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Hypophosphorylated pRb knock-in mice exhibit hallmarks of aging and vitamin C-preventable diabetes

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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. Depending on transfer agreements, referee reports obtained elsewhere may or may not be included in this compilation. Referee reports are anonymous unless the Referee chooses to sign their reports.)

Thank you again for submitting your manuscript on pRB phosphorylation site mutant knockin mice for our consideration. We have now received the reports of three expert reviewers, copied below for your information. As you will see, the referees acknowledge the importance of such in vivo dissection of the pRB phosphorylation code, and in principle appreciate the results pertaining to this aspect. However, there appear to be several substantive concerns especially with regard to the main conclusions of pRB phosphorylation links to diabetes and premature aging, raised in particular by referee 1 (whose expertise is in this area).

Should you be able to satisfactorily address these key issues, as well as the various other major points brought up in the three reports, then we would be interested in pursuing a revised version of this manuscript further for publication in The EMBO Journal. I realize that this may not be trivial and possibly require significant further time and effort, but I do feel that the study would become a much more compelling candidate for an EMBO Journal article if improved along the lines suggested by all three reviewers. I also note that the unpredictable COVID-19 pandemic situation and associated difficulties with lab access and experimental work may not allow comprehensive revision of all critical issues in a timely manner, in which case resubmission at a later point, as well as consideration of a less extensive revision for a sister journal (such as EMBO reports) would be options we might discuss. In any case, I would encourage you to contact me with a tentative point-by-point response letter and provisional revision plan already during the early stages of your revision, which we could then discuss further via email or online call.

REFEREE REPORTS

Referee #1:

In this manuscript Jiang et al present findings pointing towards a role of pRb N-terminal phosphorylation in b-cell proliferation. They develop de RbDK7 mice, which show increased fasting glucose from age 3 months, and abnormal glucose homeostasis and decreased pancreatic islet size with aging (more than 6 months). The authors state that this phenotype is due to increased DNA-damage response and senescence in pancreatic beta cells, and identify Vitamin C enriched diet, as a means to revert their phenotype. This is indeed novel, as the role of the phosphorylation of pRb directly, has never been described in vivo, and this to my knowledge the 1st mouse model allowing it.
Despite being rather well written, I find the data presented rather confusing, since the authors show a lot of results not related to their phenotype, and lack in my opinion key experiments to support their main claims. In addition, the results (in the figures) are shown in a disorganized manner, and lack key details. Out of all the images (IHC, IF, telomeres, SA-Beta-Gal), only figure 2E and 7E show scale bars, and more than half of the images have no quantification at all, despite n>3 indicated in figure legends for most experiments. Finally, the authors describe their main findings in beta-cells, but use a plethora of models, ranging from thymocytes, splenocytes, MEFs, retina, myotubes and even a breast cancer model (PyMT). I believe the findings done in those models to be of certain relevance, but they could be moved to supplementary images to free space for the findings needed to support the author's main finding.

Major concerns:

1. The author's main finding is that the homozygous presence of RbDK7 causes diabetes, and prevents pancreatic beta cells from proliferating, however, the "gold-standard" assay of streptozotocin [STZ]-induced diabetes was not performed. In addition, there is no figure showing an actual defect in either circulating insulin levels, or glucose/feeding induced insulin secretion. Similarly, no insulin IHC or IF is shown comparing RbDK7 and WT mice (corresponding to Figure 5D), with the except from the single image from 14.5 pregnant female mice in Figure 5G. Importantly, insulin is not part of the differentially regulated genes identified by microarray when comparing RbDK7 and WT mice pancreatic islets.
2. Glucose induced Insulin secretion from isolated mice was not measured either.
3. The authors claim that the diabetic phenotype RbDK7 mice is aging dependent, but if I understood correctly, most molecular and histological analysis were performed well before the phenotype appears, suggesting that other tissues than pancreatic islets are touched. By observing the single radiograph shown in figure 4D and the single skin H-E staining shown in figure 5F, I would imagine that RbDK7 mice also have enlarged liver and lipodystrophy, with total loss of skin adipose tissue. Unfortunately, the age of the animals used for those figures is not specified in the manuscript.
4. Similarly, the authors claim that the diabetic phenotype observed in RbDK7 mice is due to an increased senescence in beta cells, however, the senescence in pancreatic islets cells is not actually measured (SA-Bgal, secretion or protein of SASP proteins), see PMID 3079928.
5. Many figures lack scale bars, quantifications (and thus statistics), and clear indications of whether independent experiments were performed, making it difficult to estimate data quality/reliability.
6. Similarly, for the main findings, concerning glucose homeostasis, insulin levels, and pancreatic beta cells, the age and sex of the mice is not clearly specified in the legends or the methods (it is only specified that GSIS, ITT and GTT were performed using male mice, does it mean females were used for histology and microarray?). In addition, whereas clear differences in glucose levels are not shown before 3 months old, the analysis of pancreatic sections is performed in 10day old mice or "young mice" in others, when even specified. This is especially important since the size of pancreatic islet is known to change with age (PMID 32005707 and PMID 27284112, and the islets of RbDK7 do not actually decrease in size (Fig 5D), they rather do not show the published age dependent expansion, and even more since the authors use islet size as a readout for islet fitness.

7. Some key experiments are not at all described in the methods section, like the islet isolation and the microarray analysis. Other experiments are never detailed. Numerous key experimental details are not written, making it unlikely that the data can be reproduced in other labs.
8. Some panels show no clear staining, yet the authors draw negative conclusions: KIR6.2 in fig S5 and pS15-p53 in fig S6.

Minor concerns:
• The panels in Figure 3H appear inverted.
• The authors state that no difference in myogenesis is observed, despite only showing single images that actually point to 1: a defective myogenesis protocol in vitro, 2: a decrease of myotube differentiation in vitro, 3: a decreased in myotube thickness in vivo. The authors should either measure myogenesis properly, or remove this data from the paper, since it does not really relate to the main finding. (see PMID 23868259 and PMID 19001499)
• The experiment in figure 7A should be repeated several times, to be of sufficient quality for publication.
• What is stained red and green in figure 6G middle panels?
• The authors should at least discuss why, despite observing, according to their own words decreased proliferation, no cell cycle/proliferation gene seems to be differentially expressed in their microarray.
• The authors mention abnormal islet morphology in their discussion but show no data about that in the results section.
• The authors mention loss of beta cell organization in their discussion but show no data about that in the results section.
• The authors should show a survival curve of RbDK7 and WT male and female mice, to further support their premature aging clam.

Referee #2:

This manuscript presents an interesting mouse genetic model to study the effects of retinoblastoma protein (Rb) phosphorylation in vivo. The Rb tumor suppressor protein has been well studied for its role in various cellular processes and cancer, and its inactivation through Cdk phosphorylation is a critical mechanism driving normal and cancer cell proliferation. The authors rightly point out that to date all studies of the implications of Rb phosphorylation have been performed in vitro, and this study is the first to probe deficiencies that occur in vivo specifically from a lack of Rb phosphorylation at several sites.

The authors generated two knock-in mice with alanine substitutions at 4 (RbdeltaK4) and 7 (Rbdelta7) different S/T Cdk sites in the Rb C-terminal domain. While the mice develop normally, cells from the mice show defects in proliferation in vitro and more potent tumor suppression in vivo. Remarkably, the RbdeltaK7 mice show signs of accelerated aging and diabetes that results from a failure of differentiated pancreatic beta-cells to reenter the cell cycle. Overall, the experiments are well designed and executed, and the results are fascinating. Most importantly, the work for the first time implicates directly Rb hypophosphorylation as a mechanism for aging. While this result was anticipated from studies correlating the Cdk inhibitor p16 with aging, it is important that the phenotype is now more directly tied to Rb. The study will be of broad interest to those studying cell biology, development, aging, and cancer. For these reasons, I am enthusiastic for publication of the manuscript in EMBO, although I have several comments and suggestions the authors may wish to consider:

1) I found the last paragraph of the introduction, in which the authors discuss a potential role for the Rb pathway in aging, difficult to read and flat. The authors could more clearly articulate here the anticipation in the field that Rb hypophosphorylation is important in aging and the lack of strong data supporting that hypothesis in the literature.

