DGCR8 is Essential for Tumor Progression Following PTEN Loss in the Prostate.

Cassandra D. Belair, Alireza Paikari, Felix Moltzahn, Archana Shenoy, Christina Yau, Marc Dall’Era, Jeff Simko, Christopher Benz and Robert Blelloch

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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.)

Editor: Barbara Pauly

1st Editorial Decision 18 December 2014

Thank you very much for the submission of your research manuscript to our editorial office and for your patience while we were waiting to hear back from the referees. We have now received the full set of reviews on your manuscript.

As the detailed reports are pasted below I will only repeat the main points here. While all reviewers agree on the potential interest of the findings, they also pinpoint several aspects that should be addressed before publication of the study can be considered in EMBO reports. Most importantly, both referees 1 and 2 state that the relevance of Dgcr8 in human prostate cancer samples should be investigated. Second, reviewers 1 and 3 feel that a better understanding of which of the dysregulated miRNAs regulate prostate cancer progression would be needed. I certainly understand that the elucidation of the entire molecular pathway(s) regulating tumor cell proliferation and senescence is beyond the scope of this study. However, I do agree with the reviewers on this issue and would suggest to at least look at some potential candidates and, for example, compare miRNAs that are induced upon Pten, but not Dgcr8, loss and which might be responsible for the observed senescence phenotype and the basal cell expansion. Other aspects that would require your attention include the points raised by referee 2 on the inclusion of additional immunofluorescence images and on the distinction between epithelial and stromal cells. Reviewer 3 recommends better age-matching of the tumors, to test in which cell types the Pten-associated senescence is activated and, if possible, to investigate whether Dgcr8 is involved in overcoming the senescence barrier in younger animals as
well.

Given the reviewers' constructive comments, I would like to give you the opportunity to revise your manuscript, with the understanding that the main concerns of the referees should be addressed. I do realize that the referees ask for the addition of a significant amount of new data, but maybe you already have some of it at hand or will be able to acquire it relatively easily. Acceptance of the manuscript will depend on a positive outcome of a second round of review and I should also remind you that it is EMBO reports policy to allow a single round of revision only and that therefore, acceptance or rejection of the manuscript will depend on the completeness of your responses included in the next, final version of the manuscript.

I look forward to seeing a revised form of your manuscript when it is ready. Should you in the meantime have any questions, please do not hesitate to contact me.

REFEREE REPORTS:

Referee #1:

In this manuscript, the authors report that deletion of Dgcr8 in Pten conditional knockout mice reduces prostate tumor formation and specifically suppresses basal (but not luminal) cell expansion associated with Pten loss. Since Dgcr8/Pten double knockout prostate tumors exhibit less proliferation and more senescence compared with Pten single knockout prostate tumors, the authors concluded that microRNAs play an important role in enhancing proliferation and suppressing senescence in Pten null basal cell prostate tumors.

The phenotype found in Dgcr8/Pten double knockout mouse prostate tumors is very interesting. However, due to the lack of human relevance and mechanistic studies, the potential impact of this manuscript is weakened and the conclusion is hypothetical. Specifically:

1. Does DGCR8 play a similar role in human Pten null basal prostate tumors?
2. There are many microRNAs regulated by Dgcr8. Which one(s) is the major mediator of Dgcr8's effects in Pten null basal prostate tumor cells?
3. Does the role of Dgcr8 in basal prostate tumor cells depend on Pten deficiency? If so, what is the mechanistic link between Dgcr8 and Pten?

