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Inducible progenitor-derived Wingless regulates adult midgut regeneration in Drosophila

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

1st Editorial Decision 07 March 2012

Thanks so much for submitting your research paper on the role of Wnt-signals in the intestinal niche in response to damage/stress for consideration to The EMBO Journal editorial office.

Apologies that it took so long in the end, but with the need to assess the related paper some of the referees requested additional time to finalize their comments. As you will see from their remarks below, all appreciate the timeliness and interest in the subject. Despite highlighting some important observations, all three also indicate the rather premature status of the data that lack essential controls in places and focus mostly on the source/epistasis of Wnt's rather than characterize the actual details of the inductive progenitor niche as promised in the title. Most importantly, refs#2 and #3 rightly mention that for all the genes investigated, mutants are readily available. Thus, one wonders why the study does so heavily rely on RNAi-lines instead of exploiting these available tools to reach definitive mechanistic conclusions.
I do certainly recognize the rather harsh tone of one of the referees, but this does certainly not distract from some of the valuable critiques raised by this scientist, which are reiterated by the other, more encouraging assessments.

On face value, I do have to conclude that the current dataset though of potential interest, remains much too premature. Appreciating that the related paper does not have experimental, but minimally conceptual overlap (looking at the steady state situation versus damage response here and similar positioning the Wg- versus the Jak/STAT pathway currently presented in both studies), it would need definitive, mechanisms together with broader characterization of what determines the niche in both steady-state and challenged conditions to reach the necessary level and significance for a more general title compared to a rather more developmental-oriented journal.

Given this current, preliminary state of affairs and not to rush you into any deadlines with a potential unsatisfying outcome, I do have no other choice than to formerly reject the paper.
We do however recognize the potential and would be prepared to re-assess a thoroughly developed and reconstructed study at a later timepoint.
As this entails time-consuming additional experimentation with currently uncertain outcome(s), I would fully understand if you might find it easier to seek rapid publication elsewhere.

I am sorry that I cannot be more encouraging on this occasion was not a positive one but I hope that clearly communicating our demands and expectations might facilitate efficient further proceedings of your study.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

The role of Wg signaling in the adult fly intestine has been previously investigated (Lin, 2008; Xu, 2011) and Wg protein was shown to be to be expressed in the visceral muscle and ISC cells at low levels. Moreover, impairment of Wg signaling via TCF dominant negative expression and Frizzled 1, 2 loss of function in clones suggested a general role in ISC proliferation (Lin, 2008). Cordero and colleagues extend these findings by investigating intestinal response to damage and find that Wg is strongly induced in epithelial cells where it is required to promote proliferation in response to stress. Forced activation of Jnk signaling can induce Wg and is required for hyperproliferation in this context. Upon aging Wg expression in both the visceral muscle and epithelial cells is required to drive proliferation. The authors also identify Myc as an important effector downstream of Wg and provide evidence that Jak/Stat acts in a parallel manner to Wg in promoting ISC proliferation, both being essential in response to stress. A role for Myc in ISC proliferation has been previously noted (Amcheslavsky, 2011).

Though several of the findings here have been previously published, this work does provide new insight into how the intestine responds to injury. In addition, placing Wg genetically in respect to the other known pathways induced upon stress in the intestine is useful for the field. While the authors provide compelling evidence that Wg protein levels are strongly inducing upon various types of stress, many important controls of knock-down effects on non-stress conditions are lacking. In addition, I am not convinced that the title is appropriate, as I believe a niche should help contribute to maintenance, which is not clearly demonstrated here (though perhaps this is a bit semantic).

1. Many important controls are lacking making the authors' claims of stress-specific or ageing-specific roles of Wg signaling impossible to conclusively demonstrate. Do Wg signaling components have a role in normal proliferation or not? If they are generally required for ISC proliferation, then finding that they are also required under stress is not really too surprising. One would like to see non-stress conditions for all genetic contexts. Because there is such a low basal level of PH3+ cells at a per gut level in these authors' experiments, a role for Wg components in normal homeostasis would be best addressed in mitotic clones, preferentially with loss of function alleles, or using the esgGal4 flip-out system (Jiang, 2009).

Specifically: In Fig 1, 2, 6 . Esg>Wg-IR in unstressed conditions (MARCM clones or esg flip-out); In Fig. 6 . Esg>Wg-IR, how>wgIR, dm/+, wg/+ all in unstressed conditions. In Fig S2- wgIR; wlsIR in unstressed (cell #/ clone at a given time point relative to wild-type); Fig. S3 TCF Dominant negative in unstressed conditions; Fig. S4 esg>Myc-IR and TCF-DN in young/unstressed clones or esg flip-out; Fig. S5, esg>WgIR in young/unstressed clones or esg flip-out, and Fig. S6 esg and how>wg-IR in young/unstressed clones or esg flip-out.

In addition, the effect of MycRNAi on unstressed ISCs was previously reported to block ISC

proliferation in Amcheslavsky, 2011: "Over a short duration of 2 d, the Myc RNAi did not cause a significant change in ISC growth or division (Fig. 7, A and B). However, the Myc RNAi, for 6 d or longer, did cause a halt of division (unpublished data)". This reference to previous work on Myc in the fly intestine should be cited. Moreover, the previous work suggests that the effect seen by Cordero and colleagues on proliferation, stated as being damage-dependent, may simply a block in proliferation as the authors themselves seem to indicate on p. 9.

2. The authors use esgGal4 UAS-GFP as a marker of ISC/EB cells (Fig. 1), however upon rapid proliferation due to damage, GFP is likely present in recently produced EC cells. They should assess the nuclear size of the cells expressing Wg in the epithelia to see if young ECs can also express Wg. In addition, in the images shown in Fig 1A, C , the express of Wg in the muscle is not very clear to me (these appear to be cells between the howGal4 expressing cells?). Since the major finding here is of high level of epithelial expression of Wg upon damage, I am not sure it is extremely important whether or not some young EC cells express Wg. The authors should be cautious about their interpretation that it is completely ISC/EB specific however.

3. The knock-down of Wg using a howGal4 driver specific for the muscle seems to result in a significant reduction of PH3+ cells in response to Bleomycin in Fig. 3L. The authors do not comment on this in the text. Is there something different with Bleo damage? Also, the knock-down of Wg in the esg+ cells does not bring proliferation down to wild-type levels. Is this due to expression of Wg in another cell population, possibly even the muscles? If tubulinGal4 is used or combined esg, How Gal4 does this change?

4. In Fig. 3A-I, the authors are not measuring directly newly produced cells with an esg-flip out technique (which would be more informative), but merely esgGal4 UAS-GFP expression upon various stress and genetic manipulation. That being the case, I am not sure that panels G-I are very informative and could potentially be removed as the quantification is in 3J.
5. As mentioned above, a non-stress control is lacking for Fig. S4. It would also be useful to see a wild-type control with Delta staining as it is difficult to conclude anything from Sup. 4 A" and B" without this control.

6. There is a nice part in the discussion about regeneration vs normal homeostasis. However, since the authors do not carefully look at normal homeostasis, it is difficult to draw conclusions. At the level of Wg protein expression, clearly it is highly induced upon damage, but what are the levels of epithelial and visceral muscle expression in normal homeostatic tissues? Is it transiently expressed in response to cell death to promote normal proliferation? It is very difficult to determine this from the data presented here and from the previous publication. Some sort of quantification of Wg+ muscle and epithelial cells during normal homeostasis would be appreciated and help clarify the role of Wg, which is clearly playing an important role.

7. The authors state in the introduction that the "source and composition of Droso niche has been extensively studied" but "remains inconclusive". While they provide insight into signaling controlling proliferation, I am not really convinced they address a niche (see title).

Additional points:

In the introduction, the authors should clarify when they are speaking about the mammalian gut vs Drosophila gut as it is very unclear. Also, they should spell out what a "CRC cell line" is.

Unless I missed it, there is no reference to the arrows in Fig. 5C'.

The authors should reference Xu, 2011, which looks at ISC maintenance in response to loss of arm, and Amcheslavsky, 2011 that examines Myc IR with and without DSS treatment.

In Figure S3, the authors use negatively marked clones and quantify the number of cells per clone. In the intestine, usually the MARCM is used as the perdurance of GFP causes problems in negatively marked clones. Can the authors be sure that they can reliably identify all cells of the clone?

The manuscript was at times difficult to read with so much information in the Supplementary text

including entire sections of the results.

Note:
In light of the second manuscript which is under review elsewhere, I am not convinced these two manuscripts stand on their own. Many of the important controls I mention in point 1 are in fact present in this second manuscript and need to be included here as well. There is also much overlap in terms of understanding regulation downstream of activated Wg signaling, leading to duplicate findings on Myc and Jak/Stat acting downstream of Wg.

Referee #2:

The manuscript is straightforward, well-written and well-carried out. The authors show that Wg protein is specifically activated in ISC/Ebs during damage, but not in the surrounding muscle, which maintains its level of Wg constant. When Wg and Wls are knocked down by RNAi, they are only found to suppress damage-induced proliferation when knocked down in the ISC/Ebs. The authors confirm these findings using TCF(dominant-negative) overexpression in ISC/Ebs and Fz1/2 mutant clones. Myc, a known target of the pathway shows a role in the ISC/Ebs as would be expected. The manuscript positions the Wnt pathway as downstream of JNK, but working in parallel to Jak/STAT - two other pathways already implicated in the regeneration of this tissue. Finally, the authors show that Wg increases during ageing and that both ISC/Eb and muscle sources of Wg ligand contribute to the ageing phenotype in this tissue.

Importantly, there has been some confusion on the importance of the Wg/Wnt pathway in the Drosophila intestine. This manuscript does much to resolve the role of this pathway and the authors should be commended on their approach. For instance, the findings suggest that the muscle niche is not an important source of Wg, and only shows a weak phenotype in the aged gut.

That said, the findings should be confirmed with a second RNAi construct, in case of RNAi off-target effects, or (preferably) conventional mutant alleles for components of the pathway. Mutant alleles for all the genes studied here are available and should be tested to corroborate the authors' results.

Major Points:

1. The Wg RNAi efficacy needs to be confirmed by RT-qPCR. Overexpression of the two RNAi lines using the esg-Gal4 (during damage) versus the how-Gal (before damage) could confirm these trangenes work. Importantly, the same qPCR could be applied to confirm increases in Wg expression after damage, even when it is knocked down in the surrounding muscle cells, or enterocytes. Because both the surrounding muscle and the ISC/Ebs are sources of the ligand - it is suprising that only the ISC/Eb source is needed for the regenerative response. The levels of Wg tested by qPCR in combination with the RNAi might reveal the relative contributions of these Wg sources in this tissue.
2. The clonal analysis needs work. The authors make Fz1/2 double mutant clones (Fig S3), and quantify their sizes. Why not try this same strategy using Arm mutant clones? Because much of the manuscript relies on the RNAi lines, it would be good to corroborate these findings using the effector of the Wnt pathway: Arm/bCat. The same applies to the Myc RNAi - why not try making Myc mutant clones? These should be done using the MARCM system which is more reliable than using the loss of GFP in the intestinal epithelium, and the clones should be tested before and during damage. Second, the clone sizes are way too large for this system (reaching 70 cells/clone during damage!!), suggesting that they are actually polyclones - where two or more clones have merged into one. These quantifications should be redone carefully in order to make sure it is the size rather than the frequency of clones that is changing over time. Both clone size and frequency could be calculated easily using the MARCM system to test ISC proliferation versus ISC maintenance/self-renewal. MARCM clones for Arm and Myc will strengthen this manuscript significantly and the authors should consider moving these data to the main body of the text rather than burying them in the supplementary information.
3. If the clones do not work, would the heterozygous mutant lines used in Fig 6 (dm4/+,

dmG0139/+, wgCX4/+) confirm the results of the regeneration experiments shown in Fig 1-4?

