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Wnt activity and basal niche position sensitize intestinal stem and progenitor cells to DNA damage

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Transaction Report:

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

1st Editorial Decision 11 September 2014

Thank you very much for submitting your paper and making relevant referee reports from an earlier round of peer-review including your responses available to The EMBO Journal.

The paper and the accompanying information was now assessed by the editorial team AND an expert external advisor.

As you will recognize from the attached comments, there are concerns regarding the interpretation/precise characterization (nomenclature). As explicitly stated by this knowledgeable expert in the field, this may confound some of the conclusions that can be drawn with confidence from the presented data. After extensive further consultations also here in the editorial office, I like to summarize our main conclusions:

-some of your initial results are at least conceptually impacted by a recent publication that revealed LGR5+ cells as the relevant population for efficient regeneration upon irradiation and have thus to be seen as confirmatory;

-major value arises from the proposed function of Wnt-signals to determine radio-sensitivity in the ISC-niche that had previously been described as rather hierarchical and still relatively dynamic/possibly heterogeneous stem cell population;

The referee thus takes issue with the possibility that modulating Wnt signaling may simply switch

ratios of radioresistant vs. radiosensitive cells (thus cell fate) than alter ultimate cell behavior. Along these lines s/he remains unconvinced that the in-vitro results on Lgr5 hi/lo cells (that indicate changes in radioresistance in a Wnt-dependent manner in fig 7) and the complementary in-vivo analyses (in fig 8) that correlates PCNA-staining with cell-number and possibly position would suffice to support the major claim of your study.

On face value, s/he would request preferably genetic and further reaching quantitative analyses that discriminates between these alternative interpretations, respective a more careful presentation/integration also with regard to the by now published previous results.

Consequently, I am only able to offer further revisions for The EMBO Journal in case you were willing and able to address this concern (together with the other points raised that essentially are congruent with this line of thinking).

Alternatively, I did inquire with one of my colleagues from our sister title EMBOreports (Barbara Pauly, here in CC) that would be prepared to pursue the existing experimental dataset, though demanding a much more balanced/toned-down presentation.

Please trust that we thoroughly analyzed the current information at hand as to offer a constructive and possibly timely way for their publication.

I hope you understand however that these are remaining concerns that are not easily to be dismissed and if properly addressed, would indeed increase the overall value of your scientific contribution.

I am thus looking forward to your timely response and approach/possibly integrating already existing further results from your lab that would enable rapid presentation in one or the other EMBO title.

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REFEREE REPORTS:

Referee #1:

1. Authors interpret that Lgr5-Low cells represent a subpopulation of ISCs located above the crypt base that receive low WNT signals. However, extensive evidences indicate that Lgr5-Low cells are in fact transient amplifying cells (Sato et al. Nature. 2009; MuÒoz et al. EMBO J. 2012). They express differentiation markers of adsorptive, enteroendocrine and mucosecreting cells and display low clonogenic potential in vitro and in vivo (see below). From my experience, the +4 crypt cells that authors observe in Tert KO or upon irradiation are in fact residual Lgr5-Hi cells as they appear bright under the microscope (figure 3). Authors confound these +4/+5 Lgr5-hi cells with Lgr5-Low cells purified by FACS, which are about 10> fold GFP dimmer. This misinterpretation may invalidate several of the conclusions drawn by authors regarding levels of WNT signaling in these cells.
2. In the original papers by Clevers and colleagues, Lgr5-Low cells formed 10-100 fold less in vitro organoids that Lgr5-high cells (Sato et al. Nature 2009) as opposed to data shown by authors in Figure 2. This difference is likely explained because Sato et al. only included R-SPO in the culture media whereas Tao et al. maximize WNT signaling by adding recombinant Wnt3a. In these conditions, some transient amplifying cells, such as Dll1+ precursors (van Es et al. Nature Cell Biology. 2012), can reconvert to Lgr5-Hi ISCs. These observations supports the notion that the phenotypes described by authors after irradiation could be explained by reconversion of transient amplifying cells to ISCs as a result of increased WNT signals.
3. Several key observations included in the manuscript, particularly those that describe crypt repopulation from cells located above the crypt base, are not entirely novel and have been previously reported by others (van Es et al. Nature Cell Biology 2012; Sangirogi et al. Nat Gen. 2008).
4. In addition, author's interpretation of how crypts regenerate after irradiation is in sharp contradiction to that of Sauvage and colleagues (Metcalfe et al. Cell Stem Cell. 2014) who convincingly showed that Lgr5+ ISCs are required for crypt regeneration upon irradiation. The residual Lgr5-hi population that authors observed after irradiation (Figure3) maybe

able to regenerate the crypts (and thus unify both views).
5. The experiments of modulation of WNT signaling in vitro and in vivo (Figure 8) in association with crypt regeneration are not convincing and require additional controls. It appears as if anti-LRP6 antibodies reduce the frequency of LGR5-high cells in control mice (Figure 4DE). Also, organoids cultured in the presence of GSK3-beta inhibitors should demonstrate expansion of LGR5-hi cell compartment as a result of increased WNT signalling. Therefore, authors should exclude that modulation of WNT signaling may simple alter the ratios of radioresistant vs. radiosensitive cells in crypts (i.e. ISCs numbers) rather than altering the radiosensitivity of ISCs per se.

1st Revision - authors' response 07 October 2014

Referee #1:
1. Authors interpret that Lgr5-Low cells represent a subpopulation of ISCs located above the crypt base that receive low WNT signals. However, extensive evidences indicate that Lgr5-Low cells are in fact transient amplifying cells (Sato et al. Nature. 2009; Muñoz et al. EMBO J. 2012).They express differentiation markers of adsorptive, enteroendocrine and mucosecreting cells and display low clonogenic potential in vitro and in vivo (see below).

Response:
We agree with the reviewer that it was under debate whether LGR5^{lo} cells represent true intestinal stem cells or progenitor cells. However, we would like to briefly summarize this discussion and the evidences indicating that the LGR5^{lo} cells represent intestinal stem cells.
1.) The initial study of Sato et al showed that FACS sorted single LGR5^{lo} cells could not form organoids without Wnt3a while 6% of sorted LGR5^{hi} cells could make it under these culture conditions (Sato et al., 2009).These results suggested that LGR5^{lo} cells have no stem cell activity in culture. However, when Wnt3a was added to the culture medium, LGR5^{lo} cells could form organoids as shown by Sato et al. 2011 as well as by us (Fig. 2).
2.) Recent in vivo live-cell-imaging of LGR5-GFP-positive cells in the intestinal epithelium revealed that border LGR5 cells (LGR5^{lo} cells) self-renew and can give rise to central LGR5 cells (LGR5^{hi} cells) at the crypt bottom as well as to differentiated cells (Ritsma et al., 2014); This study provided *in vivo* proof that LGR5^{lo} border cells are stem cells albeit having an increased chance to get pushed out of the stem cell zone then undergoing differentiation and loss of stem cell potential.
3.) Microarray analysis revealed similar expression levels for a set of stem cell markers in freshly isolated LGR5^{hi} and LGR5^{lo} cells, including Msi1, Prom1, Mmp7, Bmi1, Hopx, mTERT, Wip1, and Nfat5 (Our manuscript Fig. E2 B).
Together, current data indicate that LGR5^{lo} cells contain true stem cell activity when exposed to Wnt-ligand both in culture as well as *in vivo* in the mouse intestine. *In vivo*, the self-renewal potential of LGR5^{+} cells depends on the position in the stem cell niche and thus on the gradient of Wnt signaling activity in the crypt base with the border cells having an increased chance to stochastically getting pushed out (our manuscript Fig. 2, see also Sato et al., 2011; Yin et al., 2014, van Es et al. Nature Cell Biology, 2012).