Referee #2:

In the manuscript Belair et al. focused their attention on the role of Dgcr8 in prostate cancer progression induced by Pten loss. In the paper they produced and described the murine model Pten;Dgcr8 double-knockout and they declared that results uncovered a key role of miRNA in Pten-loss tumor progression. Pten and Dgcr8 knockout murine models are not novel and are well reported in already published articles by the proponents and others. The unique novelty of the manuscript is the production of the double murine knockout model (probasin -promoter mediated) but the experiments highlighting the relevance in the field of the model are not conclusive. Prostate cancer field absolutely requires murine models for amplifying knowledge and testing new therapeutical approach. Thus, the paper may be promising but it reports several general major criticisms that reduce the novelty and the impact of the results. As general criticism, miRNA biogenesis machinery increase or decrease during prostate cancer progression is still debated and it requires further investigation. As consequence the first fundamental point that authors should solve is to evaluate the miRNA biogenesis machinery, particularly Dgcr8, expression using patients' tissues in their hands. In particular they have to correlate Dgcr8 expression and Pten-defective human tumors. Their new murine model may acquire a great value if it mirrors or simulates what happens in humans with particular attention to Pten-defective cancers. In other words they should demonstrate the relevance of their model in prostate cancer field. In Figure 1 A, authors should show (Immunohistochemistry images) both low and high magnification images (including more then one acini) indicating in legend what prostate lobes are
analyzing and clarifying if the situation is similar in all murine prostate lobes. In figure 1 (Immunofluorescence images) co-staining of Dgcr8, basal and luminal markers should be reported, both at low and high magnification. In the first paragraph authors performed miRNA profiling by multiplex qRT-PCR reaction and commented that several miRNA levels should be affected by fibroblast contamination. This experimental part is absolutely fundamental. To react at the conclusion that miRNA compartment is required for cancer progression authors should deeply investigated this part. Authors should purify epithelial (normal and neoplastic) part of murine tissues by laser microdissection and perform miRNA expression evaluation. Since published articles report miRNA which are affected and unaffected by GDCR8 loss, authors should includes them as internal control. In figure 1D, again, authors show only an high magnification images. Also if this part is already published, authors should report pAKT staining in a microscopy field with several acini and not only one. All immunohistochemistry and immunofluorescence assay should be quantify in more then one slide. In the first paragraph authors described figure 1D before of Figure 1 B and C. Please describe in the text figures with order. Figure 2 A should be associated with hematoxylin and eosin staining.

The images should report scale bars.

Minor criticism: line 19 page 3, change mir- with miR-

Figure S1 and Figure S2 should include also the images of the other genotypes and wild type mice.

Referee #3:

The manuscript by Belair et al investigates the role of DGCR8 and miRNA biogenesis in prostate tumorigenesis in the context of PTEN inactivation, using mouse models with prostate epithelium-specific ablation of both genes. They show that Dgcr8 deletion inhibits tumor progression, with reduced expansion of basal cells. Finally, they suggest that these phenotypes can be correlated to differences in cellular senescence, which has been shown to block tumor progression in this model. The findings are novel and the experimental work is generally sound. However, some mechanistic aspects - most importantly the link to senescence - need to be better substantiated and some conclusions are not fully supported by the data.

Major points:

1. The tumor data in Figure 2C groups together mice ranging from 32 to 44 weeks of age. This display may obscure differences that could be more evident if age-matched tumors are compared, as in Figure 3. The comparison of Figure 2 and Figure 3 shows some discrepancies. The data for Pten-null mice in 2B suggests that most "visible tumors" at 12 weeks are hyperplasias. However, only 28% (4/14) of double mutant mice display tumors (Fig. 2B), while 62% (5/8) show hyperplasias at the same age (Fig. 3B). This discrepancy should be explained.

2. The authors show convincingly that Dgcr8 loss blocks the expansion of basal cells typical of Pten-KO prostates. It would be interesting to try and correlate this observation with the suggested effects on senescence. Is Pten-senescence activated in luminal, basal or both cell types? The age of the mice used in this analysis should be indicated.

3. In Fig 5, the authors study SA-BetaGal and Ki-67 in late stage tumors (32-44 weeks old) and based on this data, they conclude that "Dgcr8 is required to overcome the senescence barrier enabling progression in the Pten null background". This conclusion is not fully supported by the current data. It would be more informative to perform the same study in young animals, where Pten-dependent senescence has been described (Chen et al, Nature, 2005) and determine if Dgcr8 loss does in fact impair senescence due to Pten loss. It would also be useful to test additional senescence markers linked to Pten-senescence such as p21, p19Arf or p53.