Minor Points:

1. Fig 1B and 1B': the arrows need to be corrected, the upper ones don't point out the same cells in B vs. B'.

Note:

The first study is about regeneration and the role of Wg/Myc. This second one focuses on the mutation of Apc, building on the findings from Craig Micchelli's lab. There are no duplicated figures or data. Of course since Apc is working downstream of Wg, both papers are about Wg signaling.

The main issue of concern is the overlap in positioning the Wg and Jak/STAT pathways. Here they could have put the data altogether in one manuscript. In the first paper, the authors report that Jak/STAT activation does not induce Wg expression, but that Wg activity is required for Jak/STAT-induced overproliferation. Thus, they argue that the pathways are working in parallel during regeneration. In the second paper, they report that Upd and Jak/STAT are actually activated downstream of Wg, and that Jak/STAT activity is required for Wg-induced overproliferation. The authors also position the JNK pathway upstream of Wg in the first manuscript, and Myc downstream of Wg in both manuscripts. Again, data is not duplicated between the papers, but there is significant overlap in content, particularly since overall: JNK is upstream of Wg, and Wg is upstream of Jak/STAT.

Referee #3:

The Drosophila midgut has emerged as a premiere stem cell model and there is now considerable interest in using the system to study tissue renewal and the ageing processes. Cordero et al have submitted the study entitled, "An Inductive Stem/Progenitor Niche Regulates Stem Cell Proliferation in Response to Acute and Long-Term Stress in the Drosophila adult midgut". The authors conduct an investigation of Wnt/Wg regulation using a standard molecular genetic toolkit. Despite an eye-catching title, the experiments performed offer little actual insight into the nature of the intestinal stem cell niche or the finely calibrated molecular interactions necessary to maintain tissue homeostasis. Therefore, a number of issues still remain to be addressed before publication.

Suggestions that could significantly improve the manuscript:

1. The authors claim "that production of the ligand Wg from intestinal stem cells (ISCs)/enteroblasts (EBs) is induced upon damage or stress in the midgut." However, studies were performed using the Wnt/Wg antibody, leaving open the possibility that Wg protein distribution or transport may have changed, but not the site of production. The authors should perform double fluorescent in situ analysis to test whether or not the site of Wg production in the midgut actually changes upon damage or stress. The need for additional clarification here is underscored by the apparent variability of the Wg antibody staining protocol under the conditions used by Cordero et al. If we compare controls in Fig. 1B' with those in Fig. 1G' (or Fig. 1E' with those in Fig. 1H') it is clear that the antibody staining results are quite variable even among wild type samples. Since no quantitation of the magnitude or the number of samples analyzed is presented this key claim of the manuscript is ultimately unconvincing.

Cordero et al go on to address the cellular requirement of Wnt/Wg in the midgut using Gal 4 drivers. The central problem with the authors' interpretation is that other plausible explanations of the phenotype have not been ruled out. The driver line used in these experiments (esg Gal4) and throughout the manuscript is a general one for the organism, although it exhibits some degree of spatial restriction in the gut. Thus, the reported observations are just as likely to be an indirect consequence of Wg knockdown on organismal physiology (e.g. a shift in the balance of commensal/pathogenic bacteria titer, increased nutrient consumption, alteration of circadian rhythm, etc).

Conversely, the authors conclude that "Wg ....but not the VM is required for the acute proliferative response of ISCs to damage in the Drosophila adult midgut". From the experiments performed the authors cannot exclude the role of VM. For example, how ts may simply be expressed at low levels and the RNAi effect from the two drivers differ accordingly. In fact data in the paper indicates that the wg IR transgene appears to be only mildly effect as Wg antibody is still detectable even after very long periods of transgene induction (Fig. 2B). This experiment needs a positive control.

2. The authors claim that Wg is "required for ISC proliferation during the regenerative response to acute damage." In the literature, it is now well documented that Wnt/Wg signaling is required for the maintenance of ISCs through a mechanism in which differentiation is inhibited specifically in the stem cell (Lin et al., 2008; Lin and Xi, 2008; Lee et al., 2009; Xu et al., 2011). That is to say, according to the current model, loss of Wnt/Wg leads to reduction of ISC proliferation as stem cells differentiate and are ultimately lost from the midgut. The authors spend considerable time documenting this obvious corollary to the extant literature under conditions of "acute and long term stress". Cordero's result is wholly predictable and tells us nothing new about gut homeostasis.

3. The authors claim that their "data place Wg downstream of JNK/SAP activation and upstream of Myc upregulation." It is the experimental design that is at issue here. A meaningful demonstration of genetic epistasis requires the use of null alleles, which completely remove gene function. Cordero et al. rely on RNAi, which only partially removes gene function. Thus, the data generated here is meaningless with respect to epistasis. Further, once epistasis can be established, an experimentally conservative interpretation dictates that a model in which genes act in parallel pathways also be ruled out. Thus, their conclusion is not yet supported. The authors are advised to use null alleles in constructing meaningful genetic pathways.

4. It is clear from Figure 1 that Wnt/Wg is detectable in many cells of the midgut, not just the stem cells. For example, in Fig. 1D, E, F there are clearly very large differentiated cells that are distinct from ISCs being labeled. The authors need to address this. The authors cite negative correlation with Myo1A as evidence for the specificity of the effect. From Fig. 1G, H it appears that Myo1A has a mosaic pattern of expression in enterocytes, could the authors comment on this. The fact that Wg is not detected there may also indicate that it is these fully differentiated cells that are the first to be shed in response to injury. The authors should examine Wg in the newly formed young enterocytes that still express esg to determine the specificity.

5. Can the authors rule out the possibility that the increase in Wnt/Wg signal is actually "bleed through" from the esg (green channel)? It looks like this may be a factor in some of the micrographs presented.

6. The gut epithelium turns over following acute stress. Is it therefore also possible that Wnt/Wg is simply being down regulated in cells of the midgut that are being shed and not being induced at all in esg cells. Can the authors rule this out?

7. The RT-PCR data is also highly variable and does not significantly differ from controls for most stresses assayed. Perhaps one source of Wg is from the muscle, as it appears from Fig. 1A vs. 1C that DSS treatment actually leads to an increase in the number of how +ve muscle cells.

8. It seems like the number of esg cells varies greatly in each panel (Fig. 3A vs. 3B). Why might this be? Could the esg population be heterogenous and made up of multiple cell types? Is it possible that esg expression is induced in some differentiated daughters following DSS?

9. In the legend for Fig. 3 it is stated "esgts>wgIR midguts treated with DSS showed failed regeneration (F)". Looking at the corresponding micrograph this is simply not clear. Please comment on this. What is the reader supposed to see in Fig. 3F?

10. The model presented is not supported by experimental data and is thus of limited value. For example, the model indicates that JNK non-autonomously activates Wnt transduction. What is the evidence for this? The authors need to use an additional readout for Wnt transduction.

11. If JNK acts non-autonomously to activate Wg, which autonomously activates myc, why don't we

see evidence of this non-autonomy in Fig. 4? Also, Wg is known to be required to signal to ISC to maintain them, so why is no myc detected in Fig. 7E?

12. In Fig. 6G the authors claim no difference in the number of Dl cells between gfp and WgIR at 30 days. However, a simple count from the micrographs presented Fig.6A' vs Fig. 6B' indicates a significant difference (~30 vs 15 Dl cells to my eye). The same can be said for Fig.6C' vs. Fig. 6D vs. Fig. 6E. The authors should use representative images to report their data. As it stands the micrographs and photos tell a different story.

13. In the TARGET studies the authors indicate that flies were grown at 22C and not 18C, as is commonly the case. As a result the system was not fully repressed during development and this opens the possibility that the results are complicated by disrupting earlier developmental processes (similar concerns pertain to the analysis using dm/+ and Wg/+ heterozygotes). Controls need to be presented demonstrating that the TARGET system is in fact conditional under these experimental conditions.

14. The introduction supplied is inappropriate and out of context for the study. It seems fragmented, cobbled together and fails to frame a problem.

15. What is the experimental manipulation used to provide long-term stress? How does this differ from ageing?

16. The gut appears to be a complex "layered" tissue. Since the authors are following gene expression levels in multiple layers in response to tissue specific manipulations, it would be helpful to know how deeply the tissue was imaged in each case. Are these single sections or Z sections presented in Figs. 1-6? If z sections are presented are stacks of equal size being compared in each case?

17. Micrographs presented in the paper are confusing. Orientation should be indicated. It appears from other papers published in the field that anterior is always to the left.

18. Scale bars must be included in each data panel presented.

19. The title presented is inappropriate. The running title would more accurately describe the study.

20. The authors fail to provide adequate detail on the methods included in the manuscript to completely understand (or for that matter reproduce) the data reported.

1st Resubmission 21 June 2012

We would like to start by thanking the reviewers for taking the time to carefully assess our original manuscript and for their constructive criticism, which has helped us to significantly develop our work. Please find below a summary of the experiments performed to address the main general issues expressed by all reviewers and a point-by-point answer to all the reviewer's comments.

<u>Extra data required by all reviewers</u>:

A) We used independent *wg* RNAi (*wg-IR*) lines a wls-IR line and also the *wgCX4* allele to definitively show that wg is essential to drive intestinal stem cells (ISC) proliferation during regeneration. Furthermore we unambiguously demonstrate that it is inducible Wg produced within the intestinal epithelium itself and not the visceral muscle, which is required for intestinal regeneration in response to different type of damages.

<u>The following experiments support these conclusions and are presented in new Figures 1-3 and Figures S1 and S2</u>:

1- By using a combination of RT-PCR and protein staining in the tissue we show that driving

wg-IR using the muscle specific drivers *how-gal4* or *mef2-gal4* efficiently knocked down Wg from in basal conditions. Nevertheless, epithelial Wg is upregulated in response to damage. Consistent with this, *how>wg-IR* and *mef2>wg-IR* guts showed normal regeneration in response to multiple damages. These results were confirmed by the use of two-independent *wg-IR* lines plus a *wls-IR* line.

2- Using *esg-gal4* to drive two independent *wg-IR* lines prevents Wg upregulation in response to damage and consequently results in inhibition of ISC proliferation during regeneration. This was also the case of *esg>wls-IR* guts. A combined *esg;how>wg-IR* line showed impaired regeneration comparable to *esg>wg-IR* alone further confirming insignificant contribution of visceral muscle (VM)-derived Wg in regeneration.
3- We used the temperature sensitive '*esg flip out system'* and confirmed the requirement of Wg from the esg^{+ve} cell lineage in regeneration.
4- Furthermore animal heterozygous for the null allele wg^{CX4} showed halved regenerative response.

