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PRC2 Preserves Intestinal Progenitors and Restricts Secretory Lineage Commitment

Fulvio Chiacchiera, Alessandra Rossi, SriGanesh Jammula, Marika Zanotti and Diego Pasini

Corresponding authors: Fulvio Chiacchiera and Diego Pasini, European Institute of Oncology

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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 18 May 2016

Thank you again for submitting your manuscript for consideration by The EMBO Journal. We have now heard back from two referees and their comments are included below.

As you will see, both referees appreciate the significance and timeliness of the findings reported in your manuscript, although they also point to a number of issues that would need to be addressed before they can support publication here. In particular, you will see that while ref #2 finds the current level of analysis to be too superficial s/he at the same time offers constructive criticism for you to improve the conclusiveness of the work. Furthermore, both referees ask for more insight on the PRC2-target interaction and the direct contribution to the cellular phenotype seen.

Given the referees' interest and overall positive recommendations, I would like to invite you to submit a revised version of the manuscript, addressing the comments from both reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance or rejection of your manuscript will therefore depend on the completeness of your responses in this revised version.

In addition, you will see that referee #2 is the more critical of the referees and I want to emphasise that the revised manuscript would have to convince this referee in order to be a strong candidate for publication here. Since some of the points raised by this referee are rather open - and for me to have a better grasp of the timeline for the revision - it would be very helpful if you could provide me with an outline of the experiments you would do for the revision at this stage already. This can be sent directly to me via email.

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

REFEREE COMMENTS

Referee #1:

This manuscript investigates the consequences of PRC2 loss in the adult intestine by analyzing tissue-specific depletion of EED during homeostasis and after irradiation damage. In homeostasis, rapidly dividing cells of the crypt-vilus are negatively affected upon EED loss, whereas the opposite effect is seen in secretory cells. These observations are further supported by RNAseq data from entire crypts, which show that the expression of genes required for secretion is increased in EED-/- crypts as compared to the control crypts, whereby the negative effect on proliferation of the rapidly dividing cells could possibly arise from derepression of the Cdkn2a locus.
In sum, the concept of a dual role for PRC2 in crypts-namely, that of restricting Cdkn2a expression in TA cells and repressing secretory expression program-is well supported by the experimental evidence. This manuscript is well written and could be of broad interest, since much is known about the role of Polycomb in embryonic stem cells but less about the Polycomb function in adult homeostasis.
However, there are few experiments missing that would increase the impact of this manuscript.

1. An interesting observation after PRC2 depletion is that secretory cells (mainly Goblet cells) are increased. Would it be possible to test if other secretory cell lines (such as enteroendocrine or Paneth cells) are also affected? If no specific markers for other secretory cell lines are available, this might be included in the discussion.

2. Irradiation depletes ICS, which are then replaced by secretory precursors, demonstrating the plasticity of the system. In the absence of PRC2, regeneration after irradiation is impaired, as shown by histological staining. The authors should discuss how this regenerated intestine resembles the WT/KO conditions (i.e. compare Fig 1C with Fig 3A). Some sort of quantification may be useful for the reader (see also point below).

3. The molecular section is very interesting: the fact that more that 70% of up-regulated genes are PRC2 targets is a very strong indication of a direct mechanism. However, the manuscript would benefit from including ChIP analysis in Eed-/- cells, to prove that PRC2 is displaced from affected genes (Fig. 4). Moreover, I would encourage the authors to state how many replicates or independent validations they have performed, as well as to specify in the text the fold changes considered as up- or down-regulation for genes. The PRC2 analysis in Eed-/- also refers to Fig. 6.

4. Two additional set of experiments might further improve this manuscript. Since I am aware that this might go beyond the scope of this manuscript, I leave to the authors the decision to include them or not.
A) Taking into account that the authors previously reported that that PRC1 affects self-renewal and proliferation of the intestinal stem cells (ICS), it would be interesting to show in this manuscript that the amount of ICS remains unchanged upon PRC2 depletion.
B) a rescue for the in vivo ink4a-arf null experiments.

Minor point:
1. The abstract seems to need some reorganization, since the first three sentences don't provide any preparation of the reader for the subject of the study. Indeed, the word "intestine" does not appear until the last sentence.
What are "common genes"?
2. In the main text, the references to the Fig. 5C and 5D are wrong (page 11).
3. Quantification of data presented in Fig. 1D and 5C would help.
4. It is unclear which cells/tissue were used for the ChIPseq analysis presented in Fig 4 C, D, 6B.
5. In the introduction the authors referred to EED and SUZ12 as scaffold proteins: "both catalytic subunits require the two scaffold proteins, EED and SUZ12..." which is not correct since they confer histone recognition, Zinc finger binding etc to PRC2. Text should be modified.