We have clarified this discussion point by adding the below paragraph on page 7 of the result section. In addition, we have adapted the nomenclature throughout the manuscript to reflect the current stand of understanding that LGR5^{lo} cells represent intestinal stem and progenitor cells (ISPCs): "It was long under debate whether LGR5^{lo} cells in position 4 (border cells) represent true intestinal stem cells or progenitor cells. Initial studies showed that FACS sorted single LGR5^{lo} cells could not form organoids in culture while 6% of sorted LGR5^{hi} cells could make it under these culture conditions (Sato et al, 2009). However, when Wnt3a was added to the culture medium, LGR5^{lo} cells could form organoids (Sato et al, 2011). Recent *in vivo* live-cell-imaging of LGR5-GFP-positive cells in the intestinal epithelium revealed that LGR5^{lo} border cells self-renew and give rise to central LGR5^{hi} cells at position 1 and 2 at the crypt bottom as well as to differentiated cells (Ritsma et al, 2014) indicating that LGR5^{lo} border cells are stem cells albeit having an increased chance to get pushed out of the stem cell border zone then undergoing differentiation and loss of stem cell potential. Therefore, in this manuscript, we refer to LGR5^{lo} cells as intestinal stem and progenitor cells (ISPCs) and to LGR5^{hi} cells as intestinal stem cells (ISCs)."
Accordingly, we also changed the title of the manuscript to "Niche positioning determines Wnt/β-catenin dependent sensitivity of intestinal stem and progenitor cells to DNA damage".

From my experience, the +4 crypt cells that authors observe in Tert KO or upon irradiation are in fact residual Lgr5-Hi cells as they appear bright under the microscope (figure 3). Authors confound these +4/+5 Lgr5-hi cells with Lgr5-Low cells purified by FACS, which are about 10> fold GFP dimmer. This misinterpretation may invalidate several of the conclusions drawn by authors regarding levels of WNT signaling in these cells.

Response: To clarify this point we have added new experimental data measuring the fluorescence intensity of LGR5-GFP cells according to the position of the cells in the crypt base. The new data confirm that in non-irradiated wild type mice position 4 cells exhibit reduced GFP expression intensity compared to position 1/2 cells (Fig. 1 D,E). Moreover, the analysis demonstrates that the surviving LGR5-posiitve cells in telomere dysfunctional mice are located in position 4 and contain similarly dim GFP intensity as position 4 cells in wild type mice (revised Fig. 3 H-L). Same holds true for surviving LGR5-positive cells in irradiated mice (revised Fig. E3 D).
These data are support by our FACS data showing that LGR5^{lo} cells preferentially survive after IR and in response to telomere dysfunction (Fig.3 D - G, Fig. 5 P - U). The decrease in staining intensity in FACS is more pronounced as in the stainings as the entire cell is measured and not a section through the cell.
We also provide new data showing that the *in vivo* manipulation of Wnt signaling activity results in decreases in GFP- expression intensity in position 1 and 2 ISCs (in response to Wnt inhibition by LRP6 antibodies, revised Fig. 8 A - G) or activation of the reporter in position 4 ISPCs (in response to introducing the APC^{min} mutation, new Fig. 9 A - C).

2. In the original papers by Clevers and colleagues, Lgr5-Low cells formed 10-100 fold less in vitro organoids that Lgr5-high cells (Sato et al. Nature 2009) as opposed to data shown by authors in Figure 2. This difference is likely explained because Sato et al. only included R-SPO in the culture media whereas Tao et al. maximize WNT signaling by adding recombinant Wnt3a. In these conditions, some transient amplifying cells, such as Dll1+ precursors (van Es et al. Nature Cell Biology. 2012), can reconvert to Lgr5-Hi ISCs. These observations supports the notion that the phenotypes described by authors after irradiation could be explained by reconversion of transient amplifying cells to ISCs as a result of increased WNT signals.

Response: As discussed in the previous point, the current literature as well as data from our current study stand in agreement in showing that LGR5^{lo} cells at position 4 (border cells) have true self-renewal and stem cell activity but an increased stochastic chance to get pushed out of the stem cell zone to differentiate. However, these border cells are less sensitive to DNA damage and represent the critical cell population with the LGR5-positive cell pool required for the maintenance and regeneration of the intestinal epithelium.
The reviewer is right that LGR5-negative cells can de-differentiate into LGR5-positive stem cells in response to damage. However, this is an extremely rare event and based on the study of Fred de Sauvage this de-differentiation of LGR5-negative cells is not sufficient to survive irradiation induced DNA damage (Metcalfe et al. Cell Stem Cell. 2014). We had already discussed this in our originally submitted paper. In the revised version this paragraph is on page 15/16 as follows: “This study supports a model indicating that stem and progenitor cells with intrinsically low Wnt signaling activity represent a backup population ensuring recovery of tissue maintenance and survival in response to acute or chronic DNA damage. Genetic mouse models showed that LGR5-positive cells are essential for intestinal regeneration and mouse survival in response to IR (Metcalfe et al, 2014). The current study indicates that the subpopulation of LGR5^{lo} stem and progenitor cells preferentially survives IR and may in fact represent the critical backup population for regeneration of intestinal epithelium in response to IR.”

3. Several key observations included in the manuscript, particularly those that describe crypt repopulation from cells located above the crypt base, are not entirely novel and have been previously reported by others (van Es et al. Nature Cell Biology 2012; Sangirogi et al. Nat Gen. 2008).

Response: This paper provides the first experimental evidence that the level of Wnt signaling activity and the position of LGR5-positive cells in the stem cell niche represent the key-factors that determine DNA damage sensitivity of the intestinal stem and progenitor cells. The LGR5^{lo} ISPCs at position 4 of the intestinal crypt are identified as the critical cell population required for regeneration

and maintenance of the intestinal epithelium in response to acute (IR) or chronic (telomere dysfunction) DNA damage.
Specifically our study shows:
1) $LGR5^{lo}$ subpopulation of ISCs in position 4 (border cells) is preferentially maintained upon damage; 2) the endogenous level of Wnt activity influences the survival of intestinal stem cells in response to DNA damage *in vivo*;
3) ISCs with higher Wnt activity show significant stronger DNA damage response;
4) transient upregulation of Wnt activity at early time point after IR activates a feed forward loop enhancing DNA damage response in ISCs;
5) genetic or chemical targeting of the Wnt signaling pathway changes the radio-sensitivity of ISCs both *in vitro* and *in vivo*.
Together, our data support a novel concept indicating that heterogeneity in Wnt signaling in stem cell populations (determined by the positioning in the niche) represents a decisive molecular mechanism influencing survival and selection of stem cells in the context of DNA damage.