2) The observation that specific mutation of S800/S804 leads to reduced overall Rb phosphorylation in vitro was also reported in the Brown, Phillips, and Gallie (1999) paper, so perhaps that reference should be added along with the Knudsen and Wang reference whenever that observation is discussed.

3) I have some concerns about the experiment shown in Fig. S2B and the conclusion. First, it is not clear why the experiment was not also performed using one of the mutant thymocytes as comparison. Second, in the figure the straight Western is shown for the deltaK7 thymocytes, but in the main text the experiment is described for the deltaK4 cells. This should be clarified. Finally, the implied concluding mechanism, that Rb in the deltaK4 cells is more hypophosphorylated and therefore Suv4-H20 is more inhibited, is rather weakly supported. The shown data more or less re-establish what is known: Suv4-H20 only associates with hypophosphorylated Rb. The key experiment is to somehow show that more Suv4-H20 is inhibited or bound to Rb in the mutant thermocytes compared to wild-type. Otherwise perhaps this conclusion, which is not essential for the study, should be left out.

4) The result that mutant cells still proliferate with only moderately increased G1 length and reduced proliferation could have been anticipated by and fits with past studies of the effects of phosphorylation at specific sites on proliferation, E2F activity, and Rb-E2F association. For example, a prominent role for S605 and T367 phosphorylation in modulating E2F binding and activity was observed in the Knudsen-Wang and Brown-Gallie studies as well as in structural biology studies described in the Rubin review. These sites are likely still phosphorylated in the RbdeltaK7 mutant cells, consistent with the idea that they are sufficient for Rb inactivation and E2F activation during development. The authors may wish to discuss this point.

5) The authors should reconsider how the conclusion to the experiment in Fig. 7A is stated. The experiment does not

demonstrate that Ser243/Thr246 or Ser605 phosphorylation "disrupts" association, but rather association does not occur when those sites are phosphorylated. It may be that phosphorylation at the other sites is driving the dissociation and E2F1 is not in the IP because all sites are phosphorylated in the hyperphosphorylated form. In other words, the authors should be encouraged to clarify that they are showing a correlation of phosphorylation at those sites with E2F1 binding rather than a causation.

6) It is interesting that the effect of the 7A mutation on Rb inactivation is so context dependent. The observed phenotypes imply that during embryonic development, the sites are not required to inactivate Rb for cell division, but they are required to inactivate Rb for cell cycle re-entry in a differentiated tissue. The authors are encouraged to discuss based on known regulators of Rb, in particular the Cdk network that phosphorylated Rb, why this may be the case. What is different about the upstream regulatory network in the different contexts that may explain the distinct observations?

7) The idea of a trade-off between tumor suppression and aging is very interesting and has been described previously in the literature. To direct readers for further discussion, the authors may wish to cite some reviews, for example the excellent review from Judith Campisi PMID: 15734683.

Referee #3:

Jiang et al have generated mice homozygous for RBΔK4 and RBΔK7 knock-in alleles that lack CDK-phosphorylation sites in the C-terminus of RB. Because RB is inactivated by phosphorylation during cell cycle progression, and because RB contains 14 sites for CDK phosphorylation, these alleles are expected to be partial gain-of-function alleles. In advance of these experiments, it would have been impossible to predict how "active" these mutant proteins would be, or to predict precisely what changes to expect. That, in many regards, is the appeal of this manuscript. The major conclusions of this study are:

1. Although RB protein is essential for embryonic development, homozygous RBΔK4 and RBΔK7 knock-in mice exhibit relatively normal development. Thus, phosphorylation of the C-terminus of RB is not necessary for normal development.
2. RB C-terminus phosphorylation is essential to maintain normal telomere length.
3. Alanine mutation of the 7 CDK-phosphorylation sites in the C-terminus RB causes specific CDK-phosphorylation sites elsewhere in RB to lose/retain phosphorylation.
4. RBΔK7 increases cellular senescence in MEFs.
5. RBΔK7 reduces pulmonary metastasis in a PyMT mouse model of breast cancer.
6. RBΔK7 mice exhibit type-1 like diabetes due to reduced cell cycle re-entry and reduplication of pancreatic b-cells.
7. Vitamin C attenuates diabetes in RBΔK7 mice.
8. Vitamin C attenuates senescence in cells expressing RBΔK11.

These are fascinating observations that provide new insights into the ways that inhibition of RB phosphorylation impacts its activity in vivo. It is important to remember that this experimental approach is fraught with risk. The mutation of all, or most, of the CDK-phosphorylation sites in RB gives mutant proteins that strongly arrests cell proliferation. Studies in cell culture have shown that mutation of multiple residues has compounding effects. The unknown, the gamble underlying this project, is the question of how many sites, and which, sites can be mutated to Alanine without blocking cell proliferation in vivo? The fact that the homozygous RBΔK4 and RBΔK7 mice develop relatively normally is fascinating. Similar clusters of sites are found in the same general position in RB orthologs in multiple species, and it is interesting that phosphorylation of these clusters is not essential to control cell proliferation during mouse development.

Beyond the initial surprise of the viable animals, a drawback of mutating just 4 or 7 of the 14 sites is that it is difficult to know what conclusions to draw from the mutant phenotypes. There is no evidence that phosphorylation of these groups of sites is regulated independently from the other sites on RB, and the sites do not appear to have been not mutated because they would cleanly eliminate a specific aspect of RB regulation. Moreover, the paper does not have the type of detailed molecular analysis that might link RB functions to specific binding partners. Nevertheless, this work is a significant step forward for RB research because it shows that these types of compound mutants are viable, it suggests which types of cells and RB functions are most sensitive to phosphorylation of the C-terminus, and it suggests that further mutagenesis of other combinations of phosphorylation sites in the mice may also be informative.

Having said that, I strongly recommend that the authors address the following specific points:

1. Does RBΔK4 retain phosphorylation in the rest CDK-phosphorylation sites? It is interesting that RBΔK4 inhibits telomeres length, but it seems important to know whether this is caused specifically by the loss of phosphorylation in the 4 C-terminal CDK-phosphorylation sites, or whether there are more extensive changes.

2. The description of the expression data in Table S1 and Figure 6C is unclear. What does "log_ratio" mean? RB has been reported to act mostly as a transcription repressor. It is unexpected to see that mutations that activate RB (RBΔK7) mostly enhance gene expression (228 transcripts go UP and only 13 go DOWN in RBΔK7 relative to RBWT pancreatic islets). Does

RBΔK7 bind to E2F promoters?

3. RBΔK7 inhibits S-phase entry and BrdU incorporation in pancreatic islets. How do the authors explain the upregulation of S-phase genes (Rad51, Brca2, MCM2, MCM4 and MCM5) in the same cells?

4. The link between GSEA results and SASP signature in Fig. 6A and 6B is very weak. It would be helpful if authors explain this association in more detail. In addition to this, the authors should provide the enrichment scores and the significance for the highlighted pathways.

5. The authors state that induction of SASP is suggestive of DNA replication stress and ATM/ATR activation. Why is that? Citations are needed to support this statement.

6. The authors show that Vitamin C significantly reduces serum glucose in RBΔK7 mice, apparently because insulin positive b-cells retain their normal morphology. Does Vitamin C restore phosphorylation in T350 and S601 in RBΔK7?

7. Interestingly, the authors show that N-terminus phosphorylation at Ser243/Thr246, or spacer site phosphorylation at Ser605, disrupts RB association with E2F1. Do alanine mutations in S243/T246 and S605 activate RB and inhibit E2F activity?

8. The authors show that Vitamin C attenuates senescence in cells expressing a mutant RB that contains Alanine mutations at 11 CDK-phosphorylation sites. Does Vitamin C attenuate RB activity and elevate the E2F transcription program in these cells?