B) In line with our original findings we confirm that wg secreted from either the epithelium or the VM only are redundant for normal homeostasis.

The following experiments support these conclusions and are presented in new Figure 3 and Figure S3:

1- Unchallenged how>wg-IR and esg>wg-IR intestines maintain a normal number of ISCs over time.
2- *Esg-flip out wg-IR* and *wls-IR* further confirms dispensability of epithelial Wg for normal homeostasis in unchallenged intestines.

C) Canonical Wnt signalling and Myc are required for ISC proliferation during regeneration.

The following experiments support these conclusions and are presented in new Figures 4, 5 and Figures S4 and S5:

1- MARCM clones of *fz1,fz2* loss of function alleles don't proliferate in response to damage and also show a mild decrease in homeostatic ISC proliferation.
2- Pygopus ($pygo^{S123}$) loss of function MARCM clones don't proliferate in response to damage but, unlike *fz1,fz2* clones, display normal homeostatic ISC proliferation.
3- Midguts from dm^4 and dm^{G0139} heterozygotes have impaired midgut regeneration but normal homeostasis.
4- Midguts carrying *myc-IR esg-flip* out clones showed impaired regeneration and homeostatic self-renewal.
5- Midguts carrying Tcf^{DN} esg-flip out clones showed impaired regeneration and homeostatic self-renewal.

D) We demonstrate the Wg secreted from the progenitor cell, the Enteroblast (EB), is the source of Wg, which is required for regeneration. EB-produced Wg induces Myc in ISCs and drives their proliferation during regeneration. This is the first direct demonstration that EBs provide proliferating signals to ISCs and could represent a parallel with the Paneth cell-ISC communication reported in the mammalian intestine.

The following experiments support this conclusion and are presented in new Figure 6:

1- We used the EB specific gal4 driver *Su(H)GBE-gal4* to drive *wg-IR* in the intestine and induced regeneration by feeding flies bacteria (Pe). $Su(H)GBE^{ts}$*>wg-IR* guts showed impaired Wg upregulation in response to damage and almost a complete blockade in ISC proliferation and regeneration.
2- Importantly, knocking down *myc* in EB only did not affect regeneration, further confirming the specific requirement of *myc* within ISCs.

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Point-by-point answers to reviewers' comments:

Referee #1:

Comment:
The role of Wg signalling in the adult fly intestine has been previously investigated (Lin, 2008; Xu, 2011) and Wg protein was shown to be expressed in the visceral muscle and ISC cells at low levels. Moreover, impairment of Wg signalling via TCF dominant negative expression and Frizzled 1, 2 loss of function in clones suggested a general role in ISC proliferation (Lin, 2008). Cordero and colleagues extend these findings by investigating intestinal response to damage and find that Wg is strongly induced in epithelial cells where it is required to promote proliferation in response to stress. Forced activation of Jnk signalling can induce Wg and is required for hyperproliferation in this context. Upon aging Wg expression in both the visceral muscle and epithelial cells is required to drive proliferation. The authors also identify Myc as an important effector downstream of Wg and provide evidence that Jak/Stat acts in a parallel manner to Wg in promoting ISC proliferation, both being essential in response to stress. A role for Myc in ISC proliferation has been previously noted (Amcheslavsky, 2011).
Though several of the findings here have been previously published, this work does provide new insight into how the intestine responds to injury. In addition, placing Wg genetically in respect to the other known pathways induced upon stress in the intestine is useful for the field. While the authors provide compelling evidence that Wg protein levels are strongly inducing upon various types of stress, many important controls of knock-down effects on non-stress conditions are lacking. In addition, I am not convinced that the title is appropriate, as I believe a niche should help contribute to maintenance, which is not clearly demonstrated here (though perhaps this is a bit semantic).

Answer: In light of the reviewers' comments we have re-worded our title. Recent work suggests that stem cell niches are plastic and react to environmental clues. Since secretion of Wg from the epithelial compartment is essential for ISC proliferation in response to damage we propose the presence of a 'regeneration-specific niche' represented by Wg secretion from the enteroblast cells. As described in detail below we have now included all the controls experiments that Reviewer 1 has requested.

Comment:
1. Many important controls are lacking making the authors' claims of stress-specific or ageing-specific roles of Wg signalling impossible to conclusively demonstrate. Do Wg signalling components have a role in normal proliferation or not? If they are generally required for ISC proliferation, then finding that they are also required under stress is not really too surprising. One would like to see non-stress conditions for all genetic contexts. Because there is such a low basal level of PH3+ cells at a per gut level in these authors' experiments, a role for Wg components in normal homeostasis would be best addressed in mitotic clones, preferentially with loss of function alleles, or using the esgGal4 flip-out system (Jiang et al, 2009).

Answer: We thank the reviewer for these constructive comments. We now provide data with all the above-suggested systems. We show several examples in which we have uncoupled ISC proliferation during homeostasis versus regeneration in response to damage:

a) Heterozygous mutants for loss of function alleles of *wg* and *myc* show normal homeostasis but impaired regeneration. On the other hand *esg^{ts} F/O myc-IR* blocked regeneration but also markedly affects homeostatic stem cell maintenance.

b) Additionally, we have used the inducible *esg-gal4 flip-out* (*esg^{ts} F/O*) system to drive *wg* and *wls* RNAis and show that their depletion from within the epithelial compartment does not affect homeostatic self-renewal but it does prevent regeneration.

c) We also used MARCM clonal analysis to assess the effects of deleting the *fz,fz2* receptors and *pygopus*. In both cases loss of function clones failed to proliferate in response to damage. Nevertheless only *fz,fz2* clones showed reduced homeostatic ISC proliferation.

d) *esg^{ts} F/O Tcf^{DN}* resulted in blocked regeneration but also markedly affected homeostatic stem cell maintenance.

Therefore, regenerative growth absolutely requires epithelial expression of Wg (and we have defined the EB as the secretory cell), full Myc expression (halving the dose of *myc* is

sufficient to inhibit regeneration) and all components of the canonical Wg signalling that we analysed. On the other hand homeostasis requires minimal levels of Myc (halving the dose is not sufficient to affect ISC maintenance), the Fz, Fz2 receptors and Tcf signalling.

Comment:

Specifically: In Fig 1, 2, 6 . Esg>Wg-IR in unstressed conditions (MARCM clones or esg flip-out); In Fig. 6 . Esg>Wg-IR, how>wgIR, dm/+, wg/+ all in unstressed conditions. In Fig S2- wgIR; wlsIR in unstressed (cell #/ clone at a given time point relative to wild-type); Fig. S3 TCF Dominant negative in unstressed conditions; Fig. S4 esg>Myc-IR and TCF-DN in young/unstressed clones or esg flip-out; Fig. S5, esg>WgIR in young/unstressed clones or esg flip-out, and Fig. S6 esg and how>wg-IR in young/unstressed clones or esg flip-out.

Answer: All these controls are now included and presented in new Figures 1-5, 7 and Supplementary Figures S1-S5 and S7

Comment:

In addition, the effect of MycRNAi on unstressed ISCs was previously reported to block ISC proliferation in Amcheslavsky, 2011: "Over a short duration of 2 d, the Myc RNAi did not cause a significant change in ISC growth or division (Fig. 7, A and B). However, the Myc RNAi, for 6 d or longer, did cause a halt of division (unpublished data)". This reference to previous work on Myc in the fly intestine should be cited. Moreover, the previous work suggests that the effect seen by Cordero and colleagues on proliferation, stated as being damage-dependent, may simply a block in proliferation as the authors themselves seem to indicate on p. 9.

Answer: We have now cited this work in the relevant results section. We had not mentioned it, as it appears as data not shown in that paper. Consistent with previous work, we show that sustained Myc depletion through RNAi does lead to a loss of stem cells and therefore we confirmed the permissive role for Myc in normal ISC proliferation. Nevertheless, our results with Myc heterozygotes also suggest an instructive role for Myc during midgut regeneration and ageing. Myc heterozygous intestine are normal and show a normal number of ISCs but have impaired regeneration and age-dependent ISC hyperproliferation (new Figures 5, 8 and S5). All together our results suggest that there is a level of Myc required for homeostasis and another required for efficient regeneration. These data supports the potential therapeutic benefits of partially blocking Myc activity.

Comment:

2. The authors use esgGal4 UAS-GFP as a marker of ISC/EB cells (Fig. 1), however upon rapid proliferation due to damage, GFP is likely present in recently produced EC cells. They should assess the nuclear size of the cells expressing Wg in the epithelia to see if young ECs can also express Wg. In addition, in the images shown in Fig 1A, C , the express of Wg in the muscle is not very clear to me (these appear to be cells between the howGal4 expressing cells?). Since the major finding here is of high level of epithelial expression of Wg upon damage, I am not sure it is extremely important whether or not some young EC cells express Wg. The authors should be cautious about their interpretation that it is completely ISC/EB specific however.

Answer: We now present improved images of Wg staining in the VM and showed specific knock down by protein staining and transcript levels (new Figures 1 and S1). The reviewer is correct. It is a common feature in regenerating and stressed midguts, to see *esg>gfp*$^{+ve}$ cells with intermediate size nuclei. Moreover we have now looked at the expression of *Su(H)-gal4,UAS-gfp*, a marker of EBs in control and damaged midguts and this behaves similarly to *esg>gfp*. It is really difficult to take immature ECs out of the equation because there are no specific drivers described for this cell type. Furthermore, it is not clear how different they are from EBs, both being transient progenies of the ISCs. In any case, our results unambiguously rule out mature ECs as the source of Wg during regeneration and demonstrate that EBs are an essential source of the ligand in this context (new Figure 6). We also tested the available *delta-gal4* driver (as an ISC driver) but it did not label ISCs reliably in our hands (not shown).

In any case the remarkable blockade of regeneration via knockdown of Wg in EBs (*Su(H)GBE*^{ts}*>wg-IR*) leads us to conclude that EBs are the key Wg-producing cells in regeneration. Additionally, we have now mentioned in the text that regenerating midguts show Wg staining in cells that are *esg*$^{+ve}$ and *Su(H)*$^{+ve}$ but have intermediate sized nuclei and presumably represent early enterocytes. We do not believe this majorly affects the interpretation of our data.

Comment:

3. The knock-down of Wg using a howGal4 driver specific for the muscle seems to result in a significant reduction of PH3+ cells in response to Bleomycin in Fig. 3L. The authors do not comment on this in the text. Is there something different with Bleo damage? Also, the knock-down of Wg in the esg+ cells does not bring proliferation down to wild-type levels. Is this due to expression of Wg in another cell population, possibly even the muscles? If tubulinGal4 is used or combined esg, How Gal4 does this change?