The papers cited by the reviewer however have a different focus:
(i) The Van Es et al. study showed that the de-differentiation process exists upon damage where $Dll1^{+}/LGR5^{-}$ transient amplifying cells (+5 $Dll1^{+}$ cells) regenerate intestinal epithelium after 6Gy irradiation. However, as described by the authors, this event is very rare (average of 96.1 stem cell derived tracings per entire duodenum of the mouse) (Van Es et al. Nature Cell Biology, 2012). The study by Fred de Sauvage showed that the de-differentiation of LGR5-negative cells is not sufficient to survive irradiation induced stem cell depletion. Our data demonstrate that $LGR5^{lo}$ cell population is maintained upon irradiation and serves as a major resource of small intestinal regeneration upon irradiation.
(ii) The study from Sangirogi et al. showed that Bmi1 is expressed predominantly at +4 position and demonstrate that it can serve as an intestinal stem cell marker. They showed that ablation of $Bmi1^{+}$ cells by inducing diphtheria toxin led to crypt loss. They did not study DNA damage induced stem cell loss and cell fate decision and therefore does not impact the novelty of our story. There was a report that $Bmi1^{+}$ ISCs survive irradiation(Yan et al., PNAS, 2012),however, it was then shown that Bmi1 has broad expression within crypts and is robustly expressed in $LGR5^{+}$ cells (Muñoz et al. EMBO J. 2012).

4. In addition, author's interpretation of how crypts regenerate after irradiation is in sharp contradiction to that of Sauvage and colleagues (Metcalfe et al. Cell Stem Cell. 2014) who convincingly showed that Lgr5+ISCs are required for crypt regeneration upon irradiation. The residualLgr5-hi population that authors observed after irradiation (Figure3) maybe able to regenerate the crypts (and thus unify both views).

Response: Our study stands in agreement and not in contrast with the mentioned study from the Sauvage lab. The cited study showed that $LGR5^{+}$ are indispensable for intestinal regeneration in response to IR and led to the conclusion that "at least a subset of $LGR5^{+}$ cells survives radiation" (Metcalfe et al. Cell Stem Cell, 2014). Our study now shows that it is indeed the $LGR5^{lo}$ cells that preferentially survive upon DNA damage and we determine the level of Wnt activity and the position in the crypt as being the decisive factor for this biology.

5. The experiments of modulation of WNT signaling in vitro and in vivo (Figure 8) in association with crypt regeneration are not convincing and require additional controls. It appears as if anti-LRP6 antibodies reduce the frequency of LGR5-high cells in control mice (Figure 4DE). Also, organoids cultured in the presence of GSK3-beta inhibitors should demonstrate expansion of LGR5-hi cell compartment as a result of increased WNT signaling. Therefore, authors should exclude that modulation of WNT signaling may simple alter the ratios of radioresistant vs. radiosensitive cells in crypts (i.e. ISCs numbers) rather than altering the radiosensitivity of ISCs per se.
Response: We agree that the original manuscript did not depict clearly enough how modulation of Wnt signaling influences radio-sensitivity of ISC and ISPC at position 1-4 of the basal crypt. To

clarify this point, we add new experimental data showing (i) how the interventions change LGR5-GFP expression intensity (a measure for Wnt signaling activity) in ISCs and ISPCs at position 1-4 of the basal crypts and (ii) how this correlates to increase or decrease radio-sensitivity of ISCs and ISPCs at position 1-4 of the basal crypt.
The revised Fig. 8 confirms that a single dose of anti-LRP6 antibody transiently lowered LGR5-GFP expression level in ISCs (revised Fig. 8 A-G). This inhibition of Wnt activity led to better maintenance of ISCs and ISPCs in the crypt base of irradiated mice as determined by PCNA (revised Fig. 8 J, K, L) and LGR5-positivity (revised Fig. 8 O – R).
The new Figure 9 shows that the APC^{min} mutation increase LGR5-GFP expression intensity in ISCs and ISPCs (new Fig. 9 A – C) and this associates with a significantly enhanced depletion of ISCs and ISPCs in position 1-4 of the basal crypts (new Fig. 9 D - F).
Together, the inclusion of new experimental data confirms that modulation on Wnt signaling changes the radio-sensitivity of intestinal stem and progenitor cells at position 1-4 of the basal crypts. Inhibition of Wnt by LRP6 neutralizing antibodies results in increased survival of ISC, whereas activation of Wnt-signaling by introducing an APC^{min} mutation enhances the depletion of ISCs at position 1-4 in response to IR.

2nd Editorial Decision 29 October 2014

I finally received comments from a second, so far unbiased referee on your paper. I also took the liberty to consult with both referees about the responses you had sent while the paper was still out for peer-review.

As you will recognize, both refs resonate on the impressive amount of data, some intriguing observations that are presented within the study but raise at the same time the same conceptual concern:

It appears impossible at this stage to conclude that there is indeed a Lgr5+lo radio-resistant cell population at the plus 4 position, as long as this cannot be conclusively and functionally distinguished from reverting TA and/or the general pool of dynamically Lgr5+ expressing cells.

The referees once again offer further reaching, constructive suggestions as to:

- run qPCRs on the Hi-Lo gated Lrg5 cells compared to the Hi-Hi and Lo-Lo and measure markers genes (Dll1, ChromograninA, defensin a5, Msi1, Krt20, Fabp1, dclk1) to clarify the identity of this radioresistant population in relation to previous studies (ISCs vs TA vs LRC, etc...)
- assess differential expression of (minimally) Msi1 (and possibly more) Wnt-targets along the crypt-base axis also by IHC or in-situs;
-use EM to confirm the morphological 'gap' within the ISC-niche using EM during CBC-ablation (ref#2 point 2);

- incorporate the most recent supplementary figure that outlines differential survival within the 'high-gated' Lgr5+ ISCs upon both Tert-deletion as well as radiation damage.
- be VERY precise and careful throughout the manuscript about what you can definitively conclude from the current data, as to ADD an intriguing element on heterogeneity/plasticity/functionality of ISC stem cells but to NOT further confuse this already hotly debated/divided area of research;

I realize that these are truly demanding requests! As you are very well aware, we are running an editorial policy to only invite revisions for those papers with certain, timely and definitive outcome of a limited number of experimental amendments. Based on this, I find it the most prudent approach to return the study to you at this point to consider yourself whether

(i) to seek rapid publication elsewhere
(ii) allocate the necessary time and resources to truly develop the study as to enable eventual publication in The EMBO Journal.

