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Ecdysteroids affect Drosophila ovarian stem cell niche formation and early germline differentiation

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Transaction Report:

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

1st Editorial Decision 07 December 2010

Thank you for submitting your manuscript to the EMBO Journal. Your study has now been seen by three referees and their comments to the authors are provided below.

As you can see, the referees find the analysis interesting and suitable for publication in the EMBO journal pending adequate revisions. I would therefore like to invite you to submit a revised manuscript that addresses the concerns raised in full. There are a number concerns that have to be addressed in a revised version including to sort out if taiman has a cell autonomous function independent of ecdysome signaling and to provide further support for the interpretation that overexpression of EcR leads to a dominant negative effect. There are also some issues regarding the presentation of the findings that should be resolved and lastly the Drummond-Barbosa paper that has in the meantime been published needs to be referenced and discussed in the revised manuscript.

I should also add that it is EMBO Journal policy to allow a single round of revision only and that it is therefore important to address the raised concerns in full. When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website:
http://www.nature.com/emboj/about/process.html

Thank you for the opportunity to consider your work for publication. I look forward to your revision.

Best wishes

Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

The manuscript by Klepzig et al reports the effects of genetic manipulation of ecdysone signaling on the biology of germline stem cells (GSCs) in the Drosophila ovary. Mutations in taiman, which encodes a co-activator of the ecdysone receptor, lead to expansion of the niche, and an increase in GSC number. Other genetic manipulations that reduce ecdysone signaling activity lead to an increase in single spectrosome cells (SSCs) which, on the basis of their lack of pMad expression, are interpreted as being undifferentiated cystoblasts and not GSCs. These phenotypes appear to occur as a result of reduced TGF-beta signaling and are suppressible with administration of ecdysone. Evidence is also presented that the escort stem cells (ESCs) are the critical site of EcR expression and thus the effects of EcR signaling on GSC differentiation are non-cell autonomous. In taiman mutants, ESCs take on an altered morphology and overexpress several cell adhesion proteins, possibly explaining their altered function.

Thus this manuscript reports interesting results of potentially broad interest in the area. However, I have some reservations about the manuscript in its present form.

1. My most serious concern involves chronic overstatement of the requirement for ecdysone signaling for GSC differentiation throughout the narrative. For instance, the abstract is categorical and absolute, stating that 'in the absence of the hormone cystoblasts are blocked in a single cell state which can be overcome by ecdysone supply'. Yet many experimental figures show that some proliferation and differentiation into nurse cells and oocytes take place in EcR/tai trans-hets (Fig 1H), in ecd1ts even after prolonged periods at the restrictive temperature (Fig 2) or after extended depletion of EcR or overexpression of Abrupt (most obvious in Fig 5G). Clearly ecdysone signaling is at least partially redundant with other pathways for transit-amplifying divisions and for differentiation, and this point is not sufficiently addressed in the description of the work.

2. The authors should determine whether ESCs take on a different morphology and overexpress cell adhesion molecules when ecdysone signaling is reduced by mechanisms other than mutation of taiman? I'm not completely convinced that taiman doesn't have a cell autonomous function independent of its function in ecdysone signaling.

3. The supernumerary SSCs are identified as cystoblasts based on their lack of pMad expression, but since pMad expression is reduced even in GSCs when ecdysone signaling is compromised it may not be the best cell type reporter in this context. Do these cells express high levels of Mei-P26 and/or other cystoblast markers?

4. The manuscript is extremely lengthy and could easily be cut by a third without loss of essential information. Also, many of the figures are busy and laborious to interpret.

Referee #2 (Remarks to the Author):

This manuscript reports that the systemic hormone ecdysone affects stem cell niche establishment and regulates germline development in the Drosophila ovary. The authors analyzed hypomorphic taiman mutants and observed extra cap cells, escort cells and germline stem cells in germaria. Subsequent analysis of ecd1 at the non-permissive temperature and overexpression of ecdysone receptor showed consistent results, with increased numbers of single spectrosome cells (SSC) and increased numbers of somatic cells. They further demonstrated that pMAD was down-regulated in germline stem cells following suppression of ecdysone signaling. The authors claim that excess E-

cadherin in tai mutant escort stem cells and their progeny perturbs germline-soma communication preventing germline differentiation. Finally, they generated taiman mutant clones with bric-a-brac-GAL4 driven flipase, which induced mitotic recombination in niche precursors and showed that ecdysone signaling is required to modulate niche size. Overall, this manuscript describes an important finding explaining the role of ecdysone signaling in niche formation and the coordination between escort and germline stem cells. Because of this, this work is in principle suitable for publication in EMBO. However, a few questions should be addressed prior to publication.

1: In the abstract, authors claim "roles of ecdysone signaling in the germarium have not been reported." However, "The Steroid Hormone Ecdysone Functions with Intrinsic Chromatin Remodeling Factors to Control Female Germline Stem Cells in Drosophila" was recently published by Drummond-Barbosa in Cell Stem Cell 7, 581-592, November 5, 2010. Although the authors may not have known about it before submitting their work, they should cite this paper in their revised manuscript. The main concern is that in Drummond-Barbosa's paper, GSC proliferation is reduced in ecd-ts and EcR-ts mutants but this manuscript shows an increased number of single spectrosome cells (GSC like cells). The authors should also explain the discrepancy. The authors' explanation for why both loss of function of ecd and over-expression of EcRA or EcRB1 produce similar phenotypes is that EcR over-expression functions in a dominant-negative manner. evidence in support of this is that the phenotype can be partially overcome by addition of ecdysone. However there are purely dominant-negative and constitutively active forms of EcR that could be used to confirm this interpretation.