Answer: Bleomycine has been shown to induce regeneration by damaging the intestinal epithelium and it is therefore similar to the effect of *Pe* feeding, while DSS is thought to damage the basal membrane (Amcheslavsky et al, 2009). In spite of the slight apparent decrease in the value of pH3 counts in *how>wg-IR* midguts treated with Bleomycine, statistical analysis did not show a significant difference with control midguts. Furthermore, no detectable difference was observed in *how>wls-IR* midguts treated with Bleomycine or midguts of any of the *how-gal4* driven transgenes treated with DSS or Pe (new Figure 2 and Figure S2). We have also used the alternative visceral muscle driver *mef2-gal4* to knock down Wg and seen the same as in *how>wg-IR* (Figures 2K and Figure S2C-E). Furthermore, midguts with combined knock down of Wg using an *esg-gal4, UAS-gfp; how-gal4* driver (*esg^{ts};how>wg-IR*) (new Figure S3K, L) show pH3 counts similar to those in *esg>wg-IR* midguts (new Figure 2L). Altogether these data lead us to conclude that Wg from the visceral muscle is not required for intestinal regeneration.

Comment:
4. In Fig. 3A-I, the authors are not measuring directly newly produced cells with an esg-flip out technique (which would be more informative), but merely esgGal4 UAS-GFP expression upon various stress and genetic manipulation. That being the case, I am not sure that panels G-I are very informative and could potentially be removed as the quantification is in 3J.

Answer: We have now used the *esg F/O* system and the data is presented in new Figure 5.

Comment:
5. As mentioned above, a non-stress control is lacking for Fig. S4. It would also be useful to see a wild-type control with Delta staining as it is difficult to conclude anything from Sup. 4 A" and B" without this control.

Answer: All of the requested controls have now been included as well as data on *esg F/O Tcf^{DN}* midguts. The new data is presented in new Figure 4 and Figure S4. Please note that images of Delta staining from 7 and 30 day-old control midguts are presented in new Figures S3 and S5.

Comment:
6. There is a nice part in the discussion about regeneration vs normal homeostasis. However, since the authors do not carefully look at normal homeostasis, it is difficult to draw conclusions. At the level of Wg protein expression, clearly it is highly induced upon damage, but what are the levels of epithelial and visceral muscle expression in normal homeostatic tissues? Is it transiently expressed in response to cell death to promote normal proliferation? It is very difficult to determine this from the data presented here and from the previous publication. Some sort of quantification of Wg+ muscle and epithelial cells during normal homeostasis would be appreciated and help clarify the role of Wg, which is clearly playing an important role.

Answer: We now provide substantial data on the regulation of homeostatic ISC proliferation in response to Wg knock down from the VM and epithelium. Please see our detailed answer to this issue at the beginning of the answers to the reviewer comments. We have added improved new images of Wg staining in the visceral muscle, including transversal sections, which clearly show efficient Wg knockdown via two independent muscle drivers (new Figure 1A-B''' and Figure S2C-E). Furthermore we did RT-PCR and confirmed efficient knock down of *wg* from the muscle in homeostatic conditions (Figure S1A). We have also looked at epithelial Wg in homeostatic and regenerating midguts. Importantly, we unambiguously demonstrate by antibody staining and RT-PCR that Wg upregulated in response to damage derives from the *esg^{+ve}* cells. All these new data are now presented in Figure 1 and Figure S1.

Comment:

7. The authors state in the introduction that the "source and composition of Droso niche has been extensively studied" but "remains inconclusive". While they provide insight into signalling

controlling proliferation, I am not really convinced they address a niche (see title).

Answer: The classic definition of a stem cell niche is the microenvironment that maintains ICS self-renewal. However, recent research has uncovered that niches are highly plastic and adapt to different conditions. We would like to emphasize our new data on Wg secretion from enteroblast, which contributes to a regenerative niche. This is a timely observation in the field as it provides the first direct functional evidence for a role of the EB in the regulation of ISC proliferation. It has been recently speculated that the EB is the functional equivalent of the Paneth cells, which remarkably express Wnt3 and seem to constitute a niche for the mammalian ISC (Jiang & Edgar, 2012). We accept the reviewer's criticism over what is a niche and thus have softened our claims in addition to discussing these concepts properly.

Our data strongly suggests that Wg is specifically regulated in response to damage (DSS, Bleomycine, *Pe* feeding) or stress (JNK overexpression and ageing) rather than as a general outcome of increased proliferation in the midgut. In support of the latter, overexpression of Upd or loss of Hippo, which efficiently increases ISC proliferation (Beebe et al, 2010; Jiang et al, 2009; Karpowicz et al, 2010; Ren et al, 2010; Shaw et al, 2010; Staley & Irvine, 2010) does not result in upregulation of Wg (Figure S8 and data not shown).

Comment:
Additional points:
In the introduction, the authors should clarify when they are speaking about the mammalian gut vs Drosophila gut as it is very unclear. Also, they should spell out what a "CRC cell line" is.
Unless I missed it, there is no reference to the arrows in Fig. 5C'.

Answer: We have now made these corrections

Comment:
The authors should reference Xu, 2011, which looks at ISC maintenance in response to loss of arm, and Amcheslavsky, 2011 that examines Myc IR with and without DSS treatment.

Answer: These studies are now cited and properly discussed.

Comment:
In Figure S3, the authors use negatively marked clones and quantify the number of cells per clone. In the intestine, usually the MARCM is used as the perdurance of GFP causes problems in negatively marked clones. Can the authors be sure that they can reliably identify all cells of the clone?

Answer: We have now revised our original clonal analysis and replaced it with positively marked MARCM *fz,fz2* and *pygo* clones. The conclusions are essentially the same and both component of Wg signalling are required for intestinal regeneration. The new clonal analysis is presented in Figure 4.

Comment:
The manuscript was at times difficult to read with so much information in the Supplementary text including entire sections of the results.

Answer: We have now reordered all the major results into the main figures with only confirmatory data in the supplementary text.

Comment:
Note:
In light of the second manuscript which is under review elsewhere, I am not convinced these two manuscripts stand on their own. Many of the important controls I mention in point 1 are in fact present in this second manuscript and need to be included here as well. There is also much overlap in terms of understanding regulation downstream of activated Wg signalling, leading to duplicate findings on Myc and Jak/Stat acting downstream

Answer: We have included the revised version of our manuscript on the mechanisms mediating the role of Adenomatous Polyposis Coli (Apc) in the fly midgut, which we have submitted back to Development, concurrently with our submission here. This manuscript has evolved significantly to now uncover and characterize a novel paracrine cross talk between ISCs and its progeny the enterocytes (ECs), which involves three pathways hyperactivated in colorectal cancer. Essentially, we found that loss of *Apc* leads to the upregulation of Upd/(IL)- and EGF-type of ligands in a Myc dependent manner. Importantly, we demonstrate that non-cell autonomous regulation of *Upd3* in

ECs, which mediates ISC hyperproliferation in response to high Wg signalling, is dependent on the activation of EGFR also in ECs. Furthermore, EGFR and Jak/Stat signalling activation are essential mediators of the *Apc*-dependent phenotype in the fly midgut. Additionally, our new work includes data on mouse models and human CRC, which points to conservation in the mechanisms mediating the role of Apc in intestinal proliferation. Therefore, while our Development submission focuses on the identification of the molecular mechanisms mediating intestinal hyperplasia in response to Apc loss, our revised manuscript for EMBO J address the role of endogenous Wg and downstream signalling in the regenerative response to damage as well as during homeostatic self-renewal. Therefore these two manuscripts represent independent studies. Moreover the only data in our EMBO J submission on Jak/Stat signalling indicates that, instead of downstream as in the case of Apc, it is activated in parallel to Wg signalling in response to damage in the wild type midgut epithelium. We have included this as a supplementary figure but we are happy to eventually remove it, as it will not affect any of the conclusions from this study.

Referee #2:

Comment:
The manuscript is straightforward, well-written and well-carried out. The authors show that Wg protein is specifically activated in ISC/Ebs during damage, but not in the surrounding muscle, which maintains its level of Wg constant. When Wg and Wls are knocked down by RNAi, they are only found to suppress damage-induced proliferation when knocked down in the ISC/Ebs. The authors confirm these findings using TCF(dominant-negative) overexpression in ISC/Ebs and Fz1/2 mutant clones. Myc, a known target of the pathway shows a role in the ISC/Ebs as would be expected. The manuscript positions the Wnt pathway as downstream of JNK, but working in parallel to Jak/STAT - two other pathways already implicated in the regeneration of this tissue. Finally, the authors show that Wg increases during ageing and that both ISC/Eb and muscle sources of Wg ligand contribute to the ageing phenotype in this tissue.
Importantly, there has been some confusion on the importance of the Wg/Wnt pathway in the Drosophila intestine. This manuscript does much to resolve the role of this pathway and the authors should be commended on their approach. For instance, the findings suggest that the muscle niche is not an important source of Wg, and only shows a weak phenotype in the aged gut.
That said, the findings should be confirmed with a second RNAi construct, in case of RNAi off-target effects, or (preferably) conventional mutant alleles for components of the pathway. Mutant alleles for all the genes studied here are available and should be tested to corroborate the authors' results.

Answer: Please note that we have used two independent wg RNAi transgenes (*UAS-wg-IR* and *UAS-wg-IR^{KK}*) and a *wls-IR* in all our regeneration and homeostasis experiments (new Figures 1-3 and Figures S1 and S2). Furthermore we now present new data on regeneration and homeostasis using mutant alleles of *wg, myc, fz, fz2* and *pygo*.

Comment:

Major Points:
1. The Wg RNAi efficacy needs to be confirmed by RT-qPCR. Overexpression of the two RNAi lines using the esg-Gal4 (during damage) versus the how-Gal (before damage) could confirm these trangenes work. Importantly, the same qPCR could be applied to confirm increases in Wg expression after damage, even when it is knocked down in the surrounding muscle cells, or enterocytes. Because both the surrounding muscle and the ISC/Ebs are sources of the ligand - it is surprising that only the ISC/Eb source is needed for the regenerative response. The levels of Wg tested by qPCR in combination with the RNAi might reveal the relative contributions of these Wg sources in this tissue.

Answer: Please note that, as described in detail in the general answer to all reviewers and also in the answer to reviewer 1, we have now used a combination of RT-PCR and improved protein staining to address the efficiency of the RNAi in the different domains. We show that driving wg-IR using the muscle specific drivers how-gal4 or mef2-gal4 efficiently knocked down Wg in basal conditions, while epithelial Wg is still upregulated in response to damage (new Figures 1, S1 and S2)

We have also looked at Wg levels (transcript and protein) in esg>wg-IR midguts before and after damage. Our results clearly show that upregulation of Wg in response to damage is completely

blocked in esg>wg-IR midguts (Figure 1 and S1).

We do not see any conditions in which Wg is found in (MyoIA>gfp^{+ve}) Enterocytes. Consistently, Wg remains upregulated in damaged (MyoIA>wg-IR) midguts and, in the same way as (how>wg-IR) midguts, (MyoIA>wg-IR) midguts show normal regeneration in response to all damages used (Figure 2 and Figure S2).

Comment:
2. The clonal analysis needs work. The authors make Fz1/2 double mutant clones (Fig S3), and quantify their sizes. Why not try this same strategy using Arm mutant clones? Because much of the manuscript relies on the RNAi lines, it would be good to corroborate these findings using the effector of the Wnt pathway: Arm/bCat. The same applies to the Myc RNAi - why not try making Myc mutant clones? These should be done using the MARCM system which is more reliable than using the loss of GFP in the intestinal epithelium, and the clones should be tested before and during damage. Second, the clone sizes are way too large for this system (reaching 70 cells/clone during damage!!), suggesting that they are actually polyclones - where two or more clones have merged into one. These quantifications should be redone carefully in order to make sure it is the size rather than the frequency of clones that is changing over time. Both clone size and frequency could be calculated easily using the MARCM system to test ISC proliferation versus ISC maintenance/self-renewal. MARCM clones for Arm and Myc will strengthen this manuscript significantly and the authors should consider moving these data to the main body of the text rather than burying them in the supplementary information.