4. It would be interesting to provide some insight into the mechanism by which defective miRNA biogenesis and Dgcr8 loss could blunt tumor progression in Pten-null prostates. One possibility is via specific miRNAs critical for prostate tumorigenesis and/or senescence. Studying the specific miRNAs altered in Pten-null versus wild-type prostates could provide clues.
Minor points:

1. The IHC data in Fig1A confirms loss of Dgcr8 in the prostate epithelium of both Dgcr8-null models, but it seems that Dgcr8 is increased in the stroma of Dgcr8-null mice (but not in the double Pten/Dgcr8-null). Is this a reproducible observation?

2. The published evidence that links specific miRNAs and miRNA biogenesis to senescence should be cited in the discussion.

RESPONSE TO REVIEWERS

Referee #1:

In this manuscript, the authors report that deletion of Dgcr8 in Pten conditional knockout mice reduces prostate tumor formation and specifically suppresses basal (but not luminal) cell expansion associated with Pten loss. Since Dgcr8/Pten double knockout prostate tumors exhibit less proliferation and more senescence compared with Pten single knockout prostate tumors, the authors concluded that microRNAs play an important role in enhancing proliferation and suppressing senescence in Pten null basal cell prostate tumors.

The phenotype found in Dgcr8/Pten double knockout mouse prostate tumors is very interesting. However, due to the lack of human relevance and mechanistic studies, the potential impact of this manuscript is weakened and the conclusion is hypothetical. Specifically:

1. Does DGCR8 play a similar role in human Pten null basal prostate tumors?

   We thank the reviewer for this question as it led to a very exciting finding (new Figure 7). Obviously, performing the mechanistic studies (i.e. knockouts of Pten and Dgcr8) in humans is impossible and doing the experiments on human cell lines defeats the purpose of this paper, which is an in vivo study. Therefore, we turned to the extensive profiling data that TCGA has been producing to see if we could find a correlation between activated Akt and increased Dgcr8 in prostate cancer. With help of two collaborators, Christine Yau and Christopher Benz, we used the PARADIGM tool to determine Akt activity across 334 tumor samples and correlate with Dgcr8 levels. We were thrilled to see a very convincing correlation. This correlation was confirmed when evaluating additional protein data measuring pAkt, which once again positively correlated with Dgcr8 levels. These data extend upon the previous data simply showing increased Dgcr8 levels when comparing tumor versus normal tissue. Together with our mechanistic data in mouse, the findings provide strong support for a critical role of Dgcr8 in the progression of prostate cancers associated with Akt activation.

2. There are many microRNAs regulated by Dgcr8. Which one(s) is the major mediator of Dgcr8's effects in Pten null basal prostate tumor cells?

   Of course this is a very important question. While, we feel that studying the mechanistic contribution of individual miRNAs is beyond the scope of this paper, per the advice of the editor, we instead decided to measure changes in miRNA levels associated with Pten loss providing candidate miRNAs for future studies. This is already a tricky question as due to the in vivo nature of our studies, we are dealing with a complex tissue. Thus changes in amount of tumor relative to non tumor tissue alone can lead to major changes in the miRNA population. This major caveat was brilliantly exemplified in a recent paper from the Mendell group where they show extensive in vitro studies on miR-143/145 in intestinal adenocarcinoma was based on the false premise that these miRNAs were up in tumor. A closer looked show that they were actually up in the associated stromal tissue. To avoid that trap, we felt it necessary to develop a strategy to specifically isolate the epithelial cells that we had genetically modified. This required the crossing to a R26-lox-stop-lox-YFP reporter and backcrossing to homozygose Pten. As one can imagine, this took much time.
resulting in the delayed submission of this revision. In the end, we have succeeded producing highly reproducible profiling data from the isolated epithelial cells. As expected, the result is a number of candidate miRNAs. We are now devising strategies to study the role of these miRNAs in prostate cancer in vivo.