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Referee #2:

The work investigates the role of polycomb repressive complex 2 (PRC2) in intestinal homeostasis through deletion of Eed, an essential component of the complex in an inducible mouse model. The research topic and the model chosen to investigate it are relevant and topical.

The authors seem unaware of the contribution by Benoit et al (J. Cell Sci, 125: 3454 (2012)). They ought to reference this paper and deal with the similarities with their own account, namely TA effects on differentiation and proliferation.

A clear epithelial phenotype is induced that is comprised of reduced proliferation (associated with transit amplifying populations only) and increased representation of secretory lineages. The epithelium although possessing reduced cellularity is viable and appears to reach a new homeostatic balance. This rather indirect observation is interpreted by the authors as demonstrating a lack of perturbation of the intestinal stem cell (ISC) compartment. Some considerable effort to directly confirm this using stem cell markers and measures of stem cell proliferation would appear necessary to support the interpretation.

In general throughout the manuscript there is no statement of how many samples/mice have been examined to support conclusions.

Perturbation of the stem cell compartment in the form of radiation is used to test the proliferative/regenerative capacity of ISCs. The authors find that the Eed deficient epithelium is impaired in its ability to restore the steady state. This observation is not presented convincingly with only selected low magnification H&E images presented and no quantitation attempted. Consequently it is hard to interpret:

1. Do the mice tolerate the treatment - one must assume so as there is no statement to the contrary. If so how impaired is the regenerative response?

2. In proportion to their reduced epithelial cellularity is there a more than additive response to the radiation? Counts of surviving crypts might begin to address this.

Eed deficient organoids are unable to form organoids on 3D culture but are only mildly affected when Eed is deleted in established organoid cultures. This is interpreted by the authors as supporting ISCs being functionally competent in an established epithelium but with reduced 'plasticity' on challenge. There are two criticisms:

1. The efficiency of the in vitro recombination to remove Eed has to be established to make the experiment interpretable.

2. In established cultures is 3 days long enough to allow for any phenotype to form?

3. Some quantitative end-point should be included to allow the reader to judge if the affect is mild rather than accepting a single picture.

Overall the proposal that the Eed deficient epithelium has an impaired regenerative response following damage may be correct but this would still not be described as an altered plasticity as indicated in the title. The terms implies an alteration in available lineages for stem cell commitment. There is no evidence that Eed impacts restricts lineage choices- the same cell types are present.

The authors next seek to understand the phenotype by RNAseq to profile the Eed deficient tissue. Genes with altered transcriptional abundance are identified. Given the major loss of the H3K27me3 mark (SFig1) and the emergent phenotype there seems rather few of these (167 deemed to be upregulated, 16 down regulated). The upregulated genes are taken to be mechanistically implicated in driving the phenotype because they are enriched for 'targets' for the H3K27me3 marks that are laid down by PRC2 (Fig4). Notably this is shown by comparing the intensity of H3K27me3 on the loci for upregulated genes to those on downregulated genes. The very small number of the latter

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would seem to make this unsafe. Why not establish if there is enrichment for genes with the mark by comparing upregulated genes versus all transcripts?

Further, why would the authors expect that the transcriptional changes are mediating the phenotype rather than a consequence of it? Secretory transcripts should be up and proliferative ones down- why are these not a feature of the analysis?

Cdkn2a is next investigated as a known target of PRC2 and that is likely to mediate the hypoproliferative aspect of the phenotype. The work contained in Fig 5 seems convincing in establishing that concomitant deletion of Eed and Cdkn2a rescues the Eed proliferative defect but not the secretory bias. Thus cell cycle arrest is not the key factor in both aspects of the phenotype. Notably though it does not rule out regulation of cell cycle progression by genes other than Cdkn2a in mediating the secretory bias. Some analysis of other candidate regulators in secretory cell progenitors might be revealing.

The attempt to define the cause of the secretory bias is less satisfactory. This again depends on the transcriptional profiling. The transcription factors determining secretory fate that are more abundant in the Eed knockout but are also markers of the secretory lineages and reflect the phenotype rather than informing as to the cause of it.

The genes such as Atonal, Ngn3 and Gfi1 are actively expressed within progenitors yet PRC2 is repressive for expression. Is the activity of PRC2 regulated temporally in secretory progenitors to restrict these genes specifically or is it acting to limit their subsequent amplification by cell division (by a non Cdkn2a route)?