Answer: We have now revised our original clonal analysis and replaced it with positively marked MARCM *fz,fz2* and *pygo* clones. Both components of Wg signalling are required for intestinal regeneration. The new clonal analysis is presented in Figure 4. Armadillo/β-Catenin loss of function clones have been presented in two studies in the midgut (Lin et al, 2008; Xu et al, 2011). Nevertheless, such clones are rarely recovered in any tissue where this has been attempted (Yashi Ahmed and Jean Paul Vincent, personal communication). Furthermore, since Armadillo/β-Catenin is an essential component of the adherens junctions, results from its loss of function are likely to have implications beyond an effect on Wg signalling. This is further supported by several reports on the effect of disruptions of adherens junctions in ISC proliferation and differentiation, in ways that do not involve Wnt signalling specifically but rather affect other pathways, such as Notch and Insulin signalling (Choi et al, 2011; Maeda et al, 2008).

Comment:
3. If the clones do not work, would the heterozygous mutant lines used in Fig 6 (dm4/+, dmG0139/+, wgCX4/+) confirm the results of the regeneration experiments shown in Fig 1-4?

Answer: This has been carried out and results show that midguts from animals heterozygotes for wg^{CX4}, dm^{4} and dm^{G0139} have approximately a 50% reduction in regeneration (new Figure 2M and Figure 5L)

Comment:
Minor Points:
1. Fig 1B and 1B': the arrows need to be corrected, the upper ones don't point out the same cells in B vs. B'.

Answer: All figures have been revised for this new manuscript and many completely changed. We have carefully edited the new figures to avoid these problems.

Comment:
Note:
The first study is about regeneration and the role of Wg/Myc. This second one focuses on the mutation of Apc, building on the findings from Craig Micchelli's lab. There are no duplicated figures or data. Of course since Apc is working downstream of Wg, both papers are about Wg signalling.
The main issue of concern is the overlap in positioning the Wg and Jak/STAT pathways. Here they could have put the data altogether in one manuscript. In the first paper, the authors report that Jak/STAT activation does not induce Wg expression, but that Wg activity is required for Jak/STAT-induced overproliferation. Thus, they argue that the pathways are working in parallel during regeneration. In the second paper, they report that Upd and Jak/STAT are actually activated downstream of Wg, and that Jak/STAT activity is required for Wg-induced overproliferation. The

authors also position the JNK pathway upstream of Wg in the first manuscript, and Myc downstream of Wg in both manuscripts. Again, data is not duplicated between the papers, but there is significant overlap in content, particularly since overall: JNK is upstream of Wg, and Wg is upstream of Jak/STAT.

Answer: We have extensively addressed this issue in the corresponding answer to reviewer 1. We have included the revised version of our manuscript on the mechanisms mediating the role of Adenomatous Polyposis Coli (Apc) in the fly midgut, which we have submitted back to Development, concurrently with our submission here. This manuscript has evolved significantly to now uncover and characterize a novel paracrine cross talk between Wnt/Myc, EGFR and Jak/Stat signalling in colorectal cancer. Additionally, our new work includes data on mouse models and human CRC, which points to conservation in the mechanisms mediating the role of Apc in intestinal proliferation. Critically, while our Development submission focuses on the identification of the molecular mechanisms mediating intestinal hyperplasia in response to Apc loss, our revised manuscript for EMBO J addresses the role of endogenous Wg and downstream signalling in the regenerative response to damage as well as during homeostatic self-renewal. Therefore these two manuscripts represent independent studies. Moreover the only data in our EMBO J submission on Jak/Stat signalling indicates that it is activated in parallel (instead of downstream) to Wg signalling in response to damage to wild type midgut epithelium. We have included this as a supplementary figure (Figure S8) but we are happy to remove it, as it will not affect any of the conclusions of our paper.

Referee #3:

Comment:
The Drosophila midgut has emerged as a premiere stem cell model and there is now considerable interest in using the system to study tissue renewal and the ageing processes. Cordero et al have submitted the study entitled, "An Inductive Stem/Progenitor Niche Regulates Stem Cell Proliferation in Response to Acute and Long-Term Stress in the Drosophila adult midgut". The authors conduct an investigation of Wnt/Wg regulation using a standard molecular genetic toolkit. Despite an eye-catching title, the experiments performed offer little actual insight into the nature of the intestinal stem cell niche or the finely calibrated molecular interactions necessary to maintain tissue homeostasis. Therefore, a number of issues still remain to be addressed before publication.

Answer: We have now done an in-depth characterization of the role of Wg and downstream signalling in intestinal regeneration as well as in homeostasis. We narrow down the source of epithelial Wg in regenerating midguts to the Enteroblast (EB). Furthermore we analyse the similarities and differences in the requirement for Wg signalling in homeostatic self-renewal versus during regeneration in response to damage.

Comment:
Suggestions that could significantly improve the manuscript:
1. The authors claim "that production of the ligand Wg from intestinal stem cells (ISCs)/enteroblasts (EBs) is induced upon damage or stress in the midgut." However, studies were performed using the Wnt/Wg antibody, leaving open the possibility that Wg protein distribution or transport may have changed, but not the site of production. The authors should perform double fluorescent in situ analysis to test whether or not the site of Wg production in the midgut actually changes upon damage or stress. The need for additional clarification here is underscored by the apparent variability of the Wg antibody staining protocol under the conditions used by Cordero et al. If we compare controls in Fig. 1B' with those in Fig. 1G' (or Fig. 1E' with those in Fig. 1H') it is clear that the antibody staining results are quite variable even among wild type samples. Since no quantitation of the magnitude or the number of samples analysed is presented this key claim of the manuscript is ultimately unconvincing.

Answer: The conclusions of our paper are not simply based on antibody staining data but are supported by extensive functional analysis of domain-specific knock down of Wg or by blocking its secretion using restricted drivers and two independent *wg* RNAi lines in addition to a *wls* RNAi line. We have now included new images with improved Wg staining, which confirm our previous assessment of Wg localization in the tissue. We also provide extensive data confirming the

efficiency of the gal4 drivers and RNAi transgenes used in our study. These new data is presented in new Figures 1, S1 and S2. In our hands *in-situ* hybridizations for *wg* have not worked and the available *wg-lacZ* reporter does not re-capitulate the expression pattern in the VM published (Lin et al., 2008) and instead only labels the midgut-hindgut junction (data not shown).

Tissue staining is only comparable within the same genetic background. Wg staining in *MyoIA>gfp* midgut is not necessarily comparable to that of *esg>gfp* midguts. We have been careful to include the appropriate controls for each of the experimental conditions used. Furthermore we have made an effort to appropriately refer to the figure panels that should be compared in the description of the results to better guide the reader.

Comment:
Cordero et al go on to address the cellular requirement of Wnt/Wg in the midgut using Gal 4 drivers. The central problem with the authors' interpretation is that other plausible explanations of the phenotype have not been ruled out. The driver line used in these experiments (esg Gal4) and throughout the manuscript is a general one for the organism, although it exhibits some degree of spatial restriction in the gut. Thus, the reported observations are just as likely to be an indirect consequence of Wg knockdown on organismal physiology (e.g. a shift in the balance of commensal/pathogenic bacteria titer, increased nutrient consumption, alteration of circadian rhythm, etc).

Conversely, the authors conclude that "Wg ....but not the VM is required for the acute proliferative response of ISCs to damage in the Drosophila adult midgut". From the experiments performed the authors cannot exclude the role of VM. For example, how ts may simply be expressed at low levels and the RNAi effect from the two drivers differ accordingly. In fact data in the paper indicates that the wg IR transgene appears to be only mildly effect as Wg antibody is still detectable even after very long periods of transgene induction (Fig. 2B). This experiment needs a positive control.

Answer: The *gal4* drivers we utilized in this study, in particular (*esg-gal4*), are well-established tools in the field with almost every work published on the fly midguts making use of this driver. Importantly, we have confirmed by protein staining and RT-PCR that *how-gal4* is an efficient driver. *How>wg-IR* undamaged midguts show no signs of Wg staining and have a significant knockdown in *wg* transcript levels (Figures 1 and S1). Nevertheless, in response to damage, *how>wg-IR* still showed significant upregulation of Wg in the epithelial compartment and had no defects in regeneration. Use of the alternative muscle driver *mef2>gal4* led to the same outcome (Figures 1, S1 and 2).

Comment:
2. The authors claim that Wg is "required for ISC proliferation during the regenerative response to acute damage." In the literature, it is now well documented that Wnt/Wg signalling is required for the maintenance of ISCs through a mechanism in which differentiation is inhibited specifically in the stem cell (Lin et al., 2008; Lin and Xi, 2008; Lee et al., 2009; Xu et al., 2011). That is to say, according to the current model, loss of Wnt/Wg leads to reduction of ISC proliferation as stem cells differentiate and are ultimately lost from the midgut. The authors spend considerable time documenting this obvious corollary to the extant literature under conditions of "acute and long term stress". Cordero's result is wholly predictable and tells us nothing new about gut homeostasis.

Answer: We respectfully disagree with the reviewer. Our results, including the new data in this revised manuscript, clearly demonstrate that loss of Wg specifically from either the epithelium or the visceral muscle has no impact on homeostatic stem cell maintenance. Therefore our results are not predictable in consideration of the current literature cited by the reviewer, which postulates the VM as the sole ISC niche (Lin et al., 2008). Regarding the requirement for Wg signalling in ISC differentiation proposed by (Lin et al., 2008), this was indeed not confirmed by (Lee et al, 2009) nor ourselves (Cordero et al, 2009). This issue has not being further addressed in (Lin & Xi, 2008; Xu et al., 2011). In particular, two of the papers cited by the reviewer used Armadillo/β-Catenin loss of function clones to knock down Wg signalling in the midgut (Lin et al., 2008; Xu et al, 2011). Nevertheless, such clones are rarely recovered in any other tissue where this has been attempted (Yashi Ahmed and Jean Paul Vincent, personal communication). Furthermore, since Armadillo/β-Catenin is an essential component of the adherens junctions, results from its loss of function are likely to have implications beyond an effect on Wg signalling. This is further supported by several

reports on the effect of disruptions of adherens junction in ISC proliferation and differentiation in ways that don't involve Wnt signalling but rather affect other pathways, such as Notch and Insulin signalling (Choi et al, 2011; Maeda et al, 2008).

Comment:
3. The authors claim that their "data place Wg downstream of JNK/SAP activation and upstream of Myc upregulation." It is the experimental design that is at issue here. A meaningful demonstration of genetic epistasis requires the use of null alleles, which completely remove gene function. Cordero et al. rely on RNAi, which only partially removes gene function. Thus, the data generated here is meaningless with respect to epistasis. Further, once epistasis can be established, an experimentally conservative interpretation dictates that a model in which genes act in parallel pathways also be ruled out. Thus, their conclusion is not yet supported. The authors are advised to use null alleles in constructing meaningful genetic pathways.