Please trust that I am really sorry to be unable to communicate more encouraging news. I still hope that our fairly argued and transparent decision enables constructive pursuit of this truly exciting

project.

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Referee #1:

In my opinion, new data included in this revised version do not address unequivocally my main criticism. Specifically:

1. Authors cite Ritsma et al. (Nature 2014) as the base for the present study and point that this previous work demonstrates that Lgr5-Lo cells around position +4 (the so called border cells) can give rise to Lgr5-hi cells (present at the center of the niche). As a matter of fact, Ritsma et al. did not investigate Lgr5-Lo cells (they do not even mention Lgr5-Lo cells). Ritsma et al, explored the behavior of Lgr5+ vs Lgr5- cells. Importantly, Ritsma et al. indicate that border cells are heterogeneous regarding Lgr5 levels and that they consider Lgr5-negative border cells as TA cells which most likely correspond to Dll1+ cells (as they had described in van Es et al. Nature Cell Biology. 2012) whereas Lgr5+ border cells are taken as bona-fide ISCs albeit with lower probability of long-term regeneration. Therefore, the manuscript by Ritsma et al should not be used to support author's conclusions about the identity or behavior of Lgr5-Lo cells.

2. A key problem that remains and that complicates the interpretation of the results is the identification of Lgr5-lo cells in tissue section versus Flow Cytometry. Authors include now evidence that Lgr5-Lo cells (+4 border cells) are about two fold dimmer than Lgr5-Hi cells by IF in sections (Figure 1D, E). In contrast, Lgr5-Lo cells gated in experiments of flow cytometry are >10 fold dimmer (Figure 3. Please note the logarithmic scale in FACS profiles). There is compelling evidence in the literature that Lgr5-Lo cells as gated here in flow experiments represent TA cells. As matter of fact, Lrg5-Lo cells that authors recognize by IF in tissue sections at position +4 reside very likely within the Lgr5-Hi gate in flow experiments. Authors point in the rebuttal that "the decrease in staining intensity in FACS is more pronounced as in the stainings as the entire cell is measured and not a section through the cell" but this argument is weak as changes in fluorescence intensity should be proportional in both techniques. Please also note that FACs profiles show that Lgr5-Lo cells are more abundant than Lgr5-Hi cells thus implying that Lgr5-Low cells cannot correspond to border cells in these experiments. My impression is that authors are looking at different populations depending on the technique. Are these cells TA cells located above +4 (Lgr5-Lo cells in flow experiments) or ISCs located in the border (Lgr5-weak cells by IF)? As example, there is fraction of Lgr5-hi cells that remain viable upon irradiation as assessed by FACS (Figure 3D). It is likely that these resilient Lgr5-hi cells rather than the Lgr5-Lo cells represent border cells that regenerate crypts. In contrast, in the experiments of WNT activation/inhibition, authors gate both Hi and Lo populations and therefore it is not possible to discern effects of WNT signaling over TA cells or ISCs. Therefore, the properties of Lgr5-Lo cells remain unclear and this drawback invalidates several of the conclusions drawn throughout the manuscript.

Referee #2:

Tao et al.

This manuscript examines the relationship between local Wnt signaling levels and intestinal stem cell response to injury/DNA damage. Accumulation of DNA damage in telomerase deficient Lgr5 reporter mice was associated with downregulation of Wnt signaling activity in the Lgr5+ stem cell compartment. Analysis of the stem cell reporter mice further revealed that this phenomenon was driven by selective loss of Wnthi stem cells restricted to the crypt base. In contrast, +4 position Wntlo stem cells appeared resistant to DNA damage-induced apoptosis. Ex vivo/In vivo inhibition of Wnt signaling preferentially suppressed radiation-induced apoptosis in the Wnthi stem cells. The authors conclude that distinct Lgr5+ stem cells (crypt base versus +4 position) displaying varying levels of intrinsic Wnt signaling exist within the intestinal crypts - the Wnthi, crypt base variety are most susceptible to damage-induced apoptosis, whilst the +4 Wntlo flavour are inherently resistant to injury due to their reduced Wnt signaling and are consequently able to drive crypt

survival/regeneration.

Whilst this is a carefully crafted study, with potentially important implications I have some reservations on some critical aspects of the study.

1) The assumption that the GFPlo cells at position 4 are stem cells is, in my opinion, not supported by robust functional evidence. The fact that Lgr5-GFPLo cells can be converted into organoid forming entities upon addition of Wnt3a reflects the plasticity of the lower crypt compartment. The Lgr5Lo cells simply convert to Lgr5hi stem cells in conditions of high Wnt - This assay does not establish the endogenous stem cell identity of Lgr5-GFPlo cells at position +4. I also believe the Ritsma study established that all crypt-base Lgr5-GFP+ cells can behave as stem cells -those present at the border of the Paneth cell compartment are less likely to survive long-term because they have a higher probability of being pushed out of the defining niche due to cell division within the finite niche space at the lower portions of the crypt. As far as I'm aware they never concluded that these border Lgr5+ cells are Lgr5-GFPlo. It is also somewhat puzzling to me how sorting the GFPhi versus GFPlo populations for the various profiling experiments can be extrapolated back to the endogenous GFPlo cells at the +4 position. Munoz et al (EMBOJ) previously showed that the stability of the GFP protein ensures that it is sequentially diluted from the Lgr5hi stem cell through several generations of its descendents. Given that the GFPlo fraction isolated using the relatively broad gate employed on the FACS is therefore likely to be a mixture of these different Lgr5 stem cell progeny (ie GFOlo, GFOlower, GFPverylow), it would appear impossible to conclude that the differential expression of Wnt target genes and stem cell markers (of which I believe only Msi1 from the stated list...) relates specifically to the +4 position GFPlo cells. To conclude this, candidate genes would have to be validated by IHC/In-situ to be differentially expressed between the +4 and crypt base GFP populations.

2) The depletion of the CBC compartment in the aged telomerase-deficient mice is interesting. However, I was intrigued by the fact that the crypt in Figure 1I appears to have gaps between the Paneth cells. Wouldn't the Paneth cell compartment simply become contiguous following loss of the intercalating CBC cells? Are the CBC cells really gone? Maybe an EM picture would have convincingly proven ablation of the CBC cells at the lower crypt positions. I also have doubts about whether the OLFM4+ cells around position +4 in these aged telomerase-deficient mice are really stem cells. OLFM4, whilst being highly expressed in the Lgr5+ CBC cells is also expressed on early TA cells (as shown in the original Van der Vlier Cell paper). Could these surviving OLFM4+ cells then simply be plastic Lgr5- TA cells (including the DLL1+ fraction)?