Overall the work is interesting but the analysis superficial and qualitative. There is an interesting start into understanding the proliferative phenotype, the basis of the secretory bias remains elusive.

Fig5C is not referred to in text.

1st Revision - authors' response 07 July 2016

(Begins on next page).

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Referee #1:

This manuscript investigates the consequences of PRC2 loss in the adult intestine by analyzing tissue-specific depletion of EED during homeostasis and after irradiation damage. In homeostasis, rapidly dividing cells of the crypt-vilus are negatively affected upon EED loss, whereas the opposite effect is seen in secretory cells. These observations are further supported by RNAseq data from entire crypts, which show that the expression of genes required for secretion is increased in EED-/- crypts as compared to the control crypts, whereby the negative effect on proliferation of the rapidly dividing cells could possibly arise from derepression of the Cdkn2a locus.

In sum, the concept of a dual role for PRC2 in crypts-namely, that of restricting Cdkn2a expression in TA cells and repressing secretory expression program-is well supported by the experimental evidence. This manuscript is well written and could be of broad interest, since much is known about the role of Polycomb in embryonic stem cells but less about the Polycomb function in adult homeostasis.

However, there are few experiments missing that would increase the impact of this manuscript.

Re1) we thank the referee for the positive comments and we hope that this revised version of our manuscript will address the remaining concerns.

1. An interesting observation after PRC2 depletion is that secretory cells (mainly Goblet cells) are increased. Would it be possible to test if other secretory cell lines (such as enteroendocrine or Paneth cells) are also affected? If no specific markers for other secretory cell lines are available, this might be included in the discussion.

Re2) To answer the referee's question, we do not only see a skewing toward Goblet cells but simply we find an overrepresentation of this cell type as the most abundant secretory cell along the crypt-villus axis. This is in line with a general upregulation of *Atoh1*. Indeed we had already reported in the previous version increased Lysozyme positivity (Paneth cell marker) along the crypt-villus axis, cells that can be considered intermediate secretory cells (cells expressing both Paneth and Goblet cell markers (van Es et al, 2012)). We have now extended this analysis to other more rare cell types of the secretory compartment and found that also the enteroendocrine cells are overrepresented in Eed KO epithelia (Chga staining in new figure S3B). Differently the rare population of Tuft cells, did not show an altered representation in the *Eed* KO tissue (DCLK1 staining new figure S3B). This result further strengthens the bias towards secretory commitment in absence of PRC2 activity. We believe that the preferential accumulation of Goblet cells is likely a consequence of the additive effect of the loss of PRC2 repressive layer from both *Atoh1* (a pan master regulator of secretory differentiation, (Shroyer et al, 2007)) and *Gfi1* (a Goblet specific regulator, (Shroyer et al, 2005)) promoters. It is also important to stress that Goblet cells are the most represented secretory cells in the small intestine and stochastically the progenitor environment is more prone to signal for Goblet differentiation respect to rare cell types.

2. Irradiation depletes ICS, which are then replaced by secretory precursors, demonstrating the plasticity of the system. In the absence of PRC2, regeneration after irradiation is impaired, as shown by histological staining. The authors should discuss how this regenerated intestine resembles the WT/KO conditions (i.e. compare Fig 1C with Fig 3A). Some sort of quantification may be useful for the reader (see also point below).

Re3) we do understand the point that the referee is raising, however is quite difficult to make a precise quantifications under these conditions since the entire tissue fails to regenerate. What we have now added are two critical experiments that we think clarify the referee's concern:

1) We have added Ki67 staining to further quantify tissue regeneration. As clearly shown in the new Figure 3B, the Eed KO epithelia fails to completely proliferate upon damage.
2) We have genetically rescued this phenotype with a *Cdkn2a* null background confirming the previous observation presented with organoids *in vitro* cultures. Together, this demonstrates that the proliferative capabilities of the *Eed* KO tissue is fully restored without affecting the skewing towards the accumulation of secretory cells (this is also linked with the answer to referee's question #4)

3. The molecular section is very interesting: the fact that more that 70% of up-regulated genes are PRC2 targets is a very strong indication of a direct mechanism. However, the manuscript would benefit from including ChIP analysis in Eed-/- cells, to prove that PRC2 is displaced from affected genes (Fig. 4). Moreover, I would encourage the authors to state how many replicates or independent validations they have performed, as well as to specify in the text the fold changes considered as up- or down-regulation for genes. The PRC2 analysis in Eed-/- also refers to Fig. 6.