In addition, I have a couple of suggestions that may improve the organization of the paper and may provide an extra insight into the mechanism of RB phosphorylation.

1. The introduction is disappointing because it doesn't lead the reader into the major question/hypothesis of the paper. The authors explain their decision to generate RBΔK4 and RBΔK7 mice in the first paragraph of the results section. It would much better to include this paragraph at the end of the introduction.

2. It has been reported that the RB C-terminus domain acts as a docking site for Cyclins/CDKs complexes. The evidence that i) RBΔK7 retains phosphorylation in some but not all of the remaining CDK-sites, and ii) RBΔK7 MEFs are more resistant to CDK2 but not CDK4 inhibition, suggests that RBΔK7 have altered affinities for CDK2 and CDK4. That would be easy to test. Is it true?

REBUTTAL

Referee #1:

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Despite being rather well written, I find the data presented rather confusing, since the authors show a lot of results not related to their phenotype, and lack in my opinion key experiments to support their main claims. In addition, the results (in the figures) are shown in a disorganized manner, and lack key details. Out of all the images (IHC, IF, telomeres, SA-Beta-Gal), only figure 2E and 7E show scale bars, and more than half of the images have no quantification at all, despite n>3 indicated in figure legends for most experiments. Finally, the authors describe their main findings in beta-cells, but use a plethora of models, ranging from thymocytes, splenocytes, MEFs, retina, myotubes and even a breast cancer model (PyMT). I believe the findings done in those models to be of certain relevance, but they could be moved to supplementary images to

free space for the findings needed to support the author's main finding.

We thank the reviewer for these thoughtful comments and insightful suggestions. We agree that the diabetes defect is a major abnormality in $Rb^{\Delta K7}$ mice. The additional phenotypic analysis of other defects in these mice is critically important for understanding the impact of blocking pRB phosphorylation on growth, development and homeostasis, which goes beyond the regulation of pancreatic β-cell regeneration. This includes the phosphorylation analysis performed in the thymus, which contains highly proliferating cells, the *in vitro* analysis of MEFs, and the various aging defects from telomere attrition to infertility, kyphosis and diabetes. We believe that this multifaceted analysis is a major strength of this paper as it highlights the complex, context/tissue-specific effects of constitutive expression of active, under-phosphorylated pRb.

However, we agree that the breast cancer model (PyMT) may distract from the major analysis on aging and diabetes, and have removed this results from the manuscript.

Also, as requested, scale bars and/or magnifications are provided in the revised figures and legends.

Finally, some aging phenotypes (infertility, kyphosis, diabetes) are seen in 100% of $Rb^{\Delta K7}$ mice vs 0% in control as explained in the text. For example, the kinetics of diabetes shown in Fig. 6G, left, demonstrates zero diabetes in control vs severe diabetes (>20mM blood glucose on average) in >9 month old $Rb^{\Delta K7}$ mice. In all other cases, we provided quantification either as graphs or as statistical analysis (e.g. telomere attrition/analysis of 5 pairs (Mann-Whitney-Wilcoxon test P<0.001); G:profiler-SASP analysis; (Fisher's exact test P=0.026), as described in the text. We have now added this statistical information to the revised legends as well.

Major concerns:

1. The author's main finding is that the homozygous presence of RbDK7 causes diabetes, and prevents pancreatic beta cells from proliferating, however, the "gold-standard" assay of streptozotocin [STZ]-induced diabetes was not performed. In addition, there is no figure showing an actual defect in either circulating insulin levels, or glucose/feeding induced insulin secretion. Similarly, no insulin IHC or IF is shown comparing RbDK7 and WT mice (corresponding to Figure 5D), with the except from the single image from 14.5 pregnant female mice in Figure 5G. Importantly, insulin is not part of the differentially regulated genes identified by microarray when comparing RbDK7 and WT mice pancreatic islets.

We performed two physiological assays to induce pancreatic β-cell proliferation: (i) pregnancy and (ii) treatment with exendin 4, an agonist of glucagon-like peptide 1 (GLP-1) receptor that promotes pancreatic β-cell proliferation by inducing cyclins expression (Fig. 5G). In both cases, we show that pancreatic β-cells were induced to enter the cell cycle in wildtype but not in $Rb^{\Delta K7}$ mice. These are important results as they demonstrate a role for pRb in G0-to-G1 transition in addition to the well-studied G1-to-S transition.

In contrast to these physiological or near-physiological conditions that induce pancreatic β-cell proliferation, the streptozotocin assay is a non-physiological agent that

causes high morbidity (e.g. https://www.jax.org/jax-mice-and-services/find-and-order-jax-mice/surgical-and-preconditioning-services/stz-induced-diabetes). In addition, STZ induces diabetes, already seen in $Rb^{\Delta K7}$ mice, and will probably be too toxic for them.

A related question is whether the observed attenuation of DDR and diabetes by vitamin C is accompanied by increased cell cycle re-entry of pancreatic $Rb^{\Delta K7}$ β-cells. To examine this possibility, and in response to the Reviewer concern about the regeneration potential of $Rb^{\Delta K7}$ islet cells, we investigated pregnancy-induced proliferation (Ki67 staining) in $Rb^{\Delta K7}$ and control mice fed on normal vs vitamin C chow. We now show that vitamin C increases cell-cycle re-entry in pregnant females by 3.8 folds compared to cell cycle re-entry in mice fed on regular diet (new Fig. 6J and Fig. EV2A).

Regarding "glucose/feeding induced insulin secretion" **requested by the Reviewer – we show "Glucose induced insulin secretion" analysis in Fig 5C. These results demonstrate that the primary defect in $Rb^{\Delta K7}$ is not insulin secretion. Later on, as pancreatic $Rb^{\Delta K7}$ islets degenerate through senescence and insulin expression is increased in some islet cells and or reduced in others as shown in new Figures 5D, 6D, 6H and S6B, but this is a secondary effect, not the primary defect in these mice.**

In addition to immunofluorescent staining for Insulin in mid-pregnancy (Fig. 5G), we show Insulin expression in mice fed on regular vs vitamin C chow in Fig. 6H. To address the reviewer concern, we also provide images for insulin in wt vs $Rb^{\Delta K7}$ mice as part of the initial characterization of the phenotype in new Figure 5D. Please also see Fig. 6D and Fig. EV2B.

Finally, with regards to "insulin is not part of the differentially regulated genes identified by microarray when comparing RbDK7 and WT mice pancreatic islets" **– this is true and exactly the message of the manuscript: the primary defect is NOT due to reduced insulin expression <u>per</u> cell, but due to a gradual reduction in the <u>number of</u> β-cells that express insulin.**

2. Glucose induced Insulin secretion from isolated mice was not measured either.
As noted, glucose induced insulin secretion is shown in Fig 5C.

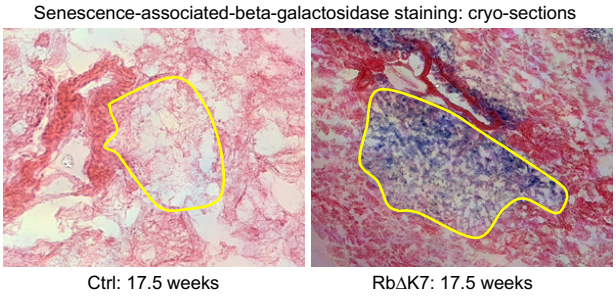
3. The authors claim that the diabetic phenotype RbDK7 mice is aging dependent, but if I understood correctly, most molecular and histological analysis were performed well before the phenotype appears, suggesting that other tissues than pancreatic islets are touched. By observing the single radiograph shown in figure 4D and the single skin H-E staining shown in figure 5F, I would imagine that RbDK7 mice also have enlarged liver and lipodystrophy, with total loss of skin adipose tissue. Unfortunately, the age of the animals used for those figures is not specified in the manuscript.
The diabetic phenotype in $Rb^{\Delta K7}$ mice is age dependent as shown in Fig. 6G.
We don't think there is a contradiction between the timing of diabetes and the age of the mice we analyzed; indeed, while high blood glucose is observed at 3 months of age (on average) and full-blown diabetes after 4-6 months (Fig. 6G), reduced pancreatic beta-cell proliferation is observed as early as 2 weeks of age (Fig. 5F). Pancreatic β-cells enter a quiescence/ differentiation state at ~1 month of age, and therefore the proliferation assays

were performed before this stage. Other experiments were performed before or after the onset of diabetes depending on the assay. The age of mice in each assay is described in the text and legends.