2: The authors claim that "Ecdysone signaling has a cell-autonomous function in germline to modulate the strength of TGF-b signaling". However, using ecdts or hs-EcR.A can not distinguish whether the results shown in Fig 3 are autonomous or non-autonomous. Figure 3E, a wild-type control should be shown for comparison. In addition, a similar conclusion was published by Ables and Drummond-Barbosa (Cell Stem Cell, 2010 Nov), so the authors should cite this paper and modify their discussion accordingly.

3: In Fig 4A, Tai antibody staining would be better than Tai-GFP to represent endogenous expression pattern of Tai. In addition, without lineage tracing or a specific marker, it is impossible to be sure whether Tai-GFP is expressed in follicle stem cells (FSC) or the immediate daughter of the FSC.

4: The authors should show the bab-Gal4 and ptc-Gal4 expression patterns.

5: Although tai transheterozygote (Fig 1D), + , EcRQ50/taiG161 , + (Fig 1H), ecdts (Fig 3A) and hs-EcR.A (Fig 3E) showed the striking SSC phenotype, none of these results demonstrates whether this is an autonomous or non-autonomous effect. To further address this, the authors should show SSC phenotype when tai is homozygous mutant in ESCs or ECs using mutant mosaic analysis.

6: Without a legend for Fig 8, it is unclear what the model represents. What does each color and shape signify? It is important for the model to clearly represent the role of ecdysone at the pre-adult stage during niche formation and during the adult stage in ESC.

Issue 7: Supplementary Fig 4 and its legend should be labeled as Supplementary Fig 3.

Referee #3 (Remarks to the Author):

This manuscript presents two types of defects in Drosophila ovaries resulting from manipulation of ecdysone-related molecules. One phenotype is the apparent failure of cystoblasts to differentiate. The second is an increase in cap cell and apparent GSC number. The former phenotype appears to derive from an initial deficit in function in the Escort cell lineage, and the latter from a deficit in cap cell precursors. Since hormonal signals are likely to be significant regulators of oogenesis (beyond what is already known) these observations are potentially fruitful and significant insights. However, I have a number of reasons for being unconvinced, at present, by the evidence and arguments offered. I will point out the major scientific issues I have encountered but I should also say that the writing is extremely unclear. Hence, it is possible that some of the issues raised are simply mis-understandings. Nevertheless....

The increase in cap cells and GSCs come in the first and last section of the Results (they might be better together) and seem close to a sound series of experiments. Both tai animals and tai clones induced by bab-GAL4 apparently show increased cap cell numbers & GSCs. I would like in each case to see clearer images illustrating this (in particular with phospho-Mad or Dad-lacZ as relatively definitive markers of GSCs, full explanation of why cap cell markers are adequate and in the case of clones, showing that the cap cell increase is only seen in tai mutant cells). Evidence for other ecdysone manipulations producing the same phenotype is fairly weak as presented. Perhaps images like those requested above, including associating cap cell phenotypes with the cell-autonomous presence of UAS-ab in flip-out clones would make this convincing enough. But why not make bab-GAL4 induced clones of EcR, just as for tai?

The failure of CB differentiation is seen in many situations but three factors are not ideal. First, the escort cell source of this phenotype is suggested partly through an analysis of tai mutations in ESCs and in the germline but I do not see any evidence that tai mutations produce a deficit in CB progression. Second, the cause of the phenotype remains unkown and indeed the definition of CBs as a target is also uncertain given that Bam is not expressed highly in these cells; the reduction in phospho-Mad staining of GSCs would likely lead to increased Bam and is therefore a distraction, at best, in understanding the CB phenotype & certainly not a cogent mechanistic hypothesis. Third, the non-intuitive dominant-negative activity of EcR overexpression adds confusion to this set of experiments. There is no marker of ecdysone responses that clearly resolves the consequences of hs-EcR, so the assertion of dominant negative activity remains largely a rationalization. If these experiments were always mentioned last regarding any specific phenotype or point I think the readers would find that they could better accept the rationalizations (& find that those experiments merely add to previously established points).

Among the many writing hazards and some issues with Figures I highlight the following:

Intro- very long and with generalizations that are so general they are clearly not accurate (counter-examples can be quoted on most points).
Explain the nature of the p[ilot screen.
Don't call "SSCs" germline stem cell-like first since that label will be refuted
"not trying to maintain stem cell identity" is one of many examples of straying from literal, accurate descriptions
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References needed for use of reporters of ecdysone responses
Tai EC phenotypes have not been causally linked to a specific consequence to justify statements of causal links in text and summary
In Discussion it is implied that reduced ecdysone signaling normally limits cap cell number, whereas data presented say the opposite (a similar contradiction between phospho-mad and Bam phenotypes is passed over as if the results might be causatively linked).