Re4) we have now included the analysis of target genes in the new figure S5B. This result is in line with the general loss of H3K27me3 reported in bulk crypt population and *in situ* staining. We have also included all the information regarding replicates and validations used to generate these results. Regarding the fold change, this was specified in the methods section and corresponds to a FC=3 for both up and down regulated genes. We have made this clear in the figure and legend.

4. Two additional set of experiments might further improve this manuscript. Since I am aware that this might go beyond the scope of this manuscript, I leave to the authors the decision to include them or not.

A) Taking into account that the authors previously reported that that PRC1 affects self-renewal and proliferation of the intestinal stem cells (ICS), it would be interesting to show in this manuscript that the amount of ICS remains unchanged upon PRC2 depletion.

B) a rescue for the in vivo ink4a-arf null experiments.

Re5) we thank the referee for both questions as we think they are really important and informative:

A) Regarding the self-renewal of ISCs, we have performed three distinct experiments that demonstrate lack of counter selection and maintenance of the Lgr5+ stem cell pool.

1) We have demonstrated that PRC2 is active in also the stem-cell compartment and not only in the TA cells showing constitutive activity among the entire crypt to villus axis (Figure 2D).

2) We have crossed our mouse model with a LacZ genetic reported and shown that upon loss of EED functions there are no sign of counter selection up to 30 days from the induction of *Eed* KO (new Figure S1C). This is consistent with the previously shown maintenance of H3K27me3 negativity in the KO tissue and lack of evident homeostasis defects (Figure 1A, S1A and S1B).

2) We have induced PRC2 loss of function in the presence of a stem cell reporter that expresses GFP specifically in Lgr5+ stem cells, demonstrating that the pool of stem cells is maintained after long-term loss (30 days) of PRC2 activity (new Figure 2E).

B) Regarding the Ink4a-arf rescue we have now shown that inactivation of *Cdkn2a* is sufficient to revert the proliferation defects in progenitor cells and failure to regenerate from irradiation damage. Importantly, *Cdkn2a* inactivation was not required to rescue the secretory linage skewing induced by loss of PRC2 activity under both homeostasis and regenerative conditions demonstrating a specific independent dual role of PRC2 in this tissue.

Minor point:

1. The abstract seems to need some reorganization, since the first three sentences don't provide any preparation of the reader for the subject of the study. Indeed, the word "intestine" does not appear until the last sentence.

What are "common genes"?

2. In the main text, the references to the Fig. 5C and 5D are wrong (page 11).

3. Quantification of data presented in Fig. 1D and 5C would help.

4. It is unclear which cells/tissue were used for the ChIPseq analysis presented in Fig 4 C, D, 6B.

5. In the introduction the authors referred to EED and SUZ12 as scaffold proteins: "both catalytic subunits require the two scaffold proteins, EED and SUZ12..." which is not correct since they confer histone recognition, Zinc finger binding etc to PRC2. Text should be modified.

Re6) we have now fixed all these minor points including abstract reorganization.

Referee #2:

The work investigates the role of polycomb repressive complex 2 (PRC2) in intestinal homeostasis through deletion of Eed, an essential component of the complex in an inducible mouse model. The research topic and the model chosen to investigate it are relevant and topical.

Re) We thank the referee for her/his positive comments and hope that the new set of data will satisfy her/his concerns

The authors seem unaware of the contribution by Benoit et al (J. Cell Sci, 125: 3454 (2012)). They ought to reference this paper and deal with the similarities with their own account, namely TA effects on differentiation and proliferation.

Re) we are sorry that this was not mention and we do are aware of the work that the referee is citing. We have now discussed this contribution in relation to our results. Although is difficult and potentially misleading to compare results obtained *in vitro* (using established cell lines or cancer cell lines) with results obtained *in vivo* (using engineered mouse models), we demonstrate that, as hypothesised by Benoit and colleagues, PRC2 activity is required for TA cells proliferation. This point has been added in the discussion section.

A clear epithelial phenotype is induced that is comprised of reduced proliferation (associated with transit amplifying populations only) and increased representation of secretory lineages. The epithelium although possessing reduced cellularity is viable and appears to reach a new homeostatic balance. This rather indirect observation is interpreted by the authors as demonstrating a lack of perturbation of the intestinal stem cell (ISC) compartment. Some considerable effort to directly confirm this using stem cell markers and measures of stem cell proliferation would appear necessary to support the interpretation.

Re) We perfectly understand the referee's concern and we think that our new set of data demonstrate clearly that intestinal stem cell are not affected by loss of PRC2 function despite PRC2 is normally active in these cells (please also refer to reply 5 to referee #1 question). In this revised version we have now shown that:

1) PRC2 is active along the entire crypt-to-villus axis including in Lgr5+ stem cells (Figure 2).