As indicated in the manuscript, a comprehensive pathology of several 9-12 month old $Rb^{\Delta K7}$ mice did not reveal additional defects, including liver pathology, beyond what we report. The lipoatrophy seen under the skin is of interest but is not seen elsewhere in the mice. This, and each of the other phenotypes we observed, e.g. male infertility and kyphosis, warrant a thorough investigation, which is beyond the scope of this manuscript. However, we have further discussed these phenotypes, the need for further analysis of each, and the possibility that additional defects may be masked by the early death from diabetes, - see Discussion (p18).

4. Similarly, the authors claim that the diabetic phenotype observed in RbDK7 mice is due to an increased senescence in beta cells, however, the senescence in pancreatic islets cells is not actually measured (SA-Bgal, secretion or protein of SASP proteins), see PMID 3079928.

We used γH2A staining, a marker for DNA damage response/DNA replication stress, as a surrogate for and a trigger of senescence. In response to the Reviewer's comment, we tried to optimize SA-βGal staining on cryo-sections. As shown below, we observed defuse SA-βGal staining in pancreatic $Rb^{\Delta K7}$ islets but not control mice, but the results are not convincing enough for publication.



However, in line with the Reviewer's suggestion, we immuno-stained for SASP markers including IL-6 and MMP-2, *bona fide* SASP marker induced in diabetic NOD mice (Thompson, et al , 2019, Cell Metabolism (PMID: 30799288), as well as Cxcl12, which is strongly induced at the mRNA level in $Rb^{\Delta K7}$ islets (Fig. 6C). We now show that these

SASP markers are expressed in a subset of pancreatic insulin+ β-cells in diabetic $Rb^{\Delta K7}$ but not in control mice (new Fig. 6D; Fig. EV2B).

5. Many figures lack scale bars, quantifications (and thus statistics), and clear indications of whether independent experiments were performed, making it difficult to estimate data quality/reliability.
All experiments were performed using at least 3 biological replicates as indicated in the text and legends. Scale bars (IF/IHC/H&E) or original magnifications (stereomicroscope) were added to figures/legends.

6. Similarly, for the main findings, concerning glucose homeostasis, insulin levels, and pancreatic beta cells, the age and sex of the mice is not clearly specified in the legends or the methods (it is only specified that GSIS, ITT and GTT were performed using male mice, does it mean females were used for histology and microarray?). In addition, whereas clear differences in glucose levels are not shown before 3 months old, the analysis of pancreatic sections is performed in 10day old mice or "young mice" in others, when even specified. This is especially important since the size of pancreatic islet is known to change with age (PMID 32005707 and PMID 27284112, and the islets of RbDK7 do not actually decrease in size (Fig 5D), they rather do not show the published age dependent expansion, and even more since the authors use islet size as a readout for islet fitness.
Mouse age is provided in text, figures and/or figure legends.

Both male and female mice develop diabetes with 100% penetrance. However, the kinetic is different with male diabetes showing a tighter window of onset. We don't know the basis for this sex-difference, but to reduce variability, all experiments were performed on males, with the exception of the cell cycle re-entry analysis during pregnancy. We made a note of this in the initial submission but have further clarified this point in the revised manuscript (p8).

As noted above, hypo-proliferation of pancreatic β-cells is observed at 2 weeks of age whereas high blood glucose and diabetes are seen at >4 months, respectively. We therefore analyzed the pancreas in most cases BEFORE the onset of severe diabetes, but other phenotypes such as SASP marker analysis and some vitamin C experiments were performed later when the phenotype is stronger and readily observed. See also response to point #3 above.

Finally, the pancreatic islets in $Rb^{\Delta K7}$ mice are clearly abnormal, not only smaller, with aberrant morphology, enlarged β-cells and expression of SASP markers.

7. Some key experiments are not at all described in the methods section, like the islet isolation and the microarray analysis. Other experiments are never detailed. Numerous key experimental details are not written, making it unlikely that the data can be reproduced in other labs.
We added additional details to the Methods section as requested.

8. Some panels show no clear staining, yet the authors draw negative conclusions: KIR6.2 in fig S5 and pS15-p53 in fig S6.

KIR6.2, which is thought to be regulated transcriptionally, was not seen in our IHC experiments or our microarray analysis. Thus, our conclusion is not based only on IHC, and likely reflects expected differences between E2F1-/- and $Rb^{\Delta K7}$ mice.

For pS15-p53, we agree we cannot draw a decisive negative conclusion based only on IHC analysis, and indeed, we merely mentioned this observation.

Minor concerns:

• The panels in Figure 3H appear inverted.
We remove this experiment as the Reviewer suggested – see response to point #1

• The authors state that no difference in myogenesis is observed, despite only showing single images that actually point to 1: a defective myogenesis protocol in vitro, 2: a decrease of myotube differentiation in vitro, 3: a decreased in myotube thickness in vivo. The authors should either measure myogenesis properly, or remove this data from the paper, since it does not really relate to the main finding. (see PMID 23868259 and PMID 19001499)
We do not observe any sign of defective myogenesis. We analysed myogenesis as it is the only tissue were Rb loss shows a clear autonomous developmental defect during embryogenesis, as we were the first to demonstrate (e.g. Genes & Dev 1996; EMBO J, 2004; JCB, 2010). However, given the fact that no significant differences in myogenesis were found between $Rb^{\Delta K7}$ vs control, we agree with the Reviewer's comment, and moved these results to the Appendix (Fig. S1).

• The experiment in figure 7A should be repeated several times, to be of sufficient quality for publication.
These challenging co-IP experiments were repeated over 3 times with consistent results.

• What is stained red and green in figure 6G middle panels?
The figure showed insulin staining in both cases with different fluorophore-secondary antibodies.
In agreement with the Reviewer's comment, we have revised the figure to show new immunofluorescent staining with the same fluorophore-secondary antibody (revised Fig. 6H).

• The authors should at least discuss why, despite observing, according to their own words decreased proliferation, no cell cycle/proliferation gene seems to be differentially expressed in their microarray.
This is a great question. In mice older than 1 month of age, only rare differentiated β-cells re-enter the cell-cycle in response to mitogens. We propose that at this stage, the observed induction of cell cycle markers in $Rb^{\Delta K7}$ islets, such as MCM4, does not indicate actual DNA replication/cell division – but reflects DNA replication stress (PMID: 15833913; PMID: 25963833). Indeed, an increase in expression of cell proliferation genes including

those associated with stress response was shown to be associated with <u>transcriptional noise</u> in aging pancreas and possibly in $Rb^{\Delta K7}$ mice (PMID: 28965763). Despite this noise, we observe a significant enrichment of pathways associated with SASP. We stated this in the text (*Induction of some of these genes likely reflects compensatory mechanisms in response to DNA replication stress and/or reduced β-cell proliferation in RbΔK7 islets*) – but have further clarified this point and provide additional references in the revised manuscript (page 10).

• The authors mention abnormal islet morphology in their discussion but show no data about that in the results section.
• The authors mention loss of beta cell organization in their discussion but show no data about that in the results section.
We showed a few images with defective islet morphology – e.g. Fig 5H (PDX1 staining) and Fig 6H. In response to the Reviewer's comment, we added new images in Fig. 5D and Fig. 6D, showing completely disorganized islets. In addition, we measured the size of pancreatic β-cells in ~9 month-old $Rb^{\Delta K7}$ vs control mice, demonstrating 1.73 fold increase in length of insulin+ β-cells in $Rb^{\Delta K7}$ mice (new Fig. 5D, right).