3. Does the role of Dgcr8 in basal prostate tumor cells depend on Pten deficiency? If so, what is the mechanistic link between Dgcr8 and Pten?
In the assays we performed, the Dgcr8 null prostates were indistinguishable from wild-type prostates. Whether the phenotype we uncover is specific to Pten loss or would be associated with alteration of other prostate cancer promoting pathways is a very interesting question. While we feel these studies are beyond the scope of the paper, we now address this point in the discussion.

Referee #2:

In the manuscript Belair et al. focused their attention on the role of Dgcr8 in prostate cancer progression induced by Pten loss. In the paper they produced and described the murine model Pten;Dgcr8 double-knockout and they declared that results uncovered a key role of miRNA in Pten-loss tumor progression. Pten and Dgcr8 knockout murine models are not novel and are well reported in already published articles by the proponents and others. The unique novelty of the manuscript is the production of the double murine knockout model (probasin - promoter mediated) but the experiments highlighting the relevance in the field of the model are not conclusive. Prostate cancer field absolutely requires murine models for amplifying knowledge and testing new therapeutical approach. Thus, the paper may be promising but it reports several general major criticisms that reduce the novelty and the impact of the results. As general criticism, miRNA biogenesis machinery increase or decrease during prostate cancer progression is still debated and it requires further investigation. As consequence the first fundamental point that authors should solve is to evaluate the miRNA biogenesis machinery, particularly Dgcr8, expression using patients' tissues in their hands. In particular they have to correlate Dgcr8 expression and Pten-defective human tumors. Their new murine model may acquire a great value if it mirrors or simulates what happens in humans with particular attention to Pten-defective cancers. In other words they should demonstrate the relevance of their model in prostate cancer field.

As discussed in response to reviewer 1 point 1, we agree this is an important question and are grateful that the reviewers led us in this direction. In addition to points made in response to reviewer one, we did also do some IHC of human samples to confirm an increase in Dgcr8 in prostate cancer samples (representative images shown in Fig. EV5. Interestingly, we found that Dgcr8 is indeed elevated relative to the predominant normal epithelial cell type (luminal cells), but interestingly is roughly the same as the basal cells. This is especially interesting as in most aspects human prostate tumor cells have a more luminal phenotype. The elevated Dgcr8 levels suggest some common features with basal cells. We also see potential increase in Dgcr8 with increasing tumor grade, but are hesitant to make any conclusion as IHC is only semi-quantitative. As for the connection of Dgcr8 to Akt activity AMONG prostate tumor samples, we did not know what to expect, but thought it was a very interesting question. Thus, as discussed above, we evaluated a large cohort of human samples using the much more quantitative measure of RNA expression and indeed found a positive correlation between Akt activity and Dgcr8 levels (Fig. 7). We thank the reviewer for suggesting the experiment.

In Figure 1 A, authors should show (Immunohistochemistry images) both low and high magnification images (including more then one acini) indicating in legend what prostate lobes are analyzing and clarifying if the situation is similar in all murine prostate lobes. We have added to the legends and methods section to indicate where the analysis was performed. We have added expanded view figures with low magnification images of pAKT, Ck5, Ck8 and Ki67 of all genotypes from both cohorts.

In figure 1 (Immunofluorescence images) co-staining of Dgcr8, basal and luminal markers should
be reported, both at low and high magnification. From the IHC images it is clear that DGCR8 is present in the luminal cells. We were also able to see this with CK8 staining. Dgcr8 and basal staining cannot be performed simultaneously for technical reasons (antibody subtypes). Due to the background (see non-nuclear staining) and generally weak staining with this antibody, low magnification images are impossible to interpret and thus were not saved.