2) There is no counter selection for EED loss of function using a genetic LacZ tracer (Figure S1C), in addition to the previously shown lack of countersanction for the loss of H3K27me3 deposition (Figure 1A, S1A and S1B).

3) The pool of Lgr5+ stem cells is maintained in the absence of PRC2 activity up to 30 days from the induction of Eed KO (Figure 2E).

In general throughout the manuscript there is no statement of how many samples/mice have been examined to support conclusions.

Re) we have now added this information

Perturbation of the stem cell compartment in the form of radiation is used to test the proliferative/regenerative capacity of ISCs. The authors find that the Eed deficient epithelium is impaired in its ability to restore the steady state. This observation is not presented convincingly with only selected low magnification H&E images presented and no quantitation attempted. Consequently it is hard to interpret:

Re) We have now performed KI67 and H3K27me3 staining showing lack of proliferation and no counter selection of H3K27me3 (Figure 1A, 1D, S1A and S1B). In addition, we have also rescued proliferation defects in the TA compartment inactivating *Cdkn2a* expression together with the inability of the *Eed* KO tissue to regenerate from damage without any sign of counter selection for PRC2 inactivation (Figure 5C, D, S5D and S5E). Importantly, the rescue of TA cell proliferation by *Cdkn2a* inactivation was not sufficient to restore normal secretory cells production (Figure 5A and S5C).

1. Do the mice tolerate the treatment - one must assume so as there is no statement to the contrary. If so how impaired is the regenerative response?

Re) No, mice do not tolerate the treatment. This treatment is well established since the 60's. We are using a lethal irradiation and mice will die within 20 days for major hematopoietic failure as well as of other organs. A good description of this can be taken from the Jax lab web page (http://www.informatics.jax.org/greenbook/chapters/chapter22.shtml).

In our case, we had to stop the experiment around day 8 for ethical reasons as the *Eed* KO mice were suffering much more then *wt* due to the failure of regenerating the intestine. Indeed, we have specifically lost half of the *Eed* KO mice in these experiments as they died before day 8. This is now clearly discussed in the results section. Moreover the lack of Ki67 positivity in the *Eed* KO tissue quantifies the failure to respond to the damage (see also the reply to the next comment).

2. In proportion to their reduced epithelial cellularity is there a more than additive response to the radiation? Counts of surviving crypts might begin to address this.

Re) We think that the genetic rescue of this phenotype by *Cdkn2a* inactivation, including the complete rescue of the Ki67 proliferation index, is the best way to address this concern. This result is further supported by our previous *in vitro* results using organoid cultures now presented in Figure S5E.

Eed deficient organoids are unable to form organoids on 3D culture but are only mildly affected when Eed is deleted in established organoid cultures. This is interpreted by the authors as

supporting ISCs being functionally competent in an established epithelium but with reduced 'plasticity' on challenge. There are two criticisms:

1. The efficiency of the in vitro recombination to remove Eed has to be established to make the experiment interpretable.

Re) We have now added western blot analysis from the organoids taken at experimental end point (day 9) confirming loss of EED expression in vitro (Figure S4D).

2. In established cultures is 3 days long enough to allow for any phenotype to form?

Re) Yes it is. To provide proof for this we have removed RSPO1 from the culture media of fully formed organoids. RSPO1 supports stem cell self-renewal via WNT signalling. Our result demonstrates that organoids homeostasis is strongly compromised already at 24h from RSPO1 removal, resulting in a complete failure at 48h (Figure S4A). Similarly, loss of global PRC1 activity, which is essential for ISC self-renewal, affects organoids with a similar timing (Chiacchiera et al, 2016). This demonstrates that compromised viability can be easily observed within our experimental window. However, we have now extended our loss of function analysis as much as possible (5 days) and confirmed our previous results (Figure S4B). This analysis cannot be extended further without splitting the organoids. However, this mimics tissue damage as organoids are broken into pieces similarly to when crypts are isolated from the tissue. Under these conditions, *Eed* KOs will no longer form viable organoids (not shown).

Overall our *in vitro* results are in perfect accordance with our old and new *in vivo* data showing full rescue of the regeneration phenotype upon *Cdkn2a* inactivation.

3. Some quantitative end-point should be included to allow the reader to judge if the affect is mild rather than accepting a single picture.

Re) we are sorry for not having included this before. We have now added low and high magnifications with multiple fields and provided quantifications of this analysis confirming our previous results (Figure 3D, S4A, S4B and S5E).