• The authors should show a survival curve of RbDK7 and WT male and female mice, to further support their premature aging clam.
As noted, <u>all</u> $Rb^{\Delta K7}$ mice succumb to severe diabetes and had to be sacrificed. In contrast, control littermate mice survive over 2 years. Although not survival curve *per se*, Figure 6G, left panel, shows the gradual increase in blood glucose/diabetes in $Rb^{\Delta K7}$ mice at 3, 4, 7 and 9 months of age, with over 20 mM blood glucose on average by 9 months of age, after which the mice were sacrificed (hence a survival curve). By ~1 year, some $Rb^{\Delta K7}$ mice that only show moderate diabetes by 9 months (8-10 mM) develop severe diabetes (20 mM). This is based on more than 100 mice from each genotype. We made a statement in the text emphasizing that $Rb^{\Delta K7}$ mice fed on regular diet exhibit age-dependent diabetes (**Fig. 6G**, left)**(page 12). Finally, <u>premature aging</u> is shown in Figures 1 (short telomere) and 4 (infertility, kyphosis, reduced hair regeneration, and diabetes).**

Referee #2:

This manuscript presents an interesting mouse genetic model to study the effects of retinoblastoma protein (Rb) phosphorylation in vivo. The Rb tumor suppressor protein has been well studied for its role in various cellular processes and cancer, and its inactivation through Cdk phosphorylation is a critical mechanism driving normal and cancer cell proliferation. The authors rightly point out that to date all studies of the implications of Rb phosphorylation have been performed in vitro, and this study is the first to probe deficiencies that occur in vivo specifically from a lack of Rb phosphorylation at several sites.

The authors generated two knock-in mice with alanine substitutions at 4 (RbdeltaK4) and 7

(Rbdelta7) different S/T Cdk sites in the Rb C-terminal domain. While the mice develop normally, cells from the mice show defects in proliferation in vitro and more potent tumor suppression in vivo. Remarkably, the RbdeltaK7 mice show signs of accelerated aging and diabetes that results from a failure of differentiated pancreatic beta-cells to reenter the cell cycle. Overall, the experiments are well designed and executed, and the results are fascinating. Most importantly, the work for the first time implicates directly Rb hypophosphorylation as a mechanism for aging. While this result was anticipated from studies correlating the Cdk inhibitor p16 with aging, it is important that the phenotype is now more directly tied to Rb. The study will be of broad interest to those studying cell biology, development, aging, and cancer. For these reasons, I am enthusiastic for publication of the manuscript in EMBO, although I have several comments and suggestions the authors may wish to consider:

1) I found the last paragraph of the introduction, in which the authors discuss a potential role for the Rb pathway in aging, difficult to read and flat. The authors could more clearly articulate here the anticipation in the field that Rb hypophosphorylation is important in aging and the lack of strong data supporting that hypothesis in the literature.
We thank the reviewer for thoughtful comments and insightful suggestions. We have revised the introduction as suggested.

2) The observation that specific mutation of S800/S804 leads to reduced overall Rb phosphorylation in vitro was also reported in the Brown, Phillips, and Gallie (1999) paper, so perhaps that reference should be added along with the Knudsen and Wang reference whenever that observation is discussed.
Agree. We referenced this paper by Brown et al., elsewhere in the manuscript but not here; we have referenced this paper again as requested.

3) I have some concerns about the experiment shown in Fig. S2B and the conclusion. First, it is not clear why the experiment was not also performed using one of the mutant thymocytes as comparison. Second, in the figure the straight Western is shown for the deltaK7 thymocytes, but in the main text the experiment is described for the deltaK4 cells. This should be clarified. Finally, the implied concluding mechanism, that Rb in the deltaK4 cells is more hypophosphorylated and therefore Suv4-H20 is more inhibited, is rather weakly supported. The shown data more or less re-establish what is known: Suv4-H20 only associates with hypo-phosphorylated Rb. The key experiment is to somehow show that more Suv4-H20 is inhibited or bound to Rb in the mutant thymocytes compared to wild-type. Otherwise perhaps this conclusion, which is not essential for the study, should be left out.
First, we used lysates from $Rb^{\Delta K7}$ thymocytes to show the migration of hypo-phosphorylated pRb. There is no difference between $Rb^{\Delta K7}$ and $Rb^{\Delta K4}$ in this regard (migration), and both can be used as control for unphosphorylated pRb. We clarified this point in the revised legend.
Second, our point is that upon phosphorylation, ppRb dissociates from Suv4-H20, whereas $pRb^{\Delta K4}$ remains bound to Suv4-H20 throughout the cell cycle, suppressing its activity. Other mechanisms such as interaction with SSU72 are possible - we have revised the text accordingly (page 5, and Discussion).

4) The result that mutant cells still proliferate with only moderately increased G1 length and

reduced proliferation could have been anticipated by and fits with past studies of the effects of phosphorylation at specific sites on proliferation, E2F activity, and Rb-E2F association. For example, a prominent role for S605 and T367 phosphorylation in modulating E2F binding and activity was observed in the Knudsen-Wang and Brown-Gallie studies as well as in structural biology studies described in the Rubin review. These sites are likely still phosphorylated in the RbdeltaK7 mutant cells, consistent with the idea that they are sufficient for Rb inactivation and E2F activation during development. The authors may wish to discuss this point.
We showed that S605 is indeed phosphorylated in proliferating $Rb^{\Delta K7}$ cells. Antibodies for mouse T367 and Ser224, are not available, but they may be phosphorylated as well and prevent interaction with E2F1. Given this comment and those from Reviewer #3, we expanded the discussion on these phosphorylation sites vis-a-vis our results and published data (revised text – page 13; Discussion – page 16).

5) The authors should reconsider how the conclusion to the experiment in Fig. 7A is stated. The experiment does not demonstrate that Ser243/Thr246 or Ser605 phosphorylation "disrupts" association, but rather association does not occur when those sites are phosphorylated. It may be that phosphorylation at the other sites is driving the dissociation and E2F1 is not in the IP because all sites are phosphorylated in the hyperphosphorylated form. In other words, the authors should be encouraged to clarify that they are showing a correlation of phosphorylation at those sites with E2F1 binding rather than a causation.
We agree, and that's what we meant: Ser243/Thr246 or Ser605 phosphorylation are surrogate for phosphorylated $pRb^{\Delta K7}$ but may not be the sites that disrupt its association with E2F (the remaining sites S242 and, more likely, T367 may be more important). We clarified this point in the text on page 13.

6) It is interesting that the effect of the 7A mutation on Rb inactivation is so context dependent. The observed phenotypes imply that during embryonic development, the sites are not required to inactivate Rb for cell division, but they are required to inactivate Rb for cell cycle re-entry in a differentiated tissue. The authors are encouraged to discuss based on known regulators of Rb, in particular the Cdk network that phosphorylated Rb, why this may be the case. What is different about the upstream regulatory network in the different contexts that may explain the distinct observations?
We thank the reviewer again for highlighting this point, and have discussed this issue/our model in the revised manuscript, Discussion p16.

7) The idea of a trade-off between tumor suppression and aging is very interesting and has been described previously in the literature. To direct readers for further discussion, the authors may wish to cite some reviews, for example the excellent review from Judith Campisi PMID: 15734683.
We agree and have now referenced this excellent review.

