* prevents *ptc* mutant CySCs from outcompeting wild type CySCs by counting them directly, as shown in Fig. 6A. Thus, we believe that GSC counts are a faithful measure of CySC competition, even though they are indirect (see above).

5. The cell cycle analysis is not ideal (Fig 5). If the authors really wise to show that cell proliferation is sped up by Stg or CycE+Stg, then they should clonal express these genes and tally the numbers of cells per clone and calculate cell doubling times from the data. The EdU and Stg-GFP pictures provided are not so convincing (even though these genes have been shown to have strong proliferative effects in other Drosophila cell types).

We thank the reviewer for this comment. We now have added new data (Fig. 6D) showing the number of labeled somatic cells within control or CycE+Stg-expressing clones. Indeed, the rate of clone growth is accelerated in CycE+Stg-expressing clones, and there are many more labeled somatic cells, consistent with results in imaginal discs (Neufeld et al, 1998). We are not able to obtain a cell doubling time from these experiments because only the stem cells are mitotically active in this lineage (Inaba et al, 2011). As a result of these experiments suggested by the reviewer, we have now shown that CycE+Stg accelerates the cell cycle in CySCs.

6. This same criticism applies to the interpretation that ptc mutant cells outcompete their neighbors due to a more rapid cell cycle. The arguments are direct, and it would be very nice to see data that directly show an accelerated cell cycle in these ptc mutant cells. The data presented is all indirect. Given the well known effects of HH and Ptc signaling on cell identities and fates and patterning, the conclusion that ptc simply affects the cell cycle is a bit fishy. Even though the data are all consistent with this idea, and no effects on other processes such as cell growth and adhesion were observed, it seems likely that ptc could affects cell competition via something other than just the cell cycle.

Very elegant work from the Bökel lab has shown a 5 fold increase in mitoses in *ptc* mutant CySCs vs control (Michel et al, 2012). We have shown that *ptc* mutant CySCs have a higher S phase index than control clones (Fig. 5E). We have shown that reducing the genetic dose of *stg* suppresses *ptc*-dependent CySC-CySC competition (Fig. 6A) and *ptc*-dependent CySC-GSC competition (Fig. 6B). We have shown that reducing the genetic dose of two other cell cycle regulators also suppresses *ptc*-dependent CySC-GSC competition (Fig. 6C). Additionally, we have shown that reducing the genetic dose of *stg* suppresses *hpo*-dependent CySC-GSC competition (Fig. 7F). Finally, we have tested by gain- and loss-of-function other potential cellular mechanisms [cellular growth (Akt, S6k, dMyc), other signaling pathways (JAK/STAT), adhesion (DE-Cad and βPS integrin), classical cell competition (apoptosis, dMyc, *Minute*) (Figs. 3, 4, 6C, S5, S6 and Table S2], and none of them induce or modify niche competition. While we cannot formally exclude that other mechanisms may impact niche competition, we submit that taken together our data strongly argue that regulation of cell cycle progression is central to niche competition by CySCs. We note that we refrain from making the claim that proliferation is the only mechanism at play.

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2nd Editorial Decision 24 June 2014

Please find attached comments from two scientists on your revised study that, in principle, support eventual publication in The EMBO Journal. There are a few remaining items with regard to presentation (ref#1 AND points 3/4 of ref#2) as well as request for a differentiation experiment in Ptc and Hpo mutants that I kindly ask you to incorporate into an ultimate version of your study.

We are also in the position to highlight the major outcome of the paper in a written, 2-4 bullet point synopsis AND a summarizing scheme for better graphical perception (like a 'graphical abstract').

I would be delighted if you were to provide such items to your earliest convenience as to ensure efficient production/publication of your study.

I look forward to receive these final amendments that should enable formal acceptance.

REFEREE REPORTS:

Referee #1:

I have read the revised version of the manuscript, and I was very satisfied with all the incorporated changes and modifications made to its original version. Therefore, in my opinion, the present manuscript is perfectly suitable for publication in The EMBO Journal.

The authors suggest that they could include two additional figures (Fig. R1 and R2) as supplementary material, should I find it helpful. I do not think this is necessary. I would instead advice that the authors include a statement indicating that they've tried their model fitting with other parameters, and summarize the key findings in text. This manuscript has already a lot of data as is, and my original concern can be amply addressed by a simple statement.

Lastly, I would like to extend my sincere, yet anonymous congratulations to the authors on their very interesting contribution to our field.

Referee #2:

The revision includes substantial new data that essentially answers the points raised in my original review. Most importantly, the theoretical treatment is better described and quantitative clonal analysis for hpo mutant CySCs is now shown in fig 6, which further support the author's conclusions in a more direct manner. I have only one major (#1) and a few minor (#2-4) points that should still be addressed:

1. The authors state that ptc mutant CySCs eventually differentiate, but don't show any data that directly supports this. Only data showing that the ptc clone frequency decreased over time is shown (Fig 8). Since a failure or delay in differentiation could help explain these results, data should be included documenting the differentiation of ptc mutant CySCs, and any defects in such. The same is true for hpo mutant CySCs - in this case the issue is simply not addressed. Since Ptc and Hpo are likely to control more than just proliferation rates, demonstrating a lack of effect on differentiation is important to support the author's contention that the hypercompetitive behavior of these cells is specific to their ability to accelerate proliferation.
2. Huang & Kalderon's paper with a similar message (J. Biol 2014) should be cited.
3. The graphs in Figs 6D and 8E should display the data-points, not just the lines.
4. Regarding my previous point #1, although it is true that EMBO J has no guidelines about tense usage in describing data, the use of the present tense to describe some (but not all) experimental results is distracting and potentially misleading. Formally speaking, an experimental result exists

only in the past, and relates only to the experiment that it came from. If the experiment were done again (in the present or future) a different result could in principle be obtained (we hope this is not the case for well-controlled studies). While many authors do use the present tense to relate results, I believe it is incorrect because it is inconstant with modern scientific logic - the theory of testing hypotheses. It also breaks into an ethical risk zone, since using the present tense implies that a particular result that was obtained will always be obtained, and is in this aspect an over-interpretation of data. Besides, the text as it stands is inconsistent: sometimes results are presented in past tense, and sometimes in the present. Thank god they didn't use the future.

2nd Revision - authors' response 02 July 2014

In response to the comments by the reviewers to the originally revised manuscript, we have:

1. provided a statement indicating that we have tried our model with other parameters and summarized the key findings in text on pp. 9-10.

2. altered the graphs in Figs. 6D and 8E to show data points in addition to the lines.

3. stated the results in past tense through the manuscript.

4. added a new expanded view figure (Fig. E3) to demonstrate that ptc and hpo mutant cyst cells differentiate normally and discussed these new data in the revised main body text.

5. added a 4 bullet point synopsis and a graphical abstract.

We hope that you find the changes satisfactory such that our paper is now acceptable for publication in EMBO J. None of the material has been published or is under consideration elsewhere.