Answer: We are not sure we understand the reviewer's request for us to use null alleles to remove all of the gene function. Null alleles of components of Wg or JNK signalling are embryonic lethal as homozygotes. The use of mitotic clones is affected by the non-autonomous roles well documented for both genes. We provide several lines of evidence, including new data, that points to Wg being downstream of JNK activation in the midgut: a) JNK activation upregulates Wg but the opposite does not happen (new Figure 7B-F). b) Wg knock down inhibits hyperproliferation in the midgut but does not affect JNK activation in conditions of stress. 30 day-old *esg>wg-IR* midguts show significant JNK activation (assessed by pJNK staining and *puckered* expression) in spite of having impaired age-dependent ISC proliferation (Figure S7). Nevertheless, since JNK is not the only mediator of ISC in damaged midguts (Jiang et al., 2009 and data not shown), we have being cautious in our conclusions and state that JNK is at least one of the signals inducing Wg in response to damage.

Comment:
4. It is clear from Figure 1 that Wnt/Wg is detectable in many cells of the midgut, not just the stem cells. For example, in Fig. 1D, E, F there are clearly very large differentiated cells that are distinct from ISCs being labelled. The authors need to address this. The authors cite negative correlation with Myo1A as evidence for the specificity of the effect. From Fig. 1G, H it appears that Myo1A has a mosaic pattern of expression in enterocytes, could the authors comment on this. The fact that Wg is not detected there may also indicate that it is these fully differentiated cells that are the first to be shed in response to injury. The authors should examine Wg in the newly formed young enterocytes that still express esg to determine the specificity.

Answer: The consensus in the field is that (Myo1A) expressed in fully differentiated enterocytes (ECs) and not in immature or early enterocytes. Therefore, highly proliferating midguts, such as those regenerating and have a significant number of newly formed ECs resulted in the patchy appearance of *MyoIA>gfp* expression. Reciprocally, it is common to see *esg>gfp*$^{+ve}$ cells with intermediate size nuclei in regenerating or stressed midguts. Moreover we have now looked at the expression of *Su(H)-gal4,UAS-gfp*, a marker of EBs in control and damaged midguts and the same applies. It is difficult to directly test the role of immature ECs because there are not specific drivers described for this cell type. Furthermore, it is not clear how different they are from EBs, both being transient progenies of the ISCs. In any case our results unambiguously rule out mature ECs as the source of Wg during regeneration. The remarkable blockade of regeneration via knockdown of Wg in EBs (*Su(H)GBE*^{ts}*>wg-IR*) leads us to conclude that EBs are the key Wg-producing cells in regeneration (new Figure 6). Additionally, we have now mentioned in the text that regenerating midguts show Wg staining in cells that are *esg*$^{+ve}$ and *Su(H)*$^{+ve}$ but have intermediate size nuclei and presumably represent early enterocytes.

Comment:
5. Can the authors rule out the possibility that the increase in Wnt/Wg signal is actually "bleed through" from the esg (green channel)? It looks like this may be a factor in some of the micrographs presented.

Answer: We can rule this out due to sequential confocal scanning methods. Furthermore, clearly in the (*MyoIA>gfp*) genotype all Wg cells are GFP negative and all GFP positive cells are Wg negative. Additionally, new Figure 1H and Figure S1I show two examples of upregulation of Wg within the epithelial compartment in cases where there is no GFP.

Comment:
6. The gut epithelium turns over following acute stress. Is it therefore also possible that Wnt/Wg is simply being down regulated in cells of the midgut that are being shed and not being induced at all in esg cells. Can the authors rule this out?

Answer: We can rule this out due to clear Wg upregulation observed by antibody staining and confirmed by qPCR. Most importantly, our functional data clearly suggest that upregulation of Wg is what drives intestinal regeneration in response to damage.

Comment:
7. The RT-PCR data is also highly variable and does not significantly differ from controls for most stresses assayed. Perhaps one source of Wg is from the muscle, as it appears from Fig. 1A vs. 1C that DSS treatment actually leads to an increase in the number of how +ve muscle cells.

Answer: We have now performed a large number of new qPCR experiments. The regulation of the *wg* message is mild but consistent. Importantly, the upregulation of Wg protein, whose specificity is confirmed by knock down with RNAi, is very robust. It should be noted that there could be also post-translational regulation of Wg. Regarding the role of the VM, experiments with two independent VM drivers now shown efficient Wg protein and mRNA knockdown, while intestinal regeneration in response to damage was not affected. The VM is a post-mitotic syncytium and cells do not proliferate.

Comment:
8. It seems like the number of esg cells varies greatly in each panel (Fig. 3A vs. 3B). Why might this be? Could the esg population be heterogenous and made up of multiple cell types? Is it possible that esg expression is induced in some differentiated daughters following DSS?

Answer: The esg^{+ve} is indeed composed by two cell types, ISCs and EBs. This population expansion in regenerating midguts is likely due to fast ISC proliferation, which overcomes the dilution and eventual disappearance of the *gfp* reporter. Therefore newly formed progeny of the ISC are also labelled by *esg.*

Comment:
9. In the legend for Fig. 3 it is stated "esgts>wgIR midguts treated with DSS showed failed regeneration (F)". Looking at the corresponding micrograph this is simply not clear. Please comment on this. What is the reader supposed to see in Fig. 3F?

Answer: We provide clear quantifications, new images, better panel labelling and clear reference to figure panels in the description of the results.

Comments:
10. The model presented is not supported by experimental data and is thus of limited value. For example, the model indicates that JNK non-autonomously activates Wnt transduction. What is the evidence for this? The authors need to use an additional readout for Wnt transduction.

Answer: Previous reports indicate that, in response to damage, stress or ageing, endogenous JNK activation assessed by *puckered* upregulation is mostly restricted to ECs or esg^{+ve} cells of abnormal nuclear size (Biteau et al, 2008). We confirmed this by pJNK staining (new Figures S6 and S7). Our data indicates that (mature) ECs do not express Wg. Thus we postulate in our model that JNK activation in ECs induces the production of Wg, not Wg pathway activity, non-autonomously.

Comment:

11. If JNK acts non-autonomously to activate Wg, which autonomously activates myc, why don't we see evidence of this non-autonomy in Fig. 4? Also, Wg is known to be required to signal to ISC to maintain them, so why is no myc detected in Fig. 7E?

Answer: Myc staining is notoriously difficult. The levels we see in regenerating guts are remarkable. The basal levels may be too low to be picked up by antibody staining, which does not mean the protein is not expressed there. We believe that the same reasons prevent us from reliably detecting non-autonomous Myc in *MyoIA>*hep^{wt} midguts (not shown). *MyoIA>*hep^{wt} midguts show a more modest proliferative phenotype than *esg>*hep^{CA} counterparts. Even though sufficient to see Wg upregulation in $MyoIA^{-ve}$ cells, Myc levels are not clearly picked up by the antibody in *MyoIA>*hep^{wt} midguts. In our hands *MyoIA>*hep^{CA} causes significant disruption of tissue integrity (not shown), which prevents reliable tissue staining. We do however see clear upregulation of Myc during regeneration, in *esg>*hep^{CA} midguts or in aged tissues. These data is now presented in (new Figure 7

and Figure S6). Furthermore we present data showing that Myc activation is exclusively required in ISCs (new Figures 5 and 6).

Comment:
12. In Fig. 6G the authors claim no difference in the number of Dl cells between gfp and WgIR at 30 days. However, a simple count from the micrographs presented Fig.6A' vs Fig. 6B' indicates a significant difference (~30 vs 15 Dl cells to my eye). The same can be said for Fig.6C' vs. Fig. 6D vs. Fig. 6E. The authors should use representative images to report their data. As it stands the micrographs and photos tell a different story.

Answer: This issue has been clarified in the corresponding result section and presented in new Figures 3-5 and Figures S3-S5. Control 30 day-old midguts do have more Delta^{+ve} cells than their young counterparts. This is consistent with the ageing phenotype previously described (Biteau et al, 2008). Consequently, all genotypes in which ageing is prevented do not show the age-dependent increase in Delta^{+ve} cells. Additionally, if midguts have impaired ISC maintenance, then the number of Delta^{+ve} cells drops from 7 to 30 days of age as in the case of *esg>myc-IR* and *esg>TCF^{DN}* midguts.

Comment:
13. In the TARGET studies the authors indicate that flies were grown at 22C and not 18C, as is commonly the case. As a result the system was not fully repressed during development and this opens the possibility that the results are complicated by disrupting earlier developmental processes (similar concerns pertain to the analysis using dm/+ and Wg/+ heterozygotes). Controls need to be presented demonstrating that the TARGET system is in fact conditional under these experimental conditions.

Answer: We have carefully tested the system at 22°C and confirmed that it is inactive at this temperature. Furthermore, for this revised version of the manuscript some of the key experiments have been repeated and all the new experiments where done switching from 18°C to 29°C. This has not affected our results.

Comment:
14. The introduction supplied is inappropriate and out of context for the study. It seems fragmented, cobbled together and fails to frame a problem.

Answer: We thank the reviewer for this constructive suggestion and have now revised the introduction.

Comment:
15. What is the experimental manipulation used to provide long-term stress? How does this differ from ageing?

Answer: It is generally accepted that ageing phenotypes are caused by long-term stress. Work form the Jasper lab has demonstrated the activation of stress-activated kinase JNK in ageing midguts. Furthermore, the Lemaitre lab has shown that the ageing phenotypes in the midgut are caused to a great extent by the growth of the gut microbiota, which ends up activating immune and stress response pathways. We nevertheless do not use ‘long term stress’ as an equivalent of ageing in the new manuscript.

Comment:

16. The gut appears to be a complex "layered" tissue. Since the authors are following gene expression levels in multiple layers in response to tissue specific manipulations, it would be helpful to know how deeply the tissue was imaged in each case. Are these single sections or Z sections presented in Figs. 1-6? If z sections are presented are stacks of equal size being compared in each case?

Answer: Images represent a maximum intensity projection of a stable number of Z-sections. This is clarified in the Methods.

Comment:
17. Micrographs presented in the paper are confusing. Orientation should be indicated. It appears from other papers published in the field that anterior is always to the left.
Answer: We have consistently oriented all images. Orientation is indicated in the figure legends

Comment:
18. Scale bars must be included in each data panel presented.

Answer: We have now included scale bars in all figures.

Comment:
19. The title presented is inappropriate. The running title would more accurately describe the study.

Answer: The main and running titles have been revised.

Comment:
20. The authors fail to provide adequate detail on the methods included in the manuscript to completely understand (or for that matter reproduce) the data reported.

Answer: We have being careful to include detailed description of the methods utilized.

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2nd Editorial Decision 06 July 2012

Thank you once more for submitting a revised version of your study that reports on the Wg-source driving ISC-proliferation during regeneration for consideration to The EMBO Journal editorial office.

Your work has been assessed by two of the original referees that are overall supportive of publication, pending a few further clarifications. I would be grateful if you incorporate the stainings requested from ref#1 (point 1) and Wg-quantifications as remarked in his/her third point.