3) The in vivo Wnt suppression experiments are potentially very interesting. However, it it would have been useful to have included some functional evidence of effects of anti-LRP6/irradiation on stem cell output/survival (ie, lineage tracing). Since this experiment was performed using the Lgr5-EGFP/Cre line, this would only require one extra breeding step to include a conditional reporter allele. Although beyond the scope of this paper, it would also have been interesting to determine whether sensitizing Lgr5+ stem cells to irradiation-induced death by conditionally deleting APC would prevent adenoma formation.

4) Although this work does nicely demonstrate the existence of cell populations within the lower crypt having distinct DNA damage senstitivities, I do not think the identity of these populations has been definitively proven. One conceptual problem I have with this work is how to relate it to the de Sauvage Lgr5DTR ablation study. Why would the +4 Lgr5 DTR+ stem/progenitor cells not be killed and prevent post-irradation regeneration in this model? If an explanation is that the +4 Lgr5+ cells express GFP-DTR below the threshold necessary to achieve their efficient ablation in vivo, then these GFPlo cells should still be present in the DT-treated crypts. However, de sauvage's data support efficient ablation of all GFP-expressing cells.

Minor comment:

1) Page 7 last paragraph - Figure 1 is depicting IF for GFP not Lgr5 as stated in the text.

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comments from further consultations:

ref#1:

It looks as if the second reviewer pointed exactly to the same concerns that I raised. The suggestions for additional experiments/modifications sound reasonable. I would also suggested to run some qPCRs on the Hi-Lo gated Lrg5 cells compared to the Hi-Hi and Lo-Lo and measure markers genes (Dll1, ChromograninA, defensin a5, Msi1, Krt20, Fabp1, dclk1). This will help clarify the identity of this radioresistant population in relation to previous studies (ISCs vs TA vs LRC, etc...).
It is a difficult paper. Some good data mixed with some conceptual drawbacks. It needs significant adjustments based on truly understanding/knowing the peculiarities of the model system.

ref#2:

I agree that there are some interesting observations in the paper, but the authors absolutely do not prove that there are Wnt lo damage-resistant stem cells restricted to the plus 4 position. At best they can claim early TA cells expressing lower levels of GFP contribute to this phenomenon.They also need to better discuss their findings in light of the Sauvage paper - it currently doesn't make sense. It needs to be accurate or it will simply add to the pile of contradictory data on intestinal stem cells.

Re-submission 29 November 2014

I finally received comments from a second, so far unbiased referee on your paper. I also took the liberty to consult with both referees about the responses you had sent while the paper was still out for peer-review.

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-use EM to confirm the morphological 'gap' within the ISC-niche using EM during CBC-ablation (ref#2 point 2);
- incorporate the most recent supplementary figure that outlines differential survival within the 'high-gated' Lgr5+ ISCs upon both Tert-deletion as well as radiation damage.
- be VERY precise and careful throughout the manuscript about what you can definitively conclude from the current data, as to ADD an intriguing element on heterogeneity/plasticity/functionality of ISC stem cells but to NOT further confuse this already hotly debated/divided area of research;

Response: As outlined below in detail, we followed the advice of the reviewers and the editor and by performing additional experiments addressing each of the above listed points. In addition, we clarified the scope of our paper and were very careful in rewording the paper. All major changes in the manuscript are highlighted in orange.

I realize that these are truly demanding requests! As you are very well aware, we are running an editorial policy to only invite revisions for those papers with certain, timely and definitive outcome of a limited number of experimental amendments. Based on this, I find it the most prudent approach

to return the study to you at this point to consider yourself whether

(i) to seek rapid publication elsewhere
(ii) allocate the necessary time and resources to truly develop the study as to enable eventual publication in The EMBO Journal.

Please trust that I am really sorry to be unable to communicate more encouraging news. I still hope that our fairly argued and transparent decision enables constructive pursuit of this truly exciting project.
Referee #1:

In my opinion, new data included in this revised version do not address unequivocally my main criticism. Specifically:
1. Authors cite Ritsma et al. (Nature 2014) as the base for the present study and point that this previous work demonstrates that Lgr5-Lo cells around position +4 (the so called border cells) can give rise to Lgr5-hi cells (present at the center of the niche). As a matter of fact, Ritsma et al. did not investigate Lgr5-Lo cells (they do not even mention Lgr5-Lo cells). Ritsma et al, explored the behavior of Lgr5+ vs Lgr5- cells. Importantly, Ritsma et al. indicate that border cells are heterogeneous regarding Lgr5 levels and that they consider Lgr5-negative border cells as TA cells which most likely correspond to Dll1+ cells (as they had described in van Es et al. Nature Cell Biology. 2012) whereas Lgr5+ border cells are taken as bona-fide ISCs albeit with lower probability of long-term regeneration. Therefore, the manuscript by Ritsma et al should not be used to support author's conclusions about the identity or behavior of Lgr5-Lo cells.

Response: We agree with the reviewer that Ritsma et al. analyzed LGR5-positive and LGR5-negative border cells (+4) without determining the LGR5-GFP-staining intensity. We also agree with the interpretation from the reviewer that the Ritsma paper showed that LGR5-positive cells in position 4 contain true stem cell/self-renewal activity and can replace central stem cells in position 1 / 2 of the niche. We specified this in the manuscript and in the revised discussion, but this does not change our main conclusions. In fact, the studies of Ritsma et al, Es et al., de Sauvage et al, and our current study do not stand in any disagreement but support our main question and conclusions:

A.) See revised introduction of the result section (page 5 lower paragraph:

"Positioning within the niche and levels of LGR5-expression discriminate intestinal ISPCs with different Wnt/β-catenin signal activity. Recent studies revealed a high plasticity of ISPCs in the basal crypts of the intestinal epithelium. It was shown that LGR5-positive ($LGR5^+$) cells at the crypt base in position 1 and 2 represent intestinal stem cells with lineage tracing activity (Barker et al. 2007). In addition, $LGR5^+$ cells in the border region (position 3 / 4 of the crypt) have true stem cell activity and can replace stem cells at the crypt base (Ritsma et al, 2014). However, it was also shown that LGR5-negative TA cells can revert to organoid-forming stem cells in culture when exposed to Wnt3A (Sato et al, 2011) as well as in response to tissue injury in vivo (van Es et al, 2012), but these events are rare and $LGR5^+$ cells were found to be essential for intestinal regeneration and mouse survival in response to IR (Metcalfe et al, 2014). The main aim of this study was to delineate the potential influence of Wnt/β-catenin signaling on the survival of the total population of ISPCs in response to DNA damage rather than to re-investigate the discrimination of intestinal stem and progenitor cells and the plasticity of early progenitors to convert into stem cells or *vice versa*. Therefore, $LGR5^+$ cells in position 1-4 cells are altogether referred to as "stem and progenitor cells (ISPCs)" from here on. "

B.) See revised discussion, page 16:

"This study supports a model indicating that stem and progenitor cells with intrinsically low Wnt signaling activity represent a backup population ensuring recovery of tissue maintenance and survival in response to acute or chronic DNA damage. It was demonstrated by live cell imaging studies that +4 cells consist of a mixture of early TA cells (LGR5-GFP-negative) and $LGR5\text{-}GFP^+$ stem cells (Ritsma et al, 2014). In addition, lineage-tracing experiments revealed that $Dll1^+$ TA cells revert to stem cells in response to severe tissue damage (van Es et al, 2012). However, $LGR5^+$ cells were shown to be essential for survival of mice in response to IR and reversion of $Dll1^+$ TA cells into stem cells is not sufficient for mouse survival in response to IR (Metcalfe et al, 2014; van Es et

al, 2012). The current study shows that within the fraction of $LGR5^{+}$ cells, the cells with low LGR5 expression and low Wnt signaling activity (low *Msi1*, low *Axin2*) preferentially survive in response to DNA damage. This holds true for FACS gated subpopulation of LGR5-high positive cells ($LGR5^{hi\text{-}high}$ cells being more sensitive to IR than $LGR5^{hi\text{-}low}$ cells) and coincides with preferential survival of GFP-positive cells in position 4 of the basal crypt. Together, these results stand in accordance with the concept that niche dependent local signals and cell intrinsic Wnt signaling modulate the survival of ISPCs in response to DNA damage."

2. A key problem that remains and that complicates the interpretation of the results is the identification of Lgr5-lo cells in tissue section versus Flow Cytometry. Authors include now evidence that Lgr5-Lo cells (+4 border cells) are about two fold dimmer than Lgr5-Hi cells by IF in sections (Figure 1D, E). In contrast, Lgr5-Lo cells gated in experiments of flow cytometry are >10 fold dimmer (Figure 3. Please note the logarithmic scale in FACS profiles). There is compelling evidence in the literature that Lgr5-Lo cells as gated here in flow experiments represent TA cells. As matter of fact, Lrg5-Lo cells that authors recognize by IF in tissue sections at position +4 reside very likely within the Lgr5-Hi gate in flow experiments. Authors point in the rebuttal that "the decrease in staining intensity in FACS is more pronounced as in the stainings as the entire cell is measured and not a section through the cell" but this argument is weak as changes in fluorescence intensity should
be proportional in both techniques. Please also note that FACs profiles show that Lgr5-Lo cells are more abundant than Lgr5-Hi cells thus implying that Lgr5-Low cells cannot correspond to border cells in these experiments. My impression is that authors are looking at different populations depending on the technique. Are these cells TA cells located above +4 (Lgr5-Lo cells in flow experiments) or ISCs located in the border (Lgr5-weak cells by IF)? As example, there is fraction of Lgr5-hi cells that remain viable upon irradiation as assessed by FACS (Figure 3D). It is likely that these resilient Lgr5-hi cells rather than the Lgr5-Lo cells represent border cells that regenerate crypts. In contrast, in the experiments of WNT activation/inhibition, authors gate both Hi and Lo populations and therefore it is not possible to discern effects of WNT signaling over TA cells or ISCs. Therefore, the properties of Lgr5-Lo cells remain unclear and this drawback invalidates several of the
conclusions drawn throughout the manuscript.

Response: GFP antibody based IHC staining and fluorescent intensity based FACS analysis could be quite different regarding to the sensitivity due to the inherent differences in both techniques. However, our immunostaining data, in line with previous studies (Itzkovitz et al, 2011), clearly show that ISCs at position +1 and +2 have higher Wnt activity than the cells located at position +4 as determined by LGR5-GFP staining as well as the Wnt target Msi1 staining (Revised Fig. 1 A-D). As suggested by the reviewers and the editor, we also add new data on Wnt target gene expression in $LGR5^{hi\text{-}high}$, $LGR5^{hi\text{-}low}$, $LGR5^{lo\text{-}high}$, and $LGR5^{lo\text{-}low}$ cells and show that LGR5-GFP expression correlates very well with the expression of several Wnt target genes, including *Axin2*, *Ascl2*, and *Msi1* (Revised Fig. 1 F-I). Together these data confirm that FACS purification of ISPCs based on the level of LGR5-GFP expression can separate intestinal stem and progenitor cells into Wnt^{hi} cells and Wnt^{lo} cells. Moreover, the immune-staining data confirm that cells at the crypt bas have higher Wnt activity compared to cells in position 4.

Because of the concerns of this reviewer about our FACS gating and about the magnitude of the difference between $LGR5^{hi}$ and $LGR5^{lo}$ cell in FACS vs. staining, we also included an analysis of subpopulations of $LGR5^{hi}$ cells gated into $LGR5^{hi\text{-}high}$ and $LGR5^{hi\text{-}low}$ cells (Fig. 1 F). The new FACS-analyses reconfirm that within the $LGR5^{hi}$ ISC population the cells with low Wnt-signaling activity preferentially survive IR induced DNA damage (Revised Fig. 5 V-X) or telomere dysfunction (Revised Fig. 3 H-J).

Furthermore, the new FACS gating reconfirms that the enhanced IR-sensitivity of $LGR5^{hi\text{-}high}$ cells compared to the $LGR5^{hi\text{-}low}$ cells is rescued by p53-deletion (Revised Fig. 6 H,J,L,N,P).

Referee #2:

Tao et al.

This manuscript examines the relationship between local Wnt signaling levels and intestinal stem cell response to injury/DNA damage. Accumulation of DNA damage in telomerase deficient Lgr5 reporter mice was associated with downregulation of Wnt signaling activity in the Lgr5+ stem cell compartment. Analysis of the stem cell reporter mice further revealed that this phenomenon was driven by selective loss of Wnthi stem cells restricted to the crypt base. In contrast, +4 position Wntlo stem cells appeared resistant to DNA damage-induced apoptosis. Ex vivo/In vivo inhibition of Wnt signaling preferentially suppressed radiation-induced apoptosis in the Wnthi stem cells. The authors conclude that distinct Lgr5+ stem cells (crypt base versus +4 position) displaying varying levels of intrinsic Wnt signaling exist within the intestinal crypts - the Wnthi, crypt base variety are most susceptible to damage-induced apoptosis, whilst the +4 Wntlo flavour are inherently resistant to injury due
to their reduced Wnt signaling and are consequently able to drive crypt survival/regeneration.

Whilst this is a carefully crafted study, with potentially important implications I have some reservations on some critical aspects of the study.

We thank the reviewer for this his/her positive judgment and the constructive suggestions to improve the study.