In the first paragraph authors performed miRNA profiling by multiplex qRT-PCR reaction and commented that several miRNA levels should be affected by fibroblast contamination. This experimental part is absolutely fundamental. To react at the conclusion that miRNA compartment is required for cancer progression authors should deeply investigated this part. Authors should purify epithelial (normal and neoplastic) part of murine tissues by laser microdissection and perform miRNA expression evaluation. Since published articles report miRNA which are affected and unaffected by DGCR8 loss, authors should includes them as internal control. See response to reviewer 1, point 2. In figure 1D, again, authors show only an high magnification images. Also if this part is already published, authors should report pAKT staining in a microscopy field with several acini and not only one. Low magnification images of pAKT staining in both cohorts of mice have been added as Figure EV1. All immunohistochemistry and immunofluorescence assay should be quantify in more then one slide. The results and methods sections have been rewritten to emphasize that quantification of expression was performed on at multiple fields of view from at least four individual mice of each genotype. In the first paragraph authors described figure 1D before of Figure 1 B and C. Please describe in the text figures with order. This had been fixed. Figure 2 A should be associated with hematoxylin and eosin staining. As stated in the results and methods sections, the young animals in the histological analysis figure 3b are a subset of figure 2b and all the animals of figure 2c were subjected to complete histological analysis in figure 3c. We have also added graphs in Figure EV2 showing all the mice of each genotype, their age and tumor status or histological category.

The images should report scale bars. Scale bars have been added to all microscopic images.

Minor criticism: line 19 page 3, change mir- with miR-

Figure S1 and Figure S2 should include also the images of the other genotypes and wild type mice. The features shown in figures S1 are histological features only found in the dysplastic prostates of the Pten null group. Figure S2 is showing that at 52 weeks of age neither the wild type nor Pten/Dgcr8 double KO had progressed to a dysplastic phenotype. We did not age Pten null mice that long in the interest of their comfort.

Referee #3:

The manuscript by Belair et al investigates the role of DGCR8 and miRNA biogenesis in prostate tumorigenesis in the context of PTEN inactivation, using mouse models with prostate epithelium-specific ablation of both genes. They show that Dgcr8 deletion inhibits tumor progression, with reduced expansion of basal cells. Finally, they suggest that these phenotypes can be correlated to differences in cellular senescence, which has been shown to block tumor progression in this model. The findings are novel and the experimental work is generally sound. However, some mechanistic aspects - most importantly the link to senescence - need to be better substantiated and some conclusions are not fully supported by the data.

Major points:

1. The tumor data in Figure 2C groups together mice ranging from 32 to 44 weeks of age. This display may obscure differences that could be more evident if age-matched tumors are compared, as in Figure 3. The comparison of Figure 2 and Figure 3 shows some discrepancies. The data for Pten-null mice in 2B suggests that most "visible tumors" at 12 weeks are hyperplasias. However, only 28% (4/14) of double mutant mice display tumors (Fig. 2B), while 62% (5/8) show hyperplasias at the same age (Fig. 3B). This discrepancy should be explained. We have added Figure EV2 to address the age range of the two cohorts. We plotted all the mice in
the study based on genotype and age and showed the tumor phenotype and histological category of each. When presented this way one can see that the double knock-out mice in the old cohort tend to be older, yet still present with less tumors and fail to progress to dysplastic phenotype.

The apparent discrepancy between tumor incidence and hyperplasia incidence mentioned by the reviewer is due to the sensitivity of the two assays. Histological analysis is more refined than the assessment of tumor based on size at dissection. There were a number of samples that appeared normal at dissection but upon histological examination showed hyperplasia.

2. The authors show convincingly that Dgcr8 loss blocks the expansion of basal cells typical of PtendKO prostates. It would be interesting to try and correlate this observation with the suggested effects on senescence. Is Ptend-senescence activated in luminal, basal or both cell types?