In summary, I think there would have to be many changes in the presentation and likely some clearer images in order to present a coherent, reasonably convincing line of reasoning. Once that is done the paper may appear considerably more attractive. However, from the material and logic presented here I don't see a strong argument that either of the main findings are of great depth or present major new insights. However,a small additional nailing down of when taiman acts and whether it delays differentiation to produce more cap cells would, I think, provide a nice paradigm for rare insight into the regulation of niche cells. Finally, after completing this review I saw a paper from Dr. Drummond-Barbosa in Cell Stem Cell with a title suggesting potential overlap. I think there is none but it is inevitable that in revision or separate submissions the authors will be asked to comment on the relationship between the two studies.

1st Revision - authors' response 17 December 2010

Thank you for your e-mail from 07.12.2010. We would also like to thank the reviewers for positive evaluation of our work, careful reading of our manuscript and helpful suggestions that we took into consideration to improve our paper. In consideration of the reviewers' comments we have now accordingly revised the manuscript.
To sort out if Tai has a cell autonomous function we compared the phenotypes caused by different components of ecdysone signaling, we down-regulated tai during adulthood in germarial soma only (UAS tai RNAi driven by somatic drivers bab1 Gal4 and ptc Gal4 combined with tub Gal80ts system, Figure 5A-B), which phenocopied EcR and ab mutants (Figure 5C-G); in addition, cell adhesion defects now also have been studied not only in tai but also in EcR and ab mutants (Figure 6). To prove that EcR also plays a role in the niche establishment we made somatic EcR clones during development using UAS EcR RNAi and the act<CD2<Gal4 system, which resulted in the appearance of extra niche-like cells within the germarium comparable with UAS ab clones (Figure 7C-D). To further prove that overexpression of EcR leads to a dominant negative effect, we additionally used EcR and usp dominant negative mutants that showed defects analogous to EcR overexpression (Figure 2 C-D, G-H, Supplementary Tables S2, S3). The Drummond-Barbosa paper on the role of ecdysone in GSCs that was recently published (Cell Stem Cell 7, 581-592, November 5, 2010) has been cited and discussed. We also changed figures and figure legends to make them easier to read.

Also as suggested by reviewer #1 we changed the title, abstract and text to avoid the overstatement of the requirement for ecdysone signaling for early germline differentiation, since our data show that ecdysone does not fully block, just delays cystoblast differentiation acting in cooperation with other pathways controlling the process.
Now we have determined that when ecdysone signaling is perturbed in somatic cells via EcR downregulation and Ab overexpression, mutant cells also do not properly change their shapes and express higher levels of the adhesion protein DE-Cadherin (Figure 6 E, F).
As suggested by the reviewer we now more carefully defined the SSC characteristics and found that supernumerary SSCs express neither a stem cell marker pMad, nor a differentiation factor Bam, which explains their inability to differentiate (Figure 2E, 3G, H).

As mentioned by reviewer #2, the paper entitled "The Steroid Hormone Ecdysone Functions with Intrinsic Chromatin Remodeling Factors to Control Female Germline Stem Cells in Drosophila" recently published by Drummond-Barbosa states that GSC maintenance and proliferation is reduced in ecd-ts and EcR-ts mutants, which does not conflict with our data. We did not performe long-term experiments on the role of ecdysone signaling in GSC maintenance, however our data (Supplementary Table 2) shows that with ecdysone deprivation the number of GSCs, defined as the most anterior germline cells containing a single spectrosome and directly touching the niche, is reduced from 2 to 1.8-1.6 after flies have been kept 3 days longer in restrictive conditions.
Unfortunately we cannot comment if an increased number of developmentally delayed SSCs has been analyzed by Drummond-Barbosa lab, since no picture was provided. In accordance with the abovementioned paper our data also show that tai loss of function in the germline has no effect on GSC maintenance (Supplementary Figure S1).
The fact that over-expression of EcR acts in a dominant-negative manner and produces phenotypes similar to loss of function mutants has been documented in different systems in Drosophila (Schubiger et al., 2005; Schubiger, Truman, 2000). We have added data on dominant-negative forms of EcR and USP that further supports our interpretation of our results.
As pointed out by the reviewer, our experiments cannot distinguish whether TGF- regulation by ecdysone signaling is cell autonomous or non-autonomous. Now we ommited the statement about cell autonomous function from the Results section, however taking into account data by Ables and Drummond-Barbosa, 2010 we commented on this possibility in the Discussion. As suggested, a wild-type control is shown for comparison in Figure 3.
As advised by the reviewer we now added the expression patterns of Tai and USP as detected by specific antibodies (Figure 4 C, D). Since we did not perform a lineage tracing nor used follicle stem cell specific markers, we now state that Tai, USP and EcR are expressed in follicle cells, not in FSCs.

The bab-Gal4 and ptc-Gal4 expression patterns are shown in Supplementary Figure S2.
To show that Tai has a cell non-autonomous effect on germline differentiation we specifically downregulated tai in soma using tai RNAi which also resulted in a supernumerary SSC phenotype (Figure 5A,B). Also in Figure 7A-B additional SSCs are shown to be present in germaria containing tai clonal somatic cells.
As suggested by the reviewer, a legend for Figure 8 has been added explaining the role of ecdysone at the pre-adult stage during niche formation and during the adult stage in ESCs.