1) The assumption that the GFPlo cells at position 4 are stem cells is, in my opinion, not supported by robust functional evidence. The fact that Lgr5-GFPLo cells can be converted into organoid forming entities upon addition of Wnt3a reflects the plasticity of the lower crypt compartment. The Lgr5Lo cells simply convert to Lgr5hi stem cells in conditions of high Wnt - This assay does not establish the endogenous stem cell identity of Lgr5-GFPlo cells at position +4. I also believe the Ritsma study established that all crypt-base Lgr5-GFP+ cells can behave as stem cells -those present at the border of the Paneth cell compartment are less likely to survive long-term because they have a higher probability of being pushed out of the defining niche due to cell division within the finite niche space at the lower portions of the crypt. As far as I'm aware they never concluded that these border Lgr5+ cells are Lgr5-GFPlo. It is also somewhat puzzling to me how sorting the GFPhi versus GFPlo populations for the various profiling experiments can be extrapolated back to the endogenous GFPlo cells at the +4 position. Munoz et al (EMBOJ) previously showed that the stability of the GFP protein ensures that it is sequentially diluted from the Lgr5hi stem cell through several generations of its descendents. Given that the GFPlo fraction isolated using the relatively broad gate employed on the FACS is therefore likely to be a mixture of these different Lgr5 stem cell progeny (ie GFOlo, GFOlower, GFPverylow), it would appear impossible to conclude that the differential expression of Wnt target genes and stem cell markers (of which I believe only Msi1 from the stated list...) relates specifically to the +4 position GFPlo cells. To conclude this, candidate genes would have to be validated by IHC/In-situ to be differentially expressed between the +4 and crypt base GFP populations.

Response: We thank the reviewer for this insightful comment, which has also been discussed by reviewer 1. We followed his/her suggestion to analyze FACS-purified subpopulation of $LGR5^{hi}$ cells (high-high and high-low) and $LGR5^{lo}$ cells (low-high and low-low) for the expression of Wnt-target genes (Revised Figure 1 F-I), and to correlate this with the analysis of Msi1 on sections (revised Figure 1 C,D). The new data confirm a gradient of Wnt-signaling activity in FACS purified cells going from $LGR^{hi\text{-}high}$ > $LGR5^{hi\text{-}low}$ > $LGR5^{lo\text{-}high}$ > $LGR5^{lo\text{-}low}$ (Revised Fig. 1 F-I). The immunofluorescence staining of Msi1 confirms that position 4 cells exhibit reduced staining of Msi1 compared to position 1 / 2 cells at the crypt base (Revised Fig. 1 C,D) standing in agreement with the data on LGR5-GFP expression (Revised Fig. 1 A,B). These data also stand in agreement with the study of Munoz et al. who showed that LGR5 transcripts were significantly less expressed in $LGR5^{lo}$ cells compared to $LGR5^{hi}$ cells (Munoz et al, 2012), and the study of Itzkovitz et al. who showed that on sections Lgr5 transcripts were significantly less expressed in position 4 cells compared to position 1/2 cells (Itzkovitz et al, 2012). Together, the new data confirm that both immunofluorescence staining and FACS purification allow depicting/separating cells with higher and lower Wnt signaling activity. Of note, we use the FACS-based subpopulation analysis ($Lgr5^{hi\text{-}high}$ vs. $Lgr5^{hi\text{-}low}$) to reconfirm that within the $LGR5^{hi}$ subpopulation the cells with lower Lgr5-expression survive DNA damage in response to IR (Revised Fig. 5 V-X) or telomere dysfunction (Revised Fig. 3 H-J).

2) The depletion of the CBC compartment in the aged telomerase-deficient mice is interesting. However, I was intrigued by the fact that the crypt in Figure 1I appears to have gaps between the Paneth cells. Wouldn't the Paneth cell compartment simply become contiguous following loss of the intercalating CBC cells? Are the CBC cells really gone? Maybe an EM picture would have convincingly proven ablation of the CBC cells at the lower crypt positions. I also have doubts about whether the OLFM4+ cells around position +4 in these aged telomerase-deficient mice are really stem cells. OLFM4, whilst being highly expressed in the Lgr5+ CBC cells is also expressed on early TA cells (as shown in the original Van der Vlier Cell paper). Could these surviving OLFM4+ cells then simply be plastic Lgr5- TA cells (including the DLL1+ fraction)?

Response: We show that the basal crypt cells in between the Paneth cells (position 1 / 2) are indeed depleted in aged telomerase-deficient mice by staining for several Wnt independent markers, including H&E, PCNA, and Olfm4 as well as Wnt-dependent marker (Msi1) (Revised Fig. 4). We now include some additional H&E stained sections to demonstrate that there are no gaps between the Paneth cells (see new H&E staining in Revised Fig. 4 A,B, and new Fig. E3). These cells have large cytoplasm and the depletion of stem cells appear as gaps in some of the immunofluorescence stainings as the Paneth cell cytoplasm does not stain for these markers.

We agree that *Olfm4* could mark early TA cells above the stem cells zone, and early TA cells have high plasticity with regard to stemness. However, it is not our intention to re-address the identity of stem cells in the highly plastic stem and progenitor cell compartment of the intestinal epithelium – as this was done by many excellent papers in the past (for example Ristma et al. 2014, van Es et al. 2012, de Sauvage et al. 2014 to name some more recent papers). We carefully went through the wording of our paper to make this very clear. The main contribution of our current study is not to show stem cell/progenitor cell identity or plasticity but to demonstrate that Wnt-signaling activity (influenced by the localization of cells in the basal crypts) represents a major determinant of stem and progenitor cells survival in response to DNA damage.

We carefully reworded the manuscript to make this very clear:

A.) See revised introduction of the result section (page 5 lower paragraph):

"Positioning within the niche and levels of LGR5-expression discriminate intestinal ISPCs with different Wnt/β-catenin signal activity. Recent studies revealed a high plasticity of ISPCs in the basal crypts of the intestinal epithelium. It was shown that LGR5-positive ($LGR5^+$) cells at the crypt base in position 1 and 2 represent intestinal stem cells with lineage tracing activity (Barker et al. 2007). In addition, $LGR5^+$ cells in the border region (position 3 / 4 of the crypt) have true stem cell activity and can replace stem cells at the crypt base (Ritsma et al, 2014). However, it was also shown that LGR5-negative TA cells can revert to organoid-forming stem cells in culture when exposed to Wnt3A (Sato et al, 2011) as well as in response to tissue injury in vivo (van Es et al, 2012), but these events are rare and $LGR5^+$ cells were found to be essential for intestinal regeneration and mouse survival in response to IR (Metcalfe et al, 2014). The main aim of this study was to delineate the potential influence of Wnt/β-catenin signaling on the survival of the total population of ISPCs in response to DNA damage rather than to re-investigate the discrimination of intestinal stem and progenitor cells and the plasticity of early progenitors to convert into stem cells or *vice versa*. Therefore, $LGR5^+$ cells in position 1-4 cells are altogether referred to as "stem and progenitor cells (ISPCs)" from here on."