We agree this is a very interesting question. We were technically unable to perform both the b-Gal senescence assay and immuno-staining for basal and/or luminal markers. We tried other markers for senescence for which there are antibodies, however we were not able to get the staining to work, probably secondary to our methods of fixation. However, we have improved our senescence staining as shown in new figure, which show broad senescence in what appears to be both compartments at the older ages.

The age of the mice used in this analysis should be indicated. We have clarified the ages in the text and have extended our analysis to multiple mice from each genotype in both age cohorts and Figure 5A now reflects this.

3. In Fig 5, the authors study SA-BetaGal and Ki-67 in late stage tumors (32-44 weeks old) and based on this data, they conclude that "Dgcr8 is required to overcome the senescence barrier enabling progression in the Pten null background". This conclusion is not fully supported by the current data. It would be more informative to perform the same study in young animals, where Pten-dependent senescence has been described (Chen et al, Nature, 2005) and determine if Dgcr8 loss does in fact impair senescence due to Pten loss. It would also be useful to test additional senescence markers linked to Pten-senescence such as p21, p19Arf or p53.

We thank the reviewer for this suggestion and have now included senescent staining on young mice (new Fig. 5a). At the younger age, Pten ko and double ko show roughly equal levels of senescence, which is below that of wt or Dgcr8 ko alone. However, the level increases in the double ko with age, reaching levels equivalent to wt and Dgcr8 knockout alone prostates. As for additional senescent markers, see previous point.

4. It would be interesting to provide some insight into the mechanism by which defective miRNA biogenesis and Dgcr8 loss could blunt tumor progression in Pten-null prostates. One possibility is via specific miRNAs critical for prostate tumorigenesis and/or senescence. Studying the specific miRNAs altered in Pten-null versus wild-type prostates could provide clues. We have added Figure 6. Small RNA sequencing analysis of Wild type versus PTEN null prostate epithelial cells revealed 15 significantly differentially expressed microRNAs five of which stand out as up-regulated in Pten null. These provide strong candidates, which we plan to follow up in the next few years. Obviously, such in vivo studies, take much time.

Minor points:

1. The IHC data in Fig1A confirms loss of Dgcr8 in the prostate epithelium of both Dgcr8-null models, but it seems that Dgcr8 is increased in the stroma of Dgcr8-null mice (but not in the double Pten/Dgcr8-null). Is this a reproducible observation?

This is not a reproducible observation, nor linked to any genotype. This staining is almost certainly secondary, possibly due to stickiness of the stroma, as the staining is throughout the cell, not nuclear. This background staining was very inconsistent. Unfortunately, neither DGC8 antibody was particularly robust; there were considerable technical hurdles to achieve specific staining without destroying the tissue during antigen retrieval.
2. The published evidence that links specific miRNAs and miRNA biogenesis to senescence should be cited in the discussion.

This is a large topic. Therefore, rather than attempt to cover the field we have added a sentence in the discussion referring to two excellent reviews on the topic. Of course, if there are specific papers that the reviewer feels are especially relevant to the topic of this paper, we would be happy to include them.

Many thanks for submitting your revised manuscript to our editorial office and for your patience while we were waiting to hear back from the original reviewers who were asked to assess the revised version.

As you will see, all referees now support publication of the study in our journal. Nevertheless, referee 3 still raises an issue with the link between DGCR8 and senescence, a point that s/he has also raised in his/her original report. Since this is likely not gonna be resolved experimentally, I would ask you to reword the interpretation of the senescence-related results and tone down your interpretations accordingly. Also maybe discuss apparent contradictions in previous reports in more depth, as suggested by this reviewer. I still think that the reported effects of DGCR8 on tumor progression are very interesting, but it seems as if the link to senescence is not yet fully understood and established.

Please do not worry about length restrictions too much as I think an insightful discussion of these points is more important than publishing a short paper in this case. Please also highlight these changes in the final version of the text, so that it will be easier for us to identify them.