As suggested by reviewer #3 we considered combining the first and last sections of the Results on the increase in CpCs and GSC numbers, however we found that it would be difficult to present the data conclusively before introducing all the players, their expression patterns and relationships.
To better explain the tai preadult phenotype we now added a representative picture of phospho-Mad staining that indicates increased GSC number (Figure 7J). LaminC and Cadherin have been previously shown to be adequate markers for niche cells (Forbes et al., Development, 1996, Song et al., Science, 2002, Xie, Spradling, Science, 2000, Ward et al., Current Biology, 2006) and normally in a wild type germarium one can find only 6-8 LaminC and Cadherin positive cells, while both tai mutant and tai clonal germaria (bab-GAL4 UAS Flp, Figure 1D, 7A) show supernumerary LaminC and Cadherin positive cells at the anterior tip of the germarium. As suggested by the reviewer we now added an image of cap cell phenotypes with the cell-autonomous presence of UAS EcR RNAi in flip out clones (Figure 5D). Unfortunately, due to technical reasons (the EcR locus is situated between the centromere and the most proximal available FRT insertions (42B)) bab1 GAL4 UAS Flp induced clones of EcR cannot be done.
To better show that the failure of early germline differentiation is caused by ecdysone signaling perturbance we (1) show that tai mutations produce a deficit in CB progression via downregulation of tai specifically in escort cells using UAS tai RNAi driven by escort cell driver ptcGal4/tubGal80ts (Figure 5B); (2) we define that delayed SSCs do not express pMad and Bam, and since the last is an essential factor that triggers a differentiation program, its absence explains the differentiation delay (Figure 2E, 3G,H); (3) as suggested by the reviewer, the dominant-negative activity of EcR overexpression has now been placed last to avoid confusion. We think that keeping this set of experiments is important since UAS EcR expression during preadult stages is the only viable combination that phenocopies the tai mutant niche enlargement phenotype, and in addition to our findings, overexpression of EcR having dominant negative activity has been previously confirmed (Kozlova, Thummel, 2003; Elke et al., 1997; Schubiger et al., 2005; Schubiger, Truman, 2000).
Also, we agree that the Intro contains some generalizations that can be counter-quoted, but taking into account the vast information on stem cells, their niches and roles of steroids we tried to express our view. We also tried to omit inaccurate statements and descriptions: "SSCs" are not called germline stem cell-like, we also agree that the statement "heterochronic" effect does require additional experiments.
As suggested by the reviewer the nature of the pilot screen has been explained, however no data were added since it is an ongoing experiment in the lab. References have been added for use of reporters of ecdysone responses and the discussion has been changed to avoid contradictions.

We hope this changes will make the manuscript acceptable to EMBO Journal.

2nd Editorial Decision 04 February 2011

Thank you for submitting your revised manuscript to the EMBO Journal. I asked the original three referees to review the revised version and I have now heard back from them. While referee #3 is still not persuaded that the manuscript is well suited for publication here, both referees #1 and 2 support publication here. Referee #2 has a few remaining issues that should be resolved prior to acceptance. Most of them concern the presentation of the data and the need for making more cautious statements and interpretations. Referee #2 also finds that the Tai antibody staining (fig 4C) is not very convincing and I agree with this. Do you have controls that the staining is specific? Referee #3 raises a number of different concerns, respond to those in the point-by-point response. Where appropriate please introduce changes in the manuscript. Once we get these last issues resolved, we

will proceed with the acceptance of the study for publication here.

When you send us your revision, please include a cover letter with an itemised list of all changes made, or your rebuttal, in response to comments from review.
When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process initiative, please visit our website:
http://www.nature.com/emboj/about/process.html

Best wishes
Editor
The EMBO Journal

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REFEREE REPORTS:

Referee #1 (Remarks to the Author):

My concerns have been fully addressed in this revised version and I now recommend publication.

Referee #2 (Remarks to the Author):

This is an interesting study showing effects of disrupting ecdysone signaling on the Drosophila ovarian niche, germline stem cells and their progeny. The authors have responded to some of the major criticisms (the inability to distinguish autonomous from non-autonomous effects) appropriately. However there remain issues of overstatement, over-interpretation, and unconvincing data that need to be addressed prior to publication.

The anti-Tai antibody staining is completely unconvincing. In later stage follicle cells, Tai is a nuclear protein, but the staining does not appear nuclear. No specificity controls are shown (staining of tai mutants for example or tai RNAi expressing cells). The staining does not appear at all enriched in ESCs or CPCs, as the authors clam. And the antibody staining looks very different from the protein trap.

Although the ecdysone activity reporters likely accurately report the somatic hormone response, they may not report germline activity even if it is present, because germline gene expression is regulated so differently and the basal promoter used in the reporter may not be permissive for germline expression even in the presence of activated hormone receptors. This caveat should be mentioned.