B.) See revised discussion, page 16:

"This study supports a model indicating that stem and progenitor cells with intrinsically low Wnt signaling activity represent a backup population ensuring recovery of tissue maintenance and survival in response to acute or chronic DNA damage. It was demonstrated by live cell imaging studies that +4 cells consist of a mixture of early TA cells (LGR5-GFP-negative) and $LGR5\text{-}GFP^+$ stem cells (Ritsma et al, 2014). In addition, lineage-tracing experiments revealed that $Dll1^+$ TA cells revert to stem cells in response to severe tissue damage (van Es et al, 2012). However, $LGR5^+$ cells were shown to be essential for survival of mice in response to IR and reversion of $Dll1^+$ TA cells into stem cells is not sufficient for mouse survival in response to IR (Metcalfe et al, 2014; van Es et al, 2012). The current study shows that within the fraction of $LGR5^+$ cells, the cells with low LGR5

expression and low Wnt signaling activity (low *Msi1*, low *Axin2*) preferentially survive in response to DNA damage. This holds true for FACS gated subpopulation of LGR5-high positive cells ($LGR5^{hi\text{-}high}$ cells being more sensitive to IR than $LGR5^{hi\text{-}low}$ cells) and coincides with preferential survival of GFP-positive cells in position 4 of the basal crypt. Together, these results stand in accordance with the concept that niche dependent local signals and cell intrinsic Wnt signaling modulate the survival of ISPCs in response to DNA damage."

3) The in vivo Wnt suppression experiments are potentially very interesting. However, it would have been useful to have included some functional evidence of effects of anti-LRP6/irradiation on stem cell output/survival (ie, lineage tracing). Since this experiment was performed using the Lgr5-EGFP/Cre line, this would only require one extra breeding step to include a conditional reporter allele. Although beyond the scope of this paper, it would also have been interesting to determine whether sensitizing Lgr5+ stem cells to irradiation-induced death by conditionally deleting APC would prevent adenoma formation.

Response: We thank the reviewer for his/her interesting comments and ideas for follow up studies. Our data on sections show that LRP6-mediated Wnt-inhibition improves survival of cells at position 1 and 2 of the basal crypt (Revised Fig.8 J-L).

4) Although this work does nicely demonstrate the existence of cell populations within the lower crypt having distinct DNA damage sensitivities, I do not think the identity of these populations has been definitively proven. One conceptual problem I have with this work is how to relate it to the de Sauvage Lgr5DTR ablation study. Why would the +4 Lgr5 DTR+ stem/progenitor cells not be killed and prevent post-irradation regeneration in this model? If an explanation is that the +4 Lgr5+ cells express GFP-DTR below the threshold necessary to achieve their efficient ablation in vivo, then these GFPlo cells should still be present in the DT-treated crypts. However, de sauvage's data support efficient ablation of all GFP-expressing cells.

Response: We agree with the reviewer that our study demonstrates the functional impact of Wnt-signaling activity (influenced by cell positioning in the basal crypt) on the survival of intestinal stem and progenitor cells in response to DNA damage. Our study does not aim to add to the discussion on stem and progenitor cell identity/plasticity in this compartment, which was covered by a series of very excellent papers in the past. We make this point very clear and have reworded the manuscript throughout (see above).
We respectfully disagree with the reviewer that our data stand in contrast with the recent work from the de Sauvage lab. In fact, our data stand in agreement with his work. De Sauvage and colleagues showed that $LGR5^{+}$ cells are completely depleted by DT-injection (including position 4 cells). Of note, the study shows that these mice cannot survive IR due to intestinal failure. The data indicate that $LGR5^{+}$ cells are indispensable for intestinal regeneration in response to IR and led to the conclusion that "at least a subset of $LGR5^{+}$ cells survives radiation" (Metcalfe et al. Cell Stem Cell, 2014). Our study stand in line with this interpretation and shows that within the fraction of LGR5-positive cells (also in the LGR5-hi subpopulation) the subpopulation of cells with low Wnt activity (low LGR5 expression) preferentially survives in response to DNA damage and this correlates with positioning of the cells in the stem cell niche (Revised Fig. 3 E, F, G-L, Revised Fig. 5 J-X).

Minor comment:

1) Page 7 last paragraph - Figure 1 is depicting IF for GFP not Lgr5 as stated in the text.

Response: corrected accordingly (Revised manuscript Page 6).

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comments from further consultations:

ref#1:

It looks as if the second reviewer pointed exactly to the same concerns that I raised. The suggestions for additional experiments/modifications sound reasonable. I would also suggested to run some

qPCRs on the Hi-Lo gated Lrg5 cells compared to the Hi-Hi and Lo-Lo and measure markers genes (Dll1, ChromograninA, defensin a5, Msi1, Krt20, Fabp1, dclk1). This will help clarify the identity of this radioresistant population in relation to previous studies (ISCs vs TA vs LRC, etc...).

Response: We followed the reviewers suggestion and included an analysis of Wnt signaling (Revised Fig. 1 F-I) and differentiation markers (new Fig. E1) in the fractions of FACS purified $LGR5^{hi\text{-}high}$, $Lgr5^{hi\text{-}low}$, $Lgr5^{lo\text{-}high}$, and $Lgr5^{lo\text{-}low}$ cells. We also included an analysis of survival of $LGR5^{hi\text{-}high}$, $Lgr5^{hi\text{-}low}$ subpopulations in response to IR (Revised Fig. 5 V-X) and telomere dysfunction (Revised Fig. 3 H-J). The analyses reconfirm that within Lgr5-hi subpopulation the fraction of Wnt-lo cells preferentially survives DNA damage.

It is a difficult paper. Some good data mixed with some conceptual drawbacks. It needs significant adjustments based on truly understanding/knowing the peculiarities of the model system.

Response: We thank the reviewer for the insightful comments and for the patience. We think that the main drawback was that we did not clearly enough describe and support the main focus of our study showing that the level of Wnt signaling represents a major determinant of stem and progenitor cells survival in response to DNA damage and that this co-segregates with positioning in the basal crypt niche. It was not our attention to re-address stem and progenitor cell identity/plasticity in this compartment, which has been addressed by several very good papers in the past. We believe that the new data and the careful rewording of our manuscript address the remaining concerns of both reviewers.

ref#2:

I agree that there are some interesting observations in the paper, but the authors absolutely do not prove that there are Wnt lo damage-resistant stem cells restricted to the plus 4 position. At best they can claim early TA cells expressing lower levels of GFP contribute to this phenomenon.They also need to better discuss their findings in light of the Sauvage paper - it currently doesn't make sense. It needs to be accurate or it will simply add to the pile of contradictory data on intestinal stem cells.

Response: We also thank this reviewer for taking the time to improve our study. We addressed his/her concerns as outlined above. The paper has clearly improved and should no longer be seen as controversial but as adding an important novel point to the existing literature.

3rd Editorial Decision 17 December 2014

Thank you very much for your thoroughly revised study, and please accept our apology for the slight delay in the subsequent external assessment, presumably based on the particular time of the year/the tight schedule of the relevant external advisor.

I am very pleased to inform you that based on your further experimentation and significant textual amendments we are very happy to proceed with formal production/publication of your study.

Referee #1:

The authors have finally addressed my criticisms. In particular, they have reworded the text and corrected references to TA cells (Lgr5-Lo cells) as ISCs. The analyses of Lgr5-Hi-Hi versus Lgr5-Hi-Lo cells have also clarified major aspects and strengthened the authors' conclusions.