Thank you very much for your cooperation and I look forward to receiving the revised manuscript as soon as it is ready, as we do not want to unnecessarily delay its publication, which, in the end, the reviewers support.

REFEREE REPORTS:

Referee #1:

The authors have added either new experiments or discussion to address previous criticisms. I recommend publishing this manuscript in EMBO Reports.

Referee #2:

The article is now suitable for publication in EMBO Reports Journal

Referee #3:

In this revised version, the authors have addressed most of the referees' comments resulting in a significantly improved version.

Regarding the link to senescence, the authors have included a new panel with SABGal staining for young prostates (Figure 5A in the new version). The authors say in their response that the new data shows that "Pten ko and double ko show roughly equal levels of senescence, which is below that of wt or Dger8 ko alone. However, the level increases in the double ko with age, reaching levels equivalent to wt and Dger8 knockout alone prostates". These results are difficult to interpret. The authors claim that "Dger8 is required to overcome the senescence barrier enabling progression in the Pten null background" (page 8 of the manuscript), however, the new data does not show any evidence of increased senescence in young Pten-KO/Dger8-wt prostates (Figure 5A), in contrast to
findings from several groups (see Chen, Nature, 2005; Toso, Cell Reports, 2014). With these results, and considering that the authors have not been able to analyse additional senescence markers, the link to senescence is weak and I would recommend to revise the mentions to senescence in Abstract, Results and Discussion to reflect more accurately the actual data, and also comment on the discrepancies with published data.

Response to Reviewers

Referee #1:

The authors have added either new experiments or discussion to address previous criticisms. I recommend publishing this manuscript in EMBO Reports.

Thank you.

Referee #2:

The article is now suitable for publication in EMBO Reports Journal

Thank you.

Referee #3:

In this revised version, the authors have addressed most of the referees' comments resulting in a significantly improved version.

Thank you.

Regarding the link to senescence, the authors have included a new panel with SABGal staining for young prostates (Figure 5A in the new version). The authors say in their response that the new data shows that "Pten ko and double ko show roughly equal levels of senescence, which is below that of wt or Dgcr8 ko alone. However, the level increases in the double ko with age, reaching levels equivalent to wt and Dgcr8 knockout alone prostates". These results are difficult to interpret. The authors claim that "Dgcr8 is required to overcome the senescence barrier enabling progression in the Pten null background" (page 8 of the manuscript), however, the new data does not show any evidence of increased senescence in young Pten-KO/Dgrc8-wt prostates (Figure 5A), in contrast to findings from several groups (see Chen, Nature, 2005; Toso, Cell Reports, 2014). With these results, and considering that the authors have not been able to analyse additional senescence markers, the link to senescence is weak and I would recommend to revise the mentions to senescence in Abstract, Results and Discussion to reflect more accurately the actual data, and also comment on the discrepancies with published data.

In response to these concerns, we have made changes to the abstract, results, and discussion, more carefully phrasing these findings. See highlighted sections in marked up version of text. The common ground between our study and those mentioned by the reviewer is that that SA-β-gal activity decreases in Pten-KO mice in conjunction with increased progression as determined by histology. The point of difference is that we consistently see SA-β-gal staining in wild type prostates that was not seen in these other studies. One potential reason for this difference is that the referenced studies analyzed the
anterior prostate while we focused more on the dorsal-lateral prostate lobes as described in the Shappell, Cancer Research 2004. It is true that we were unfortunately unable to achieve dependable staining with alternative markers such as HP1α/γ, p16, p21 or p27. These later markers are as much markers of cell cycle in general as senescence in particular, and we do show reduced Ki67, another marker of cell cycle, along with our SA-βgal. Thus, we believe these findings support the senescence conclusion. However, given the reviewers comments we have more carefully worded all references to these findings to more strictly limit conclusion to changes in SA-βgal.

3rd Editorial Decision 27 June 2015

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports.

Thank you for your contribution to EMBO reports and congratulations on a successful publication. Please consider us again in the future for your most exciting work.