Referee #3:

Jiang et al have generated mice homozygous for RBΔK4 and RBΔK7 knock-in alleles that lack CDK-phosphorylation sites in the C-terminus of RB. Because RB is inactivated by phosphorylation during cell cycle progression, and because RB contains 14 sites for CDK phosphorylation, these alleles are expected to be partial gain-of-function alleles. In advance of these experiments, it would have been impossible to predict how "active" these mutant proteins would be, or to predict precisely what changes to expect. That, in many regards, is the appeal of this manuscript. The major conclusions of this study are:

1. Although RB protein is essential for embryonic development, homozygous RBΔK4 and RBΔK7 knock-in mice exhibit relatively normal development. Thus, phosphorylation of the C-terminus of RB is not necessary for normal development.
2. RB C-terminus phosphorylation is essential to maintain normal telomere length.
3. Alanine mutation of the 7 CDK-phosphorylation sites in the C-terminus RB causes specific CDK-phosphorylation sites elsewhere in RB to lose/retain phosphorylation.
4. RBΔK7 increases cellular senescence in MEFs.
5. RBΔK7 reduces pulmonary metastasis in a PyMT mouse model of breast cancer.
6. RBΔK7 mice exhibit type-1 like diabetes due to reduced cell cycle re-entry and reduplication of pancreatic b-cells.
7. Vitamin C attenuates diabetes in RBΔK7 mice.
8. Vitamin C attenuates senescence in cells expressing RBΔK11.

These are fascinating observations that provide new insights into the ways that inhibition of RB phosphorylation impacts its activity in vivo. It is important to remember that this experimental approach is fraught with risk. The mutation of all, or most, of the CDK-phosphorylation sites in RB gives mutant proteins that strongly arrests cell proliferation. Studies in cell culture have shown that mutation of multiple residues has compounding effects. The unknown, the gamble underlying this project, is the question of how many sites, and which, sites can be mutated to Alanine without blocking cell proliferation in vivo? The fact that the homozygous RBΔK4 and RBΔK7 mice develop relatively normally is fascinating. Similar clusters of sites are found in the same general position in RB orthologs in multiple species, and it is interesting that phosphorylation of these clusters is not essential to control cell proliferation during mouse development.

Beyond the initial surprise of the viable animals, a drawback of mutating just 4 or 7 of the 14 sites is that it is difficult to know what conclusions to draw from the mutant phenotypes. There is no evidence that phosphorylation of these groups of sites is regulated independently from the other sites on RB, and the sites do not appear to have been not mutated because they would cleanly eliminate a specific aspect of RB regulation. Moreover, the paper does not have the type of detailed molecular analysis that might link RB functions to specific binding partners. Nevertheless, this work is a significant step forward for RB research because it shows that these types of compound mutants are viable, it suggests which types of cells and RB functions are most sensitive to phosphorylation of the C-terminus, and it suggests that further mutagenesis of other combinations of phosphorylation sites in the mice may also be informative.

Having said that, I strongly recommend that the authors address the following specific points:

1. Does RBΔK4 retain phosphorylation in the rest CDK-phosphorylation sites? It is interesting that RBΔK4 inhibits telomeres length, but it seems important to know whether this is caused specifically by the loss of phosphorylation in the 4 C-terminal CDK-phosphorylation sites, or whether there are more extensive changes.
We thank the reviewer for these thoughtful comments and insightful suggestions. We agree that it is important to determine the effect of RbΔK4 substitutions on the upstream phosphorylation sites, which are blocked in $Rb^{\Delta K7}$ – i.e. Thr350 and Ser601. In the revised manuscript, we present phospho-specific immunoblot analysis of proliferating RbΔK4 thymocytes as requested (new Fig. 2F). Interestingly, Thr350 phosphorylation is completely blocked. Phosphorylation of Ser601 is also dramatically inhibited - although residual phosphorylation is evident is RbΔK4 (but not RbΔK7; compare panels F and E in Fig. 2). Thus, the inhibition of telomere length in RbΔK4 mice is not only due to the $Rb^{\Delta K4}$ substitutions per se as additional upstream phosphorylation sites are also affected.

2. The description of the expression data in Table S1 and Figure 6C is unclear. What does "log_ratio" mean? RB has been reported to act mostly as a transcription repressor. It is unexpected to see that mutations that activate RB (RBΔK7) mostly enhance gene expression (228 transcripts go UP and only 13 go DOWN in RBΔK7 relative to RBWT pancreatic islets). Does RBΔK7 bind to E2F promoters?
First - "log ratio" represents the difference in gene expression between RbΔK7 and control. Thus, the array data were first normalized and then log2 transformed. Log ratio is the difference between the mean of $Rb^{\Delta K7}$ samples and the mean of control samples of the log2 normalized values for any given gene.
We clarified these terms/issues in the revised legend and Methods.

With regard to the second part – please see response to point #3 below.

3. RBΔK7 inhibits S-phase entry and BrdU incorporation in pancreatic islets. How do the authors explain the upregulation of S-phase genes (Rad51, Brca2, MCM2, MCM4 and MCM5) in the same cells?
This is a great question. In mice older than 1 month of age, only rare differentiated β-cells re-enter the cell-cycle in response to mitogens. We propose that at this stage, the observed induction of cell cycle markers in $Rb^{\Delta K7}$ islets, such as MCM4, does not indicate actual DNA replication/cell division – but reflects DNA replication stress (PMID: 15833913; PMID: 25963833). Indeed, an increase in expression of cell proliferation genes including those associated with stress response was shown to be associated with <u>transcriptional noise</u> in aging pancreas and possibly in $Rb^{\Delta K7}$ mice (PMID: 28965763). Despite this noise, we observe a significant enrichment of pathways associated with SASP. We stated this in the text (*Induction of some of these genes likely reflects compensatory mechanisms in response to DNA replication stress and/or reduced β-cell proliferation in RbΔK7 islets*) – but have further clarified this point and provide additional references in the revised manuscript (page 10).

4. The link between GSEA results and SASP signature in Fig. 6A and 6B is very weak. It would

be helpful if authors explain this association in more detail. In addition to this, the authors should provide the enrichment scores and the significance for the highlighted pathways.
We provided this additional information in the revised manuscript in the text and legends. In addition, we now show immuofluorescent staining for the SASP markers IL-6, MMP-2 and Cxcl12, showing positive staining in pancreatic islets from $Rb^{\Delta K7}$ but not control mice (new Fig. 6D, Fig. EV2B).

5. The authors state that induction of SASP is suggestive of DNA replication stress and ATM/ATR activation. Why is that? Citations are needed to support this statement.
There is extensive literature on the effect of DNA damage response on the induction of ATM/ATR, senescence and aging. We provided some key citations and reviews including PMID: 33911272 in Nature, 2021.

6. The authors show that Vitamin C significantly reduces serum glucose in RBΔK7 mice, apparently because insulin positive b-cells retain their normal morphology. Does Vitamin C restore phosphorylation in T350 and S601 in RBΔK7?
We propose that vitamin C exerts global epigenetic effects on DNA methylation and chromatin modifications that pre-empt the irreversible epigenetic state imposed by $pRb^{\Delta K7}$ in quiescent and differentiated β-cells. The Reviewer asks whether vitamin C may act through another mechanism that restores/induces phosphorylation of $pRb^{\Delta K7}$ on T350 and S601, and thereby inactivate this tumor suppressor. We think this is a remote possibility because Exendin 4, which is known to induce cyclin D1 leading to β-cell proliferation, failed to promote proliferation in $Rb^{\Delta K7}$ pancreas (Fig 5G). However, anther kinase/phosphatase may be involved. Thus, as suggested by the Reviewer, we examined the possibility that vitamin C can induce pThr350, which is completely blocked in proliferating $pRb^{\Delta K7}$ thymocytes. As shown in <u>new Appendix Fig, S6F</u>, Thr350 is not phosphorylated in $Rb^{\Delta K7}$ mice fed on either regular <u>or</u> vitamin C diet, suggesting that vitamin C does not attenuate diabetes by enforcing $pRb^{\Delta K7}$ phosphorylation.

7. Interestingly, the authors show that N-terminus phosphorylation at Ser243/Thr246, or spacer site phosphorylation at Ser605, disrupts RB association with E2F1. Do alanine mutations in S243/T246 and S605 activate RB and inhibit E2F activity?
In the revised manuscript, we provide references on published effects of S243/T246 and S605 on pRB *in vitro* and discuss potential kinases, beside CDK4/6 and CDK2, that may phosphorylate these sites (p16).