Overall the proposal that the Eed deficient epithelium has an impaired regenerative response following damage may be correct but this would still not be described as an altered plasticity as indicated in the title. The terms implies an alteration in available lineages for stem cell commitment. There is no evidence that Eed impacts restricts lineage choices- the same cell types are present.

Re) We understand the referee's point, however, it is also important to highlight that all the latest evidences have clearly demonstrated that all types of precursors (including enterocytes) can contribute to ISC regeneration upon damage and not just the secretory lineage (Tetteh et al, 2016). This suggests that the progenitor environment is highly plastic

and governed by cell positioning and exposure to specific signals. Indeed, our new data clearly demonstrates that the barrier for this plasticity is proliferation and not lineage skewing (full Figure 5 and figure S5C and S5D). These results are in line with our previous *in vitro* analysis now shown in the new Figure S5E. We think that these new *in vivo* experiments address well this issue and provide very strong evidences for the uncoupling of these two proprieties of PRC2 activity in this tissue (please also refer to the following point). We have also modified the title in accordance with this referee's criticism.

The authors next seek to understand the phenotype by RNAseq to profile the Eed deficient tissue. Genes with altered transcriptional abundance are identified. Given the major loss of the H3K27me3 mark (SFig1) and the emergent phenotype there seems rather few of these (167 deemed to be upregulated, 16 down regulated). The upregulated genes are taken to be mechanistically implicated in driving the phenotype because they are enriched for 'targets' for the H3K27me3 marks that are laid down by PRC2 (Fig4). Notably this is shown by comparing the intensity of H3K27me3 on the loci for upregulated genes to those on downregulated genes. The very small number of the latter would seem to make this unsafe. Why not establish if there is enrichment for genes with the mark by comparing upregulated genes versus all transcripts?

Re) we have now done this and the result remains the same. In addition, we have included additional controls were an identical number of promoters to the upregulated genes (167) was randomly selected from the genome, thus simulating a stochastic situation. This was repeated independently 5 times again showing no difference highlighting the specificity and directness of our results.

Further, why would the authors expect that the transcriptional changes are mediating the phenotype rather than a consequence of it? Secretory transcripts should be up and proliferative ones down- why are these not a feature of the analysis?

Re) Secretory transcripts are indeed upregulated. The GSE analysis shows exactly this. To detect cell cycle-related genes going down we need to loosen our cut off as proliferation is still occurring in the tissue and it's only mildly reduced. We rather not like to do this with RNAseq data as we start picking up a lot of noise. Indeed, we have validated all this by qPCR extending the analysis to other cell cycle related genes now presented in Figure 1E.

In relation to what is cause and what is consequence, this is a great question but an impossible task. A similar question can be applied to most, if not all, epigenetic related transcription data published so far. Losing a repressive epigenetic modification is clearly not sufficient to drive transcriptional reactivation (i.e. acute loss of PRC2 in ES cells does not result in the induction of any target genes; Riising et al, 2014), however, lack of this repressive control in combination with environmental stimuli (as it occurs in a tissue or in prolonged culturing of ES cells) results in specific transcriptional defects that might be different depending on context and tissue. This is why we observe distinct transcriptional responses depending on the tissue context that generate distinct phenotypes. This applies to many other epigenetic mechanisms that control transcription and not just to the Polycomb machinery. We have now clearly discussed this issue in the text.

Cdkn2a is next investigated as a known target of PRC2 and that is likely to mediate the hypoproliferative aspect of the phenotype. The work contained in Fig 5 seems convincing in establishing that concomitant deletion of Eed and Cdkn2a rescues the Eed proliferative defect but not the secretory bias. Thus cell cycle arrest is not the key factor in both aspects of the phenotype. Notably though it does not rule out regulation of cell cycle progression by genes other than Cdkn2a in mediating the secretory bias. Some analysis of other candidate regulators in secretory cell progenitors might be revealing.

The attempt to define the cause of the secretory bias is less satisfactory. This again depends on the transcriptional profiling. The transcription factors determining secretory fate that are more abundant in the Eed knockout but are also markers of the secretory lineages and reflect the phenotype rather than informing as to the cause of it.

Re) As described above, these are not functional markers but drivers of secretory differentiation. The single ectopic expression of *Atoh1* is sufficient to trigger secretory cells differentiation *in vivo* increasing the number of Goblet cells (as well as Spdef, which is directly induced by Atoh1 and indeed activated in Eed KOs) (Noah et al, 2010; VanDussen & Samuelson, 2010).