I would agree with this scientist to keep FigS8 in the ultimate version of your study.
Please submit a finally amended version to your earliest convenience to enable formal acceptance and efficient production.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORTS:

Referee #1:

This second version of Cordero et al., is much improved and has addressed many of my concerns in the initial version. The new versions of both papers now are significantly different enough to stand on their own. I have a few remaining questions and comments regarding some of the new data:

1. The source of Wg in homeostatic and stress conditions is still a bit confusing to me:

The authors show in Fig. 1A that Wg is present in both the muscle and esg+ ISC and EB cells, as has been previously described. In their knockdown experiment (1B) of Wg in the muscle with the howGal4ts, it looks like muscle staining is lost but esg+ ISC/EB staining is retained. Where is this Wg protein coming from? Knockdown in the esg+ population (esgts; Fig. S1F') still shows esg+ cells retaining Wg protein. While the qPCR data (Fig. S1A -note that the driver has been accidently omitted in this figure-) suggest that muscle but not esg drivers can decrease the total Wg with wg-IR, suggesting that a majority present in the ISC+EBs comes from the muscle, it is still possible that both muscles and ISC/EB cell types produce Wg under homeostatic conditions. It would thus be

useful to include the Wg staining in esgGal4, howGal4 UAS-WgIR as a control in addition to the quantification done in this background of PH3 (2L) and Delta (S3Q).

Similarly, comparing Fig. 1F' to 1C', it seems as if Wg is still somewhat induced despite esg>wgIR, which could be due to incomplete knock-down or perhaps another (muscle) source of Wg. That being said, the data in 1H showing increased epithelial expression of Wg is convincing as is the data in 2A, J and L showing a reduction in number of dividing cells upon epithelial knockdown of Wg.

2. Comment: I am not entirely convinced that the effect of Myc is somehow specific and instructive, not simply acting as a general cell cycle regulator. The data in Fig. 5I indicate that under homeostatic and stress conditions, Myc is absolutely required for cell proliferation, not playing an instructive role. However, I do agree that the understanding how levels of Myc (ie, half the dose in the case of the dm/+ experiment) could have important therapeutic implications.

3. Fig 6G' vs 6C' the lack of upregulation of Wg is not very clear. Certainly 6G' looks much higher than 6A'?? It would be nice to quantify the levels of Wg here. The failure of PH3+ cell induction in 6I using the SuHGBEts does look clear, however.

4. The data on Dl+ cells/field in esg>myc IR 30D are not very convincing-in the image in FigS5I I can count 7-8+ cells? The text referring to this on p14 states that you "observe an almost complete loss of Delta+ cells" in the esg>mycIR at 30d- ?? Can you explain this.

Additional comments:

-In the second paragraph of the introduction, I still believe the authors should indicate that they are referring to the mouse gut. Since the title includes "Drosophila", the references to mouse data will be misinterpreted.

-In Fig 4 it is unclear what the numbers on the images represent. Presumably avg cells/clone? Please clarify this in legends.

-On p14- the authors say that there is a 50% reduction in the "rate of ISC proliferation". What they measure is the number of PH3+ cells, which does not measure the rate. The text should be altered to state "number of cells undergoing mitosis" or something to that extent.

-p. 15 has many typos.

-I feel that the authors should keep Fig S8 which is important in light of their other manuscript.

Referee #2:

The authors have included data using mutant alleles of the Wg pathway (Fz, Fz2, Dm, Wg and Pygo). In particular, clonal analysis using these alleles in the MARCM system now significantly bolsters their previous findings using RNAi. A second RNAi transgene has been tested and found to exhibit the same phenotype originally reported. Overall, the manuscript therefore provides strong evidence that the Wg pathway is required for regeneration.

The qPCR results now support the upregulation of Wg during damage in the intestinal epithelium, and confirm the RNAi knockdown. The authors have resolved issues surrounding the relative contributions of the muscle versus the intestinal epithelium in this context. One minor correction needs to be made - Supplementary Fig 1A needs to be fixed because the writing on the x-axis is not visible.

The study shows that Wg is specifically expressed by Eb's in response to damage, representing an important distinction between the uninjured and injured state. This is nicely shown using the Su(H)GBE>wg-IR which reduces ISC proliferation.

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Even if these drivers are possibly expressed in other organs, the combination of the esg-Gal4, myo1A-Gal4, how-Gal4 and Su(H)GBE-Gal4 drivers and the use of mutant alleles now overwhelmingly make the authors case.

The overlap between this study and the Development paper is not significant. If the final supplementary figure contains data that is redundant with the other manuscript in press, this figure should probably be removed since it doesn't add much to this study anyway.

Editorial Correspondence 07 July 2012

Despite having sent you already comments on the revised paper, the third referee also came back with a few suggestions that I would ask you to consider for the final amendments.

As you will recognize, these come from a really knowledgeable scientist and are solely aimed at improving presentation and impact of your results.

I am very much looking forward to the ultimate version of this paper and are happy that it worked out so overwhelmingly positive in the end.

Yours sincerely,

Editor
The EMBO Journal

REFEREE REPORT:

Referee #3

I have a few relatively minor comments the authors could consider:

1. The title is flawed and should be re-written. At least the word "inductive" should be changed to "Inducible". Otherwise the actual meaning is not the intended meaning. Same problem at the bottom of p14.

2. The superscript "-ve" and "+ve" nomenclature is unfortunate and should be changed.

3. The authors have good arguments that Wg is made by EBs and required there. But it also seems to be produced by ISCs. Is this true? Can they say anything about a possible autocrine function of Wg in ISCs?

4. In Figure 6C', there are lots of Wg+ cells that are negative for the EB marker, Su(H)GBE. Could they all be ISCs? This photo seems inconsistent with the rest of the paper. Please comment.

5. Can the loss of regenerative capability caused by Wg-RNAi be rescued by overexpressed Myc? Likewise, is overexpressed Myc sufficient to drive ISC proliferation? These experiments are straightforward to do and could enhance an already excellent paper.

1st Revision - Authors' Response 03 August 2012

the Beatson
institute for cancer research

Subject: Manuscript EMBOJ-2012-82447 – Answer to reviewers' comments

We would like to thank the reviewers for their fair and constructive assessment of our work.

Referee #1:

This second version of Cordero et al., is much improved and has addressed many of my concerns in the initial version. The new versions of both papers now are significantly different enough to stand on their own. I have a few remaining questions and comments regarding some of the new data:

1. The source of Wg in homeostatic and stress conditions is still a bit confusing to me:

The authors show in Fig. 1A that Wg is present in both the muscle and esg+ ISC and EB cells, as has been previously described. In their knockdown experiment (1B) of Wg in the muscle with the howGal4ts, it looks like muscle staining is lost but esg+ ISC/EB staining is retained. Where is this Wg protein coming from? Knockdown in the esg+ population (esgts; Fig. S1F') still shows esg+ cells retaining Wg protein. While the qPCR data (Fig. S1A -note that the driver has been accidently omitted in this figure-) suggest that muscle but not esg drivers can decrease the total Wg with wg-IR, suggesting that a majority present in the ISC+EBs comes from the muscle, it is still possible that both muscles and ISC/EB cell types produce Wg under homeostatic conditions. It would thus be useful to include the Wg staining in esgGal4, howGal4 UAS-WgIR as a control in addition to the quantification

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done in this background of PH3 (2L) and Delta (S3Q). Similarly, comparing Fig. 1F' to 1C', it seems as if Wg is still somewhat induced despite esg>wgIR, which could be due to incomplete knock-down or perhaps another (muscle) source of Wg. That being said, the data in 1H showing increased epithelial expression of Wg is convincing as is the data in 2A, J and L showing a reduction in number of dividing cells upon epithelial knockdown of Wg.

Answer from authors: We have now performed the requested Wg staining in combined *esg;how>wg-IR* midguts. We have included the data in new Figures 1C-C''' and Figures S2E-F'. We find that Wg from ISCs/EBs is indeed significantly knocked down in *esg; how>wg-IR* midguts when compared to *how>wg-IR* tissues. Therefore we conclude that the VM and ISCs/EBs represent two independent sources of Wg in homeostatic midguts. Results are described in detail in the corresponding section.

2. Comment: I am not entirely convinced that the effect of Myc is somehow specific and instructive, not simply acting as a general cell cycle regulator. The data in Fig. 5I indicate that under homeostatic and stress conditions, Myc is absolutely required for cell proliferation, not playing an instructive role. However, I do agree that the understanding how levels of Myc (ie, half the dose in the case of the dm/+ experiment) could have important therapeutic implications.

Answer from authors: We believe that there is no disagreement between our conclusions and the opinion of the reviewer. We clearly show that complete or major knockdown of Myc (in either *esg>myc-IR* or *esg F/O myc-IR* midguts) results in loss of ISCs consistent with the absolute requirement of Myc to maintain ISCs. Nevertheless, Myc levels are induced in response to damage and halving the dose of Myc does not affect ISC maintainace but still results in defective intestinal regeneration. The latter allows us to uncouple the role of Myc in conditions of stress from that in basal cell proliferation. Consistently, halving the dose of Myc is also sufficient to prevent *Apc*-driven

tumourigenesis in the mouse intestine (Athineos and Sansom, 2010). Furthermore, in our work on the role of *Apc* in the fly midgut we show that downregulation of the Myc partner Max, which does not affect basal ISC proliferation on its own, suppresses *Apc*-dependent hyperplasia (Cordero et al., submitted). All together this evidence provides support for the potential therapeutic implications of a partial knock down of Myc levels and/or function.

3. Fig 6G' vs 6C' the lack of upregulation of Wg is not very clear. Certainly 6G' looks much higher than 6A'?? It would be nice to quantify the levels of Wg here. The failure of PH3+ cell induction in 6I using the SuHGBEts does look clear, however.

<u>Answer from authors</u>: The knockdown of Wg in *Pe* treated *Su(H)>wg-IR* midguts is very clear when compared to *Pe* treated control midguts. This can be more clearly seen in the insets shown for each of the corresponding figures. Most importantly, as pointed out by the reviewer, there is a clear failure to regenerate in *Su(H)>wg-IR* midguts. In any case we have now quantified this staining and data is presented in new Figure 6I. The quantification method used is described in detail in the materials and methods section and briefly in the figure legend.

4. The data on Dl+ cells/field in esg>myc IR 30D are not very convincing-in the image in FigS5I I can count 7-8+ cells? The text referring to this on p14 states that you "observe an almost complete loss of Delta+ cells" in the esg>mycIR at 30d- ?? Can you explain this.

<u>Answer from authors</u>: This issue is now clarified in the legend for Fig. S5 as follows:***. .....' Staining showed a suppression of the age-related ISC increase by myc heterozygotes (F, G) and loss of ISCs after 30 days of gene knock down in esg***^{ts}***>myc-IR midguts (H, I). The latter was confirmed by looking at esg>gfp progenitor cells (J-M). Arrow in I points to a single, Delta***$^{+ve}$ ***ISC as confirmed by its co-localization with esg>gfp (not shown). The rest of the signal did not correspond to cells of***

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either the expected size or esg>gfp^{+ve} (not shown) and therefore was interpreted as background. Please note that the loss of ISCs is also confirmed in our *'esg F/O myc-IR'* experiment in Figure 5, which includes the quantification of number of Delta^{+ve} ISCs in 7 and 30 day-old *esg>myc-IR* midguts. Therefore this result is unambiguous.