8. The authors show that Vitamin C attenuates senescence in cells expressing a mutant RB that contains Alanine mutations at 11 CDK-phosphorylation sites. Does Vitamin C attenuate RB activity and elevate the E2F transcription program in these cells?
These is a similar question as #6 above but in vitro. We addressed the question in proliferating thymocytes in vivo where $Rb^{\Delta K7}$ phosphorylation on T350 and S601 is inhibited <u>in all cells</u>. Our new results show that vitamin C does not induce phosphorylation of Thr250 (new Appendix Figure S6F). However, in the RbΔK11 transduction experiment <u>in vitro</u>, the efficiency of transduction/number of senescence cells is very low, thus hindering similar immunoblot analysis (Fig. 7F). We therefore suggest that this question, while clearly fascinating, is beyond the scope of this paper and should be addressed in

future work.

In addition, I have a couple of suggestions that may improve the organization of the paper and may provide an extra insight into the mechanism of RB phosphorylation.

1. The introduction is disappointing because it doesn't lead the reader into the major question/hypothesis of the paper. The authors explain their decision to generate RBΔK4 and RBΔK7 mice in the first paragraph of the results section. It would much better to include this paragraph at the end of the introduction.
We agree and have modified the Introduction accordingly.

2. It has been reported that the RB C-terminus domain acts as a docking site for Cyclins/CDKs complexes. The evidence that i) RBΔK7 retains phosphorylation in some but not all of the remaining CDK-sites, and ii) RBΔK7 MEFs are more resistant to CDK2 but not CDK4 inhibition, suggests that RBΔK7 have altered affinities for CDK2 and CDK4. That would be easy to test. Is it true?
This is also a fascinating question, but we suggest it is addressed experimentally in future studies together with related questions, e.g. the identification of kinases that phosphorylate Ser243/Thr246 and Ser605. We have deliberated on both issues (C-terminal docking sites; nature of kinases that phosphorylate S243/T246, S605) in the revised Discussion (p16). Notably, we tried Mass Spec analysis to identify additional sites that are aberrantly phosphorylated in $pRb^{\Delta K7}$, but for technical reasons have been so far unsuccessful (ongoing).

Thank you for submitting your revision for our consideration. All three referees have now assessed it once more, and for the most parts found their original criticisms satisfactorily addressed. However, referee 1 still retains a few specific concerns, as you will see in the report copied below. Pending adequate addressing of these remaining points, as well as various editorial issues listed below, in an additional round of revision, we would in this light be happy to eventually publish this work in The EMBO Journal.

- Please address referee 1's remaining points in the manuscript as well as in an additional point-by-point response. For any modifications of the main manuscript text, please use the attached file, leaving the "Track Changes" option activated.

REFEREE REPORTS

Referee #1:

In this revised manuscript Jian and colleagues show for the 1st time in vivo a role for the N-terminal phosphorylation of pRB. They generate a two new mouse models carrying alanine substitutions in 4 (RB delta K4) or 7 (RB delta K)of the phosphorylatable residues located in the N-Terminus of pRB.
The authors describe a dramatic phenotype, with several features reminiscent of premature aging (kyphosis, reduced fertility, hair loss) in the RB Delta K7 mice. In addition, these RB delta K7 develop type 1 diabetes, with increased fasting glucose levels detectable since age 3 months. The authors report in the rebuttal that the diabetic phenotype causes early mortality before 1y of age. Strikingly, the type 1-diabetes phenotype can be reversed with a diet intervention (vitamin C enriched diet) via an epigenetic modulation.

This revised version of the manuscript answers most of my questions, with a reorganization of both text and figures that in my opinion facilitate the understanding of the paper, and clear improvements in the methods section.

The manuscript still requires some minor changes

• the authors should show the actual circulating insulin levels, as according to their findings, these should be very reduced due to the observed decrease in Beta cell mass.
The data presented in figure 5C, with the relative insulin secretion (compared to fasting insulin), is not in my opinion the most adequate to illustrate the authors' point.

In addition, if circulating insulin levels at different timepoints are shown it would likely help shedding light on the complex kinetics the authors observe for the onset of their phenotype.
• the authors could further discuss the complex kinetics of the diabetic phenotype. Indeed, the authors detect reduced cell proliferation and DNA damage in pancreatic islets (2-3 weeks) before increased glucose levels (3 months) and much before SASP markers (9 months). This merits additional discussion.
• the authors should moderate their conclusions regarding premature aging, especially since type 1 diabetes is not usually a feature of premature aging, and since aging dependent replicative senescence and SASP in beta cells have been shown to be independent , with senescence being beneficial for insulin secretion and beta cell maturation, at least in some conditions (Arda et al 2016, Helman et al 2016, Thompson et al 2019, Li et al 2019) .

Referee #2:

In this revised manuscript, the authors have considered reviewer comments carefully and have added some more data and explanation to address concerns. Reasonable justifications are provided in cases for which recommendations were not taken. Overall, the manuscript is improved, particularly in its clarity, and I believe the main conclusions are adequately supported and explained. I have no more remaining major concerns. The results are interesting and significant, and the work will surely inspire follow up studies. Publication in EMBO is strongly recommended.

Referee #3:

Jiang et al have done a lot of work to improve the manuscript in response to the reviewer's comments. However, the answers to most of the mechanistic questions are still vague. Part of the reason for this is that the mechanism of RB action seems to much more complicated than has been described previously. It appears that we just don't have sufficient knowledge about the action of RB to be able to explain the results that the authors find in their in vivo experiments.

The major conclusion from this manuscript is that although RBΔK4 and RBΔK7 act as gain of function alleles in vitro, in early stages of development RBΔK4 and RBΔK7 alleles do not inhibit cell cycle progression and the corresponding knock-in mice exhibit relatively normal development. Only in late stages of development does the RBΔK7 allele exhibit defects, and these are related to failure of quiescent b-cells to re-enter the cell cycle. However, the mechanism behind this phenotype is unclear and additional studies will be needed to explain this. The authors observations suggest that RBΔK4 and RBΔK7 are barely phosphorylated on their additional CDK sites and the reduced ability of these proteins to inhibit cell cycle progression in specific tissues seems unlikely to be due to increased CDK activity. Instead, it seems more likely that epigenetic modifications reduce the activity of the mutant RB alleles. Two of the authors observations lead to this conclusion: 1) Although RBΔK7 is almost completely unphosphorylated, its expression does not inhibit the expression of the classic E2F-targeted genes. 2) Vitamin C attenuates the senescence/SASP phenotype that is induced by RBΔK7 without inactivating the mutant RB protein by phosphorylation.

In my opinion, these are important and provocative observations and the manuscript should be accepted for publication. The results make it clear that a great deal more needs to be done before we can claim that we understand the mechanism of RB action in vivo.

Rebuttal

• the authors should show the actual circulating insulin levels, as according to their findings, these should be very reduced due to the observed decrease in Beta cell mass.
The data presented in figure 5C, with the relative insulin secretion (compared to fasting insulin), is not in my opinion the most adequate to illustrate the authors' point.
In addition, if circulating insulin levels at different timepoints are shown it would likely help shedding light on the complex kinetics the authors observe for the onset of their phenotype.

Response: To address the issue raised by the Reviewer, we determined the levels of circulating insulin in diabetic $Rb^{\Delta K7}$ mice versus control littermates. As shown in new Fig. 5E in the revised manuscript, the level of insulin in fasting, diabetic $Rb^{\Delta K7}$ mice is 1.5 fold lower than control. Given that the glucose level in these fasting $Rb^{\Delta K7}$ mice is very high (17mM on average), the reduced insulin levels relative to normal mice, with average glucose level of 3.2 mM, is remarkable, and consistent with the increased senescence of pancreatic β-cells and diminished islet mass.

• the authors could further discuss the complex kinetics of the diabetic phenotype. Indeed, the authors detect reduced cell proliferation and DNA damage in pancreatic islets (2-3 weeks) before increased glucose levels (3 months) and much before SASP markers (9 months). This merits additional discussion.