The genes such as Atonal, Ngn3 and Gfi1 are actively expressed within progenitors yet PRC2 is repressive for expression. Is the activity of PRC2 regulated temporally in secretory progenitors to restrict these genes specifically or is it acting to limit their subsequent amplification by cell division (by a non Cdkn2a route)?

Re) PRC2 activity is not regulated temporally or spatially (as for instance suggested in Benoit et al.). Our new data further demonstrate that PRC2 is active along the entire crypt to villus axis including in the Lgr5+ the stem cell compartment (new figure S6C). Consistent with our model, PRC2 already associates to *Atoh1* and *Gfi1* promoters already in the stem cells (new Figure S6B). Our model proposes that PRC2 is an epigenetic barrier that cells have to deal with to activate these master regulators of secretory differentiation. Thus, the lack of this repressive barrier favours their activation when exposed to specific signals (ie. Notch, WNT, BMPs…). This facilitates transcriptional activation resulting in an unbalanced production of these lineages. We could in theory rescue this phenotype with *Atoh1* loss of function, but the result is fully expected as *Atoh1* KO mice lack completely the entire secretory lineage from the epithelium already in a PRC2 proficient background making this time consuming experiment useless and out of the manuscript scope.

To demonstrate that cells have to overcome this barrier during secretory differentiation, we have treated mice with the NOTCH inhibitor DBZ to stimulate the accumulation of secretory cell progenitors within the intestinal crypts. Consistent with our model, accumulation of secretory cells by NOTCH inhibition (Figure 6C) resulted in a reduced accumulation of H3K27me3 deposition at the *Atoh1* and *Gfi1* promoters from crypts bulk (Figure 6D).

Overall the work is interesting but the analysis superficial and qualitative. There is an interesting start into understanding the proliferative phenotype, the basis of the secretory bias remains elusive.

Re) we thank the referee for the comments and we think that our new set of data strengthen our findings with clear quantitative analysis by dissecting the two PRC2 dependent phenotypes (proliferation and lineage skewing) using genetic tools and by providing additional evidences in support of our mechanistic model.

Fig5C is not referred to in text.

Re) This figure is now referenced in the text.

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2nd Editorial Decision 04 August 2016

Thank you for submitting a revised version of your manuscript; it has now been seen by the two original referees and their comments are shown below.

As you will see they both find that all criticisms have been sufficiently addressed and recommend the manuscript for publication in The EMBO Journal. However, before we can go on to officially accept your study there are a few editorial issues concerning text and figures that I need you to address in a final version of the manuscript.

REFEREE COMMENTS

Referee #1:

The authors have taken in consideration all the criticisms previously raised by this referee. The new data confirm and extend the original findings. In my opinion, this new version will make a compelling EMBO J paper.

Referee #2:

The authors responses to previous critique are detailed and sufficient. I believe that the manuscript is significantly strengthened particularly in respect of the second part of secretory differentiation effects that were my main concern previously.

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PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Corresponding Author Name: Diego Pasini
Journal Submitted to: EMBO Journal
Manuscript Number: EMBOJ-2016-94550

Reporting Checklist For Life Sciences Articles (Rev. July 2015)

This checklist is used to ensure good reporting standards and to improve the reproducibility of published results. These guidelines are consistent with the Principles and Guidelines for Reporting Preclinical Research issued by the NIH in 2014. Please follow the journal's authorship guidelines in preparing your manuscript.

A- Figures
1. Data
The data shown in figures should satisfy the following conditions:

the data were obtained and processed according to the field's best practice and are presented to reflect the results of the experiments in an accurate and unbiased manner.
figure panels include only data points, measurements or observations that can be compared to each other in a scientifically meaningful way.
graphs include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical replicates.
if n< 5, the individual data points from each experiment should be plotted and any statistical test employed should be justified
Source Data should be included to report the data underlying graphs. Please follow the guidelines set out in the author ship guidelines on Data Presentation.

2. Captions
Each figure caption should contain the following information, for each panel where they are relevant:

a specification of the experimental system investigated (eg cell line, species name).
the assay(s) and method(s) used to carry out the reported observations and measurements
an explicit mention of the biological and chemical entity(ies) that are being measured.
an explicit mention of the biological and chemical entity(ies) that are altered/varied/perturbed in a controlled manner.

the exact sample size (n) for each experimental group/condition, given as a number, not a range;
a description of the sample collection allowing the reader to understand whether the samples represent technical or biological replicates (including how many animals, litters, cultures, etc.).
a statement of how many times the experiment shown was independently replicated in the laboratory.
definitions of statistical methods and measures:
- common tests, such as t-test (please specify whether paired vs. unpaired), simple χ2 tests, Wilcoxon and Mann-Whitney tests, can be unambiguously identified by name only, but more complex techniques should be described in the methods section;
- are tests one-sided or two-sided?
- are there adjustments for multiple comparisons?
- exact statistical test results, e.g., P values = x but not P values < x;
- definition of 'center values' as median or average;
- definition of error bars as s.d. or s.e.m.

