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Re-equilibration of imbalanced NAD metabolism ameliorates the impact of telomere dysfunction

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1st Editorial Decision 8th Oct 2019

Thank you for submitting your manuscript on CD38 and NAD dysregulation in Dyskeratosis Congenita to The EMBO Journal. I have now received reports from three expert referees, copied below for your information. I am afraid to say that none of these three referees is currently in favor of publication of the study. Although they appreciate the potential interest of this work, they all remain unconvinced that major conclusions in the title and abstract are sufficiently supported by the present set of data. In particular, with the beneficial effects of nicotinamide riboside treatment by themselves not appearing highly unexpected, all referees note that the specific importance of CD38 would need to be decisively established by characterizing its levels as well as NAD flux, by complementing CD38 inhibitor treatment also with genetic approaches targeting CD38, and finally by showing beneficial effects of CD38 inhibition/targeting also in the physiological paradigms on DC cells in the second part of the paper. Other key concerns include the lack of analyses of telomere length, and possible confounding effects of cellular senescence or of non-physiological nicotinamide riboside concentrations.

Given these shared major reservations and the lack of strong enthusiasm from any of our three expert reviewers, we unfortunately have to conclude that the study is not a sufficiently strong candidate for EMBO Journal publication at the present stage, and that we therefore cannot offer concrete further consideration at this point. I am sorry that the reports do not allow me to be more positive on this occasion, but hope that you will nevertheless find the referees' comments and suggestions helpful for considering your further proceedings with this study. Thank you once more for having had the opportunity to consider this work for publication.

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Referee #1:

This is an interesting manuscript that proposes that telomere dysfunction leads to NAD decline. The authors further propose that NAD decline is mediated by the consuming enzyme CD38. Furthermore, the authors show that busting NAD can ameliorate some of the features associated with the telomere dysfunction including telomere damage and mitochondrial dysfunction.

Although this is an interesting manuscript there are several aspects that are not completely convincing and need to be validated.
Major points:
In the first part of the manuscript the authors demonstrate that there is an NAD decline in cells derived from DC patients and that the expression of enzymes involved on the synthesis of NAD do not decline. Furthermore, they propose that two NAD consuming enzymes PARP1 and SIRT1 decrease.
However, changes in CD38 expression are not demonstrated. These would have to be documented. Imaging or flow may increase the sensitivity. Furthermore, PCR for the enzymes of the synthesis and degradation of NAD would help including CD38.
In addition, flux studies are necessary to demonstrate that these cells have increased turnover of NAD via its degradation.
Use of inhibitor of PARP and SIRT1 could help defining their contributions.
But the most important is that one should be able to show increase in CD38 if that is the case. The CD38 NADase activity could also be detected over several hours. Finally, the use of molecular biology approaches to decrease CD38 expression should increase NAD levels and promote similar effects as the small molecule CD38 inhibitor and also NR.

In figure 2 the gel data for some of the aspects described are not very convincing and need further validation. Positive and negative controls would help. For example using PARP1 and SIRt1 inhibitors or stimulators.

In figure 3 a dose response of the inhibitor is necessary. In addition, comparison with another way to increase NAD levels should be done side by side. Why the 48 hrs was done separate in not clear and it is difficult to compare the results.
In figure 4. Imaging to determine the specific cellular compartment where CD38 is increased is necessary. The use of CD38 KO as a control for validate the antibody would be appreciated. expression of SARM1 (another NADase) should be explored.
The effect of the small molecule CD38 inhibitor or the use of a CD38 KO for in vivo functional studies is necessary to validate the hypothesis. Looking at least at mitochondrial function and telomere damage would be enough.

Figures 5 abd 6 are convincing. But if the goal is to demonstrate the role of CD38 one needs to show the effect of inhibitors or knockdown CD38 in the cells.
Figure 7. The same as above. In addition, figure b is not extremely easy to evaluate. It appears that there are co-stain areas on the NR treated that are not highlighted. The graph C could include only the data with the more than 3 TIF (it would be easier to read). The complete graph could go to supplementary data.
Figure 8 could also be repeated with CD38 inhibitor. Recently, it has also been demonstrated that CD38 can be induced by the SASP of senescent cells. This should be discussed as a potential cause for the proposed increase in CD38.

Referee #2:

Wang et al.
"Elevated CD38 is associated with NAD dysregulation in a human disease model of telomere dysfunction"

In this study, the authors showed that primary skin fibroblasts isolated from patients with dyskeratosis congenita (DC) exhibited lower NAD+ and ATP levels, compared to fibroblasts from age-matched healthy individuals. Interestingly, these DC fibroblasts also exhibited reduced protein and activity levels of PARP1 and SIRT1, and supplementation of 3mM nicotinamide riboside (NR) was able to increase NAD+ levels and PARP1 and SIRT1 activities. Additionally, the authors assessed protein and activity levels of CD38 but failed to detected them. Nonetheless, a specific CD38 inhibitor, 78c, significantly increased NAD+ levels and PARP1 and SIRT1 activities in these DC fibroblasts. To further support their claims, they turned to brains from Tert-/- mice at generations 1 and 3 (G1 and G3). They found that G3 Tert-/- brains showed lower NAD+ levels and higher CD38 protein and activity levels, compared to those