Response: We demonstrated induction of SASP markers by gene-set enrichment analysis (GSEA) and G:profiler in 4 and 10 week old mice (Fig. 6A). Thus, the sequence of events seems to be (i) hypo-proliferation, (ii) senescence, (iii) increased glucose level/diabetes and (iv) gradual loss of islet mass, as described in the manuscript. We performed the proliferation assays at 2-3 weeks because this is when pancreatic β-cells actively proliferate. IHC for SASP markers was analyzed at 9 months to facilitate the detection of senescent (SASP+) cells, but as noted, they are detected by pathway analysis at 4-10 weeks and likely accumulate with time as mitogenic signals attempt to induce cell cycle re-entry, which elicits a DDR and senescence in pancreatic RbΔK7 β-cells (model).

• the authors should moderate their conclusions regarding premature aging, especially since type 1 diabetes is not usually a feature of premature aging, and since aging dependent replicative senescence and SASP in beta cells have been shown to be independent, with senescence being beneficial for insulin secretion and beta cell maturation, at least in some conditions (Arda et al 2016, Helman et al 2016, Thompson et al 2019, Li et al 2019).

Response: RbΔK7 mice exhibits various hallmarks of premature aging in addition to diabetes. This includes short telomeres, male infertility, kyphosis and reduced hair regeneration. These are, in our view, critically important and independent phenotypes that should be highlighted as they underscore the context-dependent impact of blocking pRb phosphorylation on homeostasis.

With regards to diabetes, the phenotype of RbΔK7 mice is reminiscent of both T1D and T2D as both exhibit senescence and progressive loss of β-cells as discussed in the manuscript (Aguayo-Mazzucato et al., 2019; Thompson et al., 2019; other references). Our observation that vitamin C reduces DDR and senescence, and attenuates diabetes suggests that the excessive DDR and senescence seen in RbΔK7 mice are not beneficial for pancreatic β-cells in this context. Direct elimination of senescent cells via senolytics may further clarify this issue, and is an area of investigation worth pursuing (Discussion).

Thank you for submitting your final revised manuscript for our consideration. I am pleased to inform you that we have now accepted it for publication in The EMBO Journal.

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YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

Eldad Zacksenhaus
EMBO J
2020-106825

Reporting Checklist For Life Sciences Articles (Rev. June 2017)

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.

In the pink boxes below, please ensure that the answers to the following questions are reported in the manuscript itself. Every question should be answered. If the question is not relevant to your research, please write NA (non applicable). We encourage you to include a specific subsection in the methods section for statistics, reagents, animal models and human subjects.

B- Statistics and general methods

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

1.a. How was the sample size chosen to ensure adequate power to detect a pre-specified effect size?	Power calculations using different parameters. All experiments show significant differences. For experimens involving vitamin C effects on diabetes and senescence we used over 20-30 mice as individual mce develop diabetes at different ages.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	At least three biological replicates, but in many experiments we used more replicates - e.g. for vitamin C rescue, over 30 mice per group were used to ensure reproducibility. In addition, when samples were collected (e.g. pancrease tissues that were sectioned and stained) - multiple islets were analyzed.
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	We didn't exclude data even when some points appeared like outliers - see actual results.
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.	Mice of particular genotypes were randomly selected and fed on regular or vitamin C diet
For animal studies, include a statement about randomization even if no randomization was used.	In some experiments when animals of the same genotype were fed different diets - mice were randomly seperated into two groups.
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.	In most experiments, the person who performed the analysis was blinded to the experimental details/setup; whereas the investigator who analyzed the results did not perform the experiment (e.g. IHC)
4.b. For animal studies, include a statement about blinding even if no blinding was done	Most experiments were performed double blinded - see above.
5. For every figure, are statistical tests justified as appropriate?	Statistical tests ste provided for each graph.
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	In most experiments – the results show normal distribution. For the kinetic of diabetes, vitamin C vs regular diet and effects of DDR and proliferation, control mice show normal distribution throughout the analysis. The RbΔK7 mice show normal distribution at 3 and 9 month but not at the intermediate periods as different individual mice develop diabetes at different stages. Likewise, the ki67 staining (Fig 6J) – included islets with vs without any staining making the distribution non-normal. Nevertheless, since we used over 20 samples per analysis – we used ANOVA to assess statistical differences. We also used Wilcoxon analysis which doesn't assume normality – and similar differences were obtained (not shown).

USEFUL LINKS FOR COMPLETING THIS FORM

http://www.antibodypedia.com
http://1degreebio.org
http://www.equator-network.org/reporting-guidelines/improving-bioscience-research-repor

http://grants.nih.gov/grants/olaw/olaw.htm
http://www.mrc.ac.uk/Ourresearch/Ethicsresearchguidance/Useofanimals/index.htm
http://ClinicalTrials.gov
http://www.consort-statement.org
http://www.consort-statement.org/checklists/view/32-consort/66-title

http://www.equator-network.org/reporting-guidelines/reporting-recommendations-for-tumo

http://datadryad.org

http://figshare.com

http://www.ncbi.nlm.nih.gov/gap

http://www.ebi.ac.uk/ega

http://biomodels.net/

http://biomodels.net/miriam/
http://jij.biochem.sun.ac.za
https://osp.od.nih.gov/biosafety-biosecurity-and-emerging-biotechnology/
http://www.selectagents.gov/

Is there an estimate of variation within each group of data?	Error bars are shown to indicate one standard deviation (SD) from the mean.
Is the variance similar between the groups that are being statistically compared?	See also above regarding normal distribution. For Fig 6G for example - the variance (VAR.P) for the two control groups and for the two K7 groups are close but the VAR.P for control vs K7 is larger.

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).	Information about antibody/reagents cat # - company is provided in M&M.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	We used primary cells (MEFs) derived from our mice, and a single, mycoplasma free cell line.

* 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.	Yes - see M&M.
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Statement of compliance - first senetence in M&M.
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.	Confirmed.

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	n/a
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.	n/a
13. For publication of patient photos, include a statement confirming that consent to publish was obtained.	n/a
14. Report any restrictions on the availability (and/or on the use) of human data or samples.	n/a
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	n/a
16. For phase II and III randomized controlled trials, please refer to the CONSORT flow diagram (see link list at top right) and submit the CONSORT checklist (see link list at top right) with your submission. See author guidelines, under 'Reporting Guidelines'. Please confirm you have submitted this list.	n/a
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.	n/a

F- Data Accessibility

18: Provide a "Data Availability" section at the end of the Materials & Methods, listing the accession codes for data generated in this study and deposited in a public database (e.g. RNA-Seq data: Gene Expression Omnibus GSE39462, Proteomics data: PRIDE PXD000208 etc.) Please refer to our author guidelines for '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	Data availability section included: microarray data accession number will be provided upon acceptance.
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).	ok
20. Access to human clinical and genomic datasets should be provided with as few restrictions as possible while respecting ethical obligations to the patients and relevant medical and legal issues. If practically possible and compatible with the individual consent agreement used in the study, such data should be deposited in one of the major public access-controlled repositories such as dbGAP (see link list at top right) or EGA (see link list at top right).	n/a
21. Computational models that are central and integral to a study should be shared without restrictions and provided in a machine-readable form. The relevant accession numbers or links should be provided. When possible, standardized format (SBML, CellML) should be used instead of scripts (e.g. MATLAB). Authors are strongly encouraged to follow the MIRIAM guidelines (see link list at top right) and deposit their model in a public database such as Biomodels (see link list at top right) or JWS Online (see link list at top right). If computer source code is provided with the paper, it should be deposited in a public repository or included in supplementary information.	n/a

G- Dual use research of concern

22. Could your study fall under dual use research restrictions? Please check biosecurity documents (see link list at top right) and list of select agents and toxins (APHIS/CDC) (see link list at top right). According to our biosecurity guidelines, provide a statement only if it could.	n/a