Any descriptions too long for the figure legend should be included in the methods section and/or with the source data.

Please ensure that the answers to the following questions are reported in the manuscript itself. We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

In the pink boxes below, provide the page number(s) of the manuscript draft or figure legend(s) where the information can be located. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable).

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B- Statistics and general methods

Please fill out these boxes (Do not worry if you cannot see all your text once you press return)

Question	Answer
1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	NA
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	For all analysis with animals a minimum number of 6-10 aged and sex matched animals have been used.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	NA
3. Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. randomization procedure)? If yes, please describe.	NA
For animal studies, include a statement about randomization even if no randomization was used.	sex match mice were randomly selected from the same litters for each experimental condition. Multiple litters were always used for each experiment. Selected mice were randomly grouped and treated accordingly to experimental conditions.
4.a. Were any steps taken to minimize the effects of subjective bias during group allocation or/and when assessing results (e.g. blinding of the investigator)? If yes please describe.	NA
4.b. For animal studies, include a statement about blinding even if no blinding was done	All phenotypic analysis including staining of organ sections were analysed blindly with coded samples by two independent scientists.
5. For every figure, are statistical tests justified as appropriate?	YES
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	NA
Is there an estimate of variation within each group of data?	NA
Is the variance similar between the groups that are being statistically compared?	NA

C- Reagents

6. To show that antibodies were profiled for use in the system under study (assay and species), provide a citation, catalog number and/or clone number, supplementary information or reference to an antibody validation profile. e.g., Antibodypedia (see link list at top right), 1DegreeBio (see link list at top right).	References and/or catalog numbers for each antibody used in this study are clearly indicated in the method section of the manuscript
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	NA

* for all hyperlinks, please see the table at the top right of the document

D- Animal Models

8. Report species, strain, gender, age of animals and genetic modification status where applicable. Please detail housing and husbandry conditions and the source of animals.	All experiments were performed with Mus musculus in C57/B6 background. All experiments were performed with sex match animals of 8-12 weeks of age. All genetically modified mice strains were developed in house and maintained by the SPF animal facility of COGENTECH S.c.a.r.l (www.cogentech.it) and fed at libitum.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Mice were housed accordingly to the guidelines set out in the European Commission Recommendation 2007/526/EC, June 18, 2007, for the accommodation and care of animals used in experimental and other scientific purposes. All experiments were performed in accordance to the Italian Law (D.L.vo 116/92 and following additions), which enforces the EU 86/609 Directive (Council Directive 86/609/EEC of 24 November 1986 on the approximation of laws, regulations, and administrative provisions of the Member States regarding the protection of animals used for experimental and other scientific purposes).
10. We recommend consulting the ARRIVE guidelines (see link list at top right) (PLoS Biol. 8(6), e1000412, 2010) to ensure that other relevant aspects of animal studies are adequately reported. See author guidelines, under 'Reporting Guidelines'. See also: NIH (see link list at top right) and MRC (see link list at top right) recommendations. Please confirm compliance.	Confirm

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	NA
12. Include a statement confirming that informed consent was obtained from all subjects and that the experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report.	NA
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	NA
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	NA
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NA
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17. For tumor marker prognostic studies, we recommend that you follow the REMARK reporting guidelines (see link list at top right). See author guidelines, under 'Reporting Guidelines'. Please confirm you have followed these guidelines.	NA

F- Data Accessibility

18. Provide accession codes for deposited data. See author guidelines, under 'Data Deposition'. Data deposition in a public repository is mandatory for: a. Protein, DNA and RNA sequences b. Macromolecular structures c. Crystallographic data for small molecules d. Functional genomics data e. Proteomics and molecular interactions	GEO database at accession: GSE80539
19. Deposition is strongly recommended for any datasets that are central and integral to the study; please consider the journal's data policy. If no structured public repository exists for a given data type, we encourage the provision of datasets in the manuscript as a Supplementary Document (see author guidelines under 'Expanded View' or in unstructured repositories such as Dryad (see link list at top right) or Figshare (see link list at top right).	data were deposited at the GEO database
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G- Dual use research of concern

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