The statement in the abstract that control of progression through the early stages of germ cell development by ecdysone "is accomplished via adjustment of TGF-b signaling in the germline and regulation of cell adhesion complexes and cytoskeletal proteins in somatic escort cells" overstates what the authors show. They show that TGF-b signaling is reduced, but not that restoring it rescues any aspect of the phenotype. Similarly they show altered cell adhesion and morphology are present, but not that restoring normal cell adhesion or morphology corrects the germline phenotypes. More cautious wording is necessary both in the abstract and at the end of the introduction.

In the discussion too the authors make claims such as "Ecdysone pathway
deficiency causes escort cell shape defects that affect the escort cell - germline cyst contacts leading to the delay of early germline differentiation." However it is not clear that the cell shape defects are the cause of the germline differentiation defects.

In general the discussion is too long and overly speculative.

The authors added a wild-type control to Figure 3, as suggested but all figures should include a control. And in Figure 1, the authors show a control in panel B stained with pMad whereas the tai

mutant germarium is stained with BamC. Both markers should be shown for both genotypes in order to be able to compare them.

Referee #3 (Remarks to the Author):

I have compared new and old manuscripts and looked through the answers to the criticisms I had previously. A few have been answered satisfactorily, including some writing points. However, as can be seen from my listed reactions to specific responses to criticisms below, I still find this paper unattractive to read, without a good logical flow and without clear illustration of some of the key points. The findings are the beginnings of defining roles for ecdysone that are potentially interesting, but not yet shown to be of great significance or readily incorporated into a clear insight into normal development. Were I the only reviewer, I would still not recommend the paper for publication in EMBO J, which I would expect to publish clearer, more digestible stories of better developed significance. However, I see that I was originally the strongest critic so, if other reviewers and the Editor, having seen my initial comments are satisfied that the paper is now acceptable I would not seek to veto that consensus.

Both tai animals and tai clones induced by bab-GAL4 apparently show increased cap cell numbers & GSCs. I would like in each case to see clearer images illustrating this (in particular with phospho-Mad or Dad-lacZ as relatively definitive markers of GSCs, full explanation of why cap cell markers are adequate and in the case of clones, showing that the cap cell increase is only seen in tai mutant cells).
Only Fig. 7J has been added to show pMad staining, revealing one example of four apparent GSCs. No such data added for the consequence of tai clones, nor for a cell autonomous effect of tai clones producing extra cap cells.

Evidence for other ecdysone manipulations producing the same phenotype is fairly weak as presented. Perhaps images like those requested above, including associating cap cell phenotypes with the cell-autonomous presence of UAS-ab in flip-out clones would make this convincing enough. But why not make bab-GAL4 induced clones of EcR, just as for tai?
The authors have added UAS EcR RNAi flip out clones in Fig 7D but I see no evidence of increased cap cells or GSCs (the relevant markers are not used) and the flipped-out cells are surprisingly rare. The images for the other Ecdysone manipulations share the problem of not defining the additional somatic and single spectrosome germline cells.

The failure of CB differentiation is seen in many situations but three factors are not ideal. First, the escort cell source of this phenotype is suggested partly through an analysis of tai mutations in ESCs and in the germline but I do not see any evidence that tai mutations produce a deficit in CB progression.
This is now added to Fig. 5 and, although not quantified like all other similar tests in the bar graphs, the phenotype seems clear (assuming it is frequent and reproducible).
Second, the cause of the phenotype remains unkown and indeed the definition of CBs as a target is also uncertain given that Bam is not expressed highly in these cells; the reduction in phospho-Mad staining of GSCs would likely lead to increased Bam and is therefore a distraction, at best, in understanding the CB phenotype & certainly not a cogent mechanistic hypothesis.
It remains true that no mechanism is offered and the TGFb signaling section remains a distraction with no known relevance to any of the described phenotypes.
Third, the non-intuitive dominant-negative activity of EcR overexpression adds confusion to this set of experiments. There is no marker of ecdysone responses that clearly resolves the consequences of hs-EcR, so the assertion of dominant negative activity remains largely a rationalization. If these experiments were always mentioned last regarding any specific phenotype or point I think the readers would find that they could better accept the rationalizations (& find that those experiments merely add to previously established points).
The addition of other dominant-negative approaches and re-organization solves the hs-EcR problem. It also reveals for the first time that the reporter system for EcR activity is actually dominant negative and therefore not ideal (but I find the description of expression patterns altogether not very definitive or revealing).

Among the many writing hazards and some issues with Figures I highlight the following:

Intro- very long and with generalizations that are so general they are clearly not accurate (counter-examples can be quoted on most points).
Most offending material removed.
Explain the nature of the p[ilot screen.
Not really accomplished because it is not clear in which cell types clones are made.
Don't call "SSCs" germline stem cell-like first since that label will be refuted
"not trying to maintain stem cell identity" is one of many examples of straying from literal, accurate descriptions
"Over-activation of EcR" is eventually followed by saying that overexpression actually reduces ecdysone responses!
Why does "heterochronic" ever appear in text & summary? It implies far more than is shown.
References needed for use of reporters of ecdysone responses
Corrected

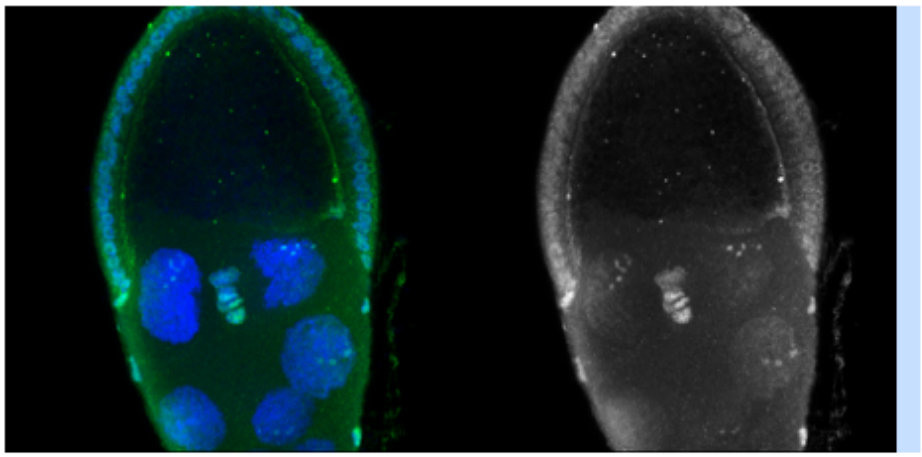
Tai EC phenotypes have not been causally linked to a specific consequence to justify statements of causal links in text and summary
It remains true that no such links have been established for this or TGFb signaling so the sentence summary below is not appropriate or justified:
"Control of this process is accomplished via adjustment of TGF- signaling in the germline and regulation of cell adhesion complexes and cytoskeletal proteins in somatic escort cells."
In Discussion it is implied that reduced ecdysone signaling normally limits cap cell number, whereas data presented say the opposite (a similar contradiction between phospho-mad and Bam phenotypes is passed over as if the results might be causatively linked).
The specific contradiction has been removed but the actual proposed contribution to regulation of cap cell number is never clearly stated (let alone proven). Are ecdysone levels proposed to be modulated critically at a precise time or the responsiveness of just a small subset of cells altered (in a manner that has nothing to do with regulation originating in hormone levels)? Evidence is presented that artificially decreasing ecdysone response has a couple of specific consequences but that is not sufficient to figure out if ecdysone normally has a regulatory role (or for the authors to propose a model of how such regulation might be imposed). The Discussion remains too long for what is said.

In summary, I think there would have to be many changes in the presentation and likely some clearer images in order to present a coherent, reasonably convincing line of reasoning. Once that is done the paper may appear considerably more attractive.
The paper is slightly more coherent than before but it still requires a lot of changes to make it moderately understandable and almost grammatically correct.
However, from the material and logic presented here I don't see a strong argument that either of the main findings are of great depth or present major new insights.
I still believe that there is no clear story or bottom-line about what ecdysone signaling contributes to normal development..
However,a small additional nailing down of when taiman acts and whether it delays differentiation to produce more cap cells would, I think, provide a nice paradigm for rare insight into the regulation of niche cells.
Delayed differentiation has not been shown and I still believe that the changes in GSC number are not shown very clearly, but there is some improvement here.

2nd Revision - authors' response 15 February 2011

Thank you for your e-mail from 04.02.2011. In consideration on the reviewers' comments we revised the manuscript as follows:
1. We have chosen another picture that better represents the anti-Tai antibody staining pattern (Figure 4C). In addition to confirm the specificity of Tai expression in escort cells, we performed anti-Tai antibody staining in clonal germaria containing tai loss of function escort cells (Supplementary Figure 2). Also, the previously described Tai expression pattern in border cells (Bai

et al., Cell, 2000) has been detected and is shown here below:



2. To prove that tai clonal niche cells are functional we did an additional experiment to calculate the number of GSCs marked by pMad in tai clonal germaria where tai was mutated specifically in the niche (Fig. 7J and text).
3. We added more panels for the controls (Fig. 1A, C, E, Fig. 7E) to make observed phenotypes easier to recognize.
4. Now all figures have pictures and/or calculations of appropriate controls and the results of the statistical analyses are shown in all bargraphs.
5. We introduced changes in the text to carefully state and interpret our findings.

Point-by-point response to reviewers' comments:

Referee #1 (Remarks to the Author):
My concerns have been fully addressed in this revised version and I now recommend publication.

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This is an interesting study showing effects of disrupting ecdysone signaling on the Drosophila ovarian niche, germline stem cells and their progeny. The authors have responded to some of the major criticisms (the inability to distinguish autonomous from non-autonomous effects) appropriately. However there remain issues of overstatement, over-interpretation, and unconvincing data that need to be addressed prior to publication.
1. The anti-Tai antibody staining is completely unconvincing. In later stage follicle cells, Tai is a nuclear protein, but the staining does not appear nuclear. No specificity controls are shown (staining of tai mutants for example or tai RNAi expressing cells). The staining does not appear at all enriched in ESCs or CPCs, as the authors clam. And the antibody staining looks very different from the protein trap.