Additional comments:

-In the second paragraph of the introduction, I still believe the authors should indicate that they are referring to the mouse gut. Since the title includes "Drosophila", the references to mouse data will be misinterpreted.

Answer from authors: This has now been clarified at the beginning of the corresponding paragraph.

-In Fig 4 it is unclear what the numbers on the images represent. Presumably avg cells/clone? Please clarify this in legends.

Answer from authors: That is correct and this has now been clarified in the corresponding Figure legend.

-On p14- the authors say that there is a 50% reduction in the "rate of ISC proliferation". What they measure is the number of PH3+ cells, which does not measure the rate. The text should be altered to state "number of cells undergoing mitosis" or something to that extent.

Answer from authors: This has been changed following the reviewer's advice.

-p. 15 has many typos.

Answer from authors: This has been fixed.

-I feel that the authors should keep Fig S8 which is important in light of their other manuscript.

Answer from authors: Following the Editor's and Reviewer's advice we have kept this figure, which is also usefully to address some of the issues raised by Reviewer 3.

Referee #2:

Revision: EMBOJ-2012-80900

Inductive progenitor-derived Wingless regulates stem cell proliferation during regeneration of the Drosophila adult midgut.

Julia B. Cordero 1#, Rhoda K. Stefanatos 2#, Alessandro Scopelliti 1,2, Marcos Vidal 2 and Owen J. Sansom 1**

The authors have included data using mutant alleles of the Wg pathway (Fz, Fz2, Dm, Wg and Pygo). In particular, clonal analysis using these alleles in the MARCM system now significantly bolsters their previous findings using RNAi. A second RNAi transgene has been tested and found to exhibit the same phenotype originally reported. Overall, the manuscript therefore provides strong evidence that the Wg pathway is required for regeneration.

The qPCR results now support the upregulation of Wg during damage in the intestinal epithelium, and confirm the RNAi knockdown. The authors have resolved issues surrounding the relative contributions of the muscle versus the intestinal epithelium in this context. One minor correction needs to be made -

Supplementary Fig 1A needs to be fixed because the writing on the x-axis is not visible.

Answer from authors: This has been fixed. We have now increased the font size in all axes.

The study shows that Wg is specifically expressed by Eb's in response to damage, representing an important distinction between the uninjured and injured state. This is nicely shown using the Su(H)GBE>wg-IR which reduces ISC proliferation.

Even if these drivers are possibly expressed in other organs, the combination of the esg-Gal4, myo1A-Gal4, how-Gal4 and Su(H)GBE-Gal4 drivers and the use of mutant alleles now overwhelmingly make the authors case.

The overlap between this study and the Development paper is not significant. If the final supplementary figure contains data that is redundant with the other manuscript in press, this figure should probably be removed since it doesn't add much to this study anyway.

Answer from Authors: We have decided to keep this figure as it does not have any data already presented in our other manuscript and it helps to address some of the issues raised by Reviewer 3.

....

Ref#3

I have a few relatively minor comments the authors could consider:

1. The title is flawed and should be re-written. At least the word "inductive" should be changed to "Inducible". Otherwise the actual meaning is not the intended meaning. Same problem at the bottom of p14.

Answer from Authors: We have now made the suggested changes and replaced "Inductive" with "Inducible" in the title and "inductive" with "instructive" at the end of page 14.

2. The superscript "-ve" and "+ve" nomenclature is unfortunate and should be changed.

Answer from Authors: We have now either removed these nomenclatures in places where it would not cause further confusion and describe their meaning the first time they were used.

3. The authors have good arguments that Wg is made by EBs and required there. But it also seems to be produced by ISCs. Is this true? Can they say anything about a possible autocrine function of Wg in ISCs?

Answer from Authors: An autocrine role of Wg is possible and we cannot formally rule it out. Nevertheless, we cannot formally test it either with the available genetic tools. As mentioned in our previous response to the reviewers there is not a reliable ISC-only *gal4* driver. Based on the similar blockade in regeneration when using either *esg-gal4* or *Su(H)-gal4* to knock down *wg*, the most parsimonious explanation for the data is that EBs produce Wg to signal to ISCs and turn on the pathway. This can therefore explain the Wg staining observed in both EBs and ISCs.

4. In Figure 6C', there are lots of Wg+ cells that are negative for the EB marker, Su(H)GBE. Could they all be ISCs? This photo seems inconsistent with the rest of the paper. Please comment.

Answer from Authors: Please note that the Wg staining shown here does not directly reflect the *wg*

expression domain but rather depicts were the Wg protein is found in the midgut. This is also relevant to the previous comment from the reviewer. Since Wg is a secreted factor, the only reliable way to address the source of the protein and its functional domain of it is by performing all the functional tests we present in our work (e.g. knocking down the gene in a cell specific manner, do the staining then and assay the functional result of gene knockdown). Therefore we cannot determine whether the other cells that show Wg are ISCs unless we use a cell specific driver to knockdown the gene only in those cells. Also, the pattern of expression of the *Su(H)-gal4* driver cannot be directly compared with that of the *esg>gal4* driver used in the rest of the paper. The latter also labels the ISCs, which increase in number in response to damage (data not shown and Nicolas Buchon, personal communication). Therefore one would expect to see more cells labeled by *esg-gal4* than with *Su(H)-gla4.* Furthermore it's not really known how stable is one driver compared with the other. In principle, EBs are transient cells when compared to ISCs. What we can confidently conclude from our results is that knocking down *wg* from EBs only results in a blockade in intestinal regeneration comparable to that observed by knocking down the gene in ISCs and EBs.

5. Can the loss of regenerative capability caused by Wg-RNAi be rescued by overexpressed Myc? Likewise, is overexpressed Myc sufficient to drive ISC proliferation? These experiments are straightforward to do and could enhance an already excellent paper.

<u>Answer from the authors</u>: These are indeed interesting possibilities, which we have tested. Our results showed that, sole ectopic overexpression of Myc is not sufficient to drive ISC proliferation and cannot overcome Wg knockdown in the context of intestinal regeneration (Figure for Reviewers). Consistently, Fig S8 C, D', shows that upregulation of Myc is not sufficient to induce regeneration in

Stat deficient midguts: *esg*^{ts}>*stat-IR* midguts do not proliferate in response to damage in spite of having efficient upregulation of Myc. Furthermore, *esg>myc* midguts fail to regenerate in response to damage (Figure for Reviewers). Together these results are consistent with previous reports in the mouse intestine (Murphy et al., 2008; Finch et al., 2009). Forced overexpression of Myc in *Drosophila* and vertebrate models has been proven to be toxic and causes apoptosis in part due to activation of p53 (Montero et al., 2008; Murphy et al., 2008; Finch et al., 2009 and Eduardo Moreno, personal communication). Therefore driving ectopic Myc by transgene overexpression is unlikely to mimic the effects of upregulation of endogenous Myc. A summary of these results has now been added to the discussion section (page 21).

References:

Athineos, D. and Sansom, O. J. (2010) 'Myc heterozygosity attenuates the phenotypes of APC deficiency in the small intestine', *Oncogene* 29(17): 2585-90.

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Figure for Reviewers: Ectopic *myc* transgene overexpression in the *Drosophila* midgut. (A-B) Myc overexpression is not sufficient to drive ISC proliferation in the adult *Drosophila* midgut. (A, A') Posterior midguts overexpressing Myc in progenitor cells for 14 days (esg^{ts}>*myc*) and stained with anti-GFP (green; A) and anti-Myc (red; A and white; A'). (C) Quantification of ISC proliferation from regenerating midguts of the indicated genotypes represented by the number of $pH3^{+ve}$ cells/posterior midgut. Transgenes were overexpressed for 6 days followed by 1 day of either Sucrose (Suc) or *Pe* feeding (***p<0.0001 one-way ANOVA with Bonferroni's Multiple Comparison Test).

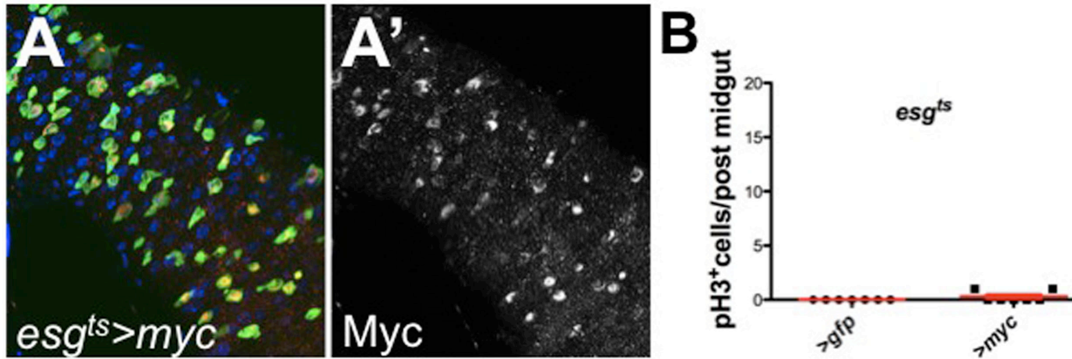
Genotypes used on Figure for Reviewers:

yw; escargot-gal4, UAS-gfp/+; tub-gal80^{ts} /+

yw; escargot-gal4, UAS-gfp/+; tub-gal80^{ts} /UAS-myc

UAS-dicer2/+; escargot-gal4, UAS-gfp/ UAS-wg-IR; +/ tub-gal80^{ts}

UAS-dicer2/+; escargot-gal4, UAS-gfp/ UAS-wg-IR; UAS-myc/ tub-gal80^{ts}



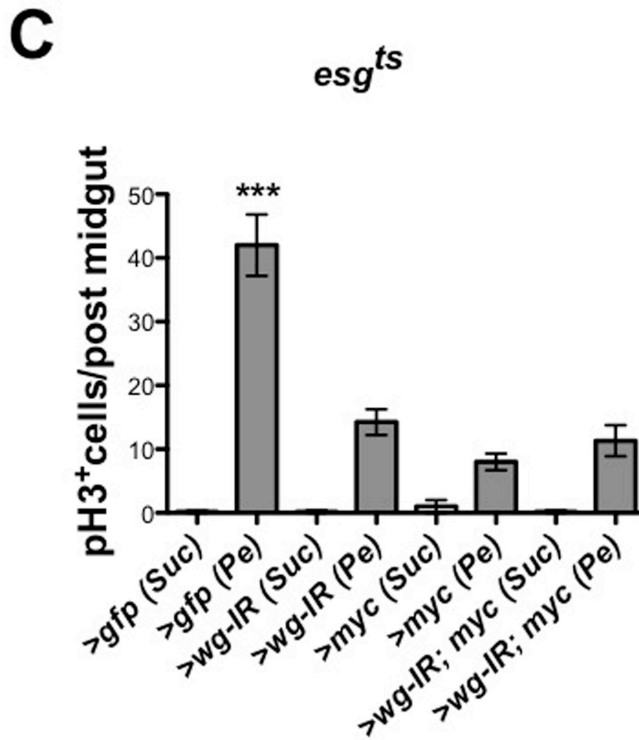


Figure for Reviewers