Thank you very much for pointing this out. We agree that the Tai staining pattern was not properly presented. Now we corrected this and show a picture where nuclear staining in somatic cells (follicle and escort) is clearly seen. As suggested by the reviewer staining in tai mutants has been performed and a specific reduction of the expression levels in tai clonal cells has been observed to confirm the specificity of the antibody staining (Supplementary Fig.2)

2. *Although the ecdysone activity reporters likely accurately report the somatic hormone response, they may not report germline activity even if it is present, because germline gene expression is regulated so differently and the basal promoter used in the reporter may not be*

permissive for germline expression even in the presence of activated hormone receptors. This caveat should be mentioned.

We are aware of this and we mention this in the text "The ecdysone pathway activity was detected mainly in ESCs and ECs analyzed using a somatically expressed UASt lacZ transgene (Figure 4E-F)".

3. The statement in the abstract that control of progression through the early stages of germ cell development by ecdysone "is accomplished via adjustment of TGF-b signaling in the germline and regulation of cell adhesion complexes and cytoskeletal proteins in somatic escort cells" overstates what the authors show. They show that TGF-b signaling is reduced, but not that restoring it rescues any aspect of the phenotype. Similarly they show altered cell adhesion and morphology are present, but not that restoring normal cell adhesion or morphology corrects the germline phenotypes. More cautious wording is necessary both in the abstract and at the end of the introduction.

As suggested by the reviewer we changed the respective sentences of the text describing our results.

4. In the discussion too the authors make claims such as "Ecdysone pathway deficiency causes escort cell shape defects that affect the escort cell - germline cyst contacts leading to the delay of early germline differentiation." However it is not clear that the cell shape defects are the cause of the germline differentiation defects.
5. In general the discussion is too long and overly speculative.

We agree with the reviewer that presented data cannot prove that the cell shape defects are the cause of the germline differentiation defects. Causative statements have been omitted and the discussion has been shortened.

6. The authors added a wild-type control to Figure 3, as suggested but all figures should include a control.
7. And in Figure 1, the authors show a control in panel B stained with pMad whereas the tai mutant germarium is stained with BamC. Both markers should be shown for both genotypes in order to be able to compare them.

Additional control has been added to Figure 1, now we have pMad and Bam stainings for both, wild type and mutant germaria. We think that different staining patterns in control germaria shown in Fig. 1C, E and a schematic drawing of the wild type germarium Fig. 1A are easily accessible to be used for comparison of described mutant phenotypes. In other cases additional control pictures and the frequency of mutant phenotype calculations are used. In addition we added the picture of a normal looking niche to have a better comparison with enlarged niche phenotype (Fig. 7 E and F).

Referee #3 (Remarks to the Author):

I have compared new and old manuscripts and looked through the answers to the criticisms I had previously. A few have been answered satisfactorily, including some writing points. However, as can be seen from my listed reactions to specific responses to criticisms below, I still find this paper unattractive to read, without a good logical flow and without clear illustration of some of the key points. The findings are the beginnings of defining roles for ecdysone that are potentially interesting, but not yet shown to be of great significance or readily incorporated into a clear insight into normal development. Were I the only reviewer, I would still not recommend the paper for publication in EMBO J, which I would expect to publish clearer, more digestible stories of better developed significance. However, I see that I was originally the strongest critic so, if other reviewers and the Editor, having seen my initial comments are satisfied that the paper is now acceptable I would not seek to veto that consensus.

Both tai animals and tai clones induced by bab-GAL4 apparently show increased cap cell numbers & GSCs. I would like in each case to see clearer images illustrating this (in particular with phospho-Mad or Dad-lacZ as relatively definitive markers of GSCs, full explanation of why cap cell markers are adequate and in the case of clones, showing that the cap cell increase is only seen in tai

mutant cells). Only Fig. 7J has been added to show pMad staining, revealing one example of four apparent GSCs. No such data added for the consequence of tai clones, nor for a cell autonomous effect of tai clones producing extra cap cells.

Now we added the new data where the increased number of GSCs has been confirmed in tai somatic clones with pMad staining (Fig. 7J and text). Tai clones producing extra cap cells cell automously is shown in Fig. 7A-B.

Evidence for other ecdysone manipulations producing the same phenotype is fairly weak as presented. Perhaps images like those requested above, including associating cap cell phenotypes with the cell-autonomous presence of UAS-ab in flip-out clones would make this convincing enough. But why not make bab-GAL4 induced clones of EcR, just as for tai?
The authors have added UAS EcR RNAi flip out clones in Fig 7D but I see no evidence of increased cap cells or GSCs (the relevant markers are not used) and the flipped-out cells are surprisingly rare. The images for the other Ecdysone manipulations share the problem of not defining the additional somatic and single spectrosome germline cells.

Here we used Adducin as a marker for SSCs that clearly shows that upon UAS EcR RNAi and UAS ab clonal induction in escort cells the number of SSCs is increased (Fig. 7C-D). Adducin staining is shown in separate panels for better illustration of the phenotype. For ecdysone manipulations UAS EcR expressed by the niche specific driver bab1Gal4 was analyzed. We show the enlarged niche using a niche specific marker Cadherin (Fig. 7F) and increased number of GSCs is shown using a stem cell specific marker pMad (Fig. 7K) and a differentiation factor BamC (Fig. 7I). The number of pMad positive cells is also counted and given in the text. As a "control" the germarium with a normal niche where UAS EcR.A is driven by ptcGal4, a driver that is not niche cell specific is shown (Fig. 7E).

We agree that the phenotype is difficult to depict: it requires a perfect orientation of the germarium and more often it is easier to count it than to show. The most representative and the best for comprehension images are shown. In addition, the frequency of all countable described phenotypes is included to make it easier for the reader who is not familiar with the architecture of the Drosophila germarium.

The failure of CB differentiation is seen in many situations but three factors are not ideal. First, the escort cell source of this phenotype is suggested partly through an analysis of tai mutations in ESCs and in the germline but I do not see any evidence that tai mutations produce a deficit in CB progression. This is now added to Fig. 5 and, although not quantified like all other similar tests in the bar graphs, the phenotype seems clear (assuming it is frequent and reproducible).

Second, the cause of the phenotype remains unkown and indeed the definition of CBs as a target is also uncertain given that Bam is not expressed highly in these cells; the reduction in phospho-Mad staining of GSCs would likely lead to increased Bam and is therefore a distraction, at best, in understanding the CB phenotype & certainly not a cogent mechanistic hypothesis.
It remains true that no mechanism is offered and the TGFb signaling section remains a distraction with no known relevance to any of the described phenotypes.

We agree with the reviewer that our data does not provide the answer for the mechanism of how exactly TGF-beta signaling is regulated (we just speculate on it in the discussion), but we think that our analysis of TGF-beta components in our "delayed" SSCs describes their nature and explains the ecdysone signaling deficit phenotype.

Third, the non-intuitive dominant-negative activity of EcR overexpression adds confusion to this set of experiments. There is no marker of ecdysone responses that clearly resolves the consequences of hs-EcR, so the assertion of dominant negative activity remains largely a rationalization. If these experiments were always mentioned last regarding any specific phenotype or point I think the readers would find that they could better accept the rationalizations (& find that those experiments merely add to previously established points).
The addition of other dominant-negative approaches and re-organization solves the hs-EcR problem. It also reveals for the first time that the reporter system for EcR activity is actually dominant negative and therefore not ideal (but I find the description of expression patterns

altogether not very definitive or revealing).

The dominant-negative effect of these reporters has been shown before (Kozlova and Thummel, Development, 2002).
We added new pictures to better show the expression pattern (Supplementary Fig 2, Fig. 4).

Among the many writing hazards and some issues with Figures I highlight the following:
Intro- very long and with generalizations that are so general they are clearly not accurate (counter-examples can be quoted on most points).
Most offending material removed.

Explain the nature of the p[ilot screen.
Not really accomplished because it is not clear in which cell types clones are made.

Clones were made using hsFlp that has no cell specificity and we add this info to the text now.

Don't call "SSCs" germline stem cell-like first since that label will be refuted "not trying to maintain stem cell identity" is one of many examples of straying from literal, accurate descriptions "Over-activation of EcR" is eventually followed by saying that overexpression actually reduces ecdysone responses! Why does "heterochronic" ever appear in text & summary? It implies far more than is shown. References needed for use of reporters of ecdysone responses Corrected Tai EC phenotypes have not been causally linked to a specific consequence to justify statements of causal links in text and summary It remains true that no such links have been established for this or TGFb signaling so the sentence summary below is not appropriate or justified: "Control of this process is accomplished via adjustment of TGF-β signaling in the germline and regulation of cell adhesion complexes and cytoskeletal proteins in somatic escort cells." In Discussion it is implied that reduced ecdysone signaling normally limits cap cell number, whereas data presented say the opposite (a similar contradiction between phospho-mad and Bam phenotypes is passed over as if the results might be causatively linked).
The specific contradiction has been removed but the actual proposed contribution to regulation of cap cell number is never clearly stated (let alone proven). Are ecdysone levels proposed to be modulated critically at a precise time or the responsiveness of just a small subset of cells altered (in a manner that has nothing to do with regulation originating in hormone levels)?
Evidence is presented that artificially decreasing ecdysone response has a couple of specific consequences but that is not sufficient to figure out if ecdysone normally has a regulatory role (or for the authors to propose a model of how such regulation might be imposed). The Discussion remains too long for what is said.
In summary, I think there would have to be many changes in the presentation and likely some clearer images in order to present a coherent, reasonably convincing line of reasoning. Once that is done the paper may appear considerably more attractive.
The paper is slightly more coherent than before but it still requires a lot of changes to make it moderately understandable and almost grammatically correct. However, from the material and logic presented here I don't see a strong argument that either of the main findings are of great depth or present major new insights. I still believe that there is no clear story or bottom-line about what ecdysone signaling contributes to normal development. However,a small additional nailing down of when taiman acts and whether it delays differentiation to produce more cap cells would, I think, provide a nice paradigm for rare insight into the regulation of niche cells.
Delayed differentiation has not been shown and I still believe that the changes in GSC number are not shown very clearly, but there is some improvement here.

We agree with the reviewer that analysis of the precise function and timing of ecdysone signaling in regulation of the niche formation is a very interesting biological question, which has to be studied in more detail further.

Finally we would like to thank our reviewers again for careful reading and helpful suggestions that motivated us to rethink our data interpretation and presentation. We hope that our revised and improved manuscript in now acceptable for publication.

Thank you very much for your help, time and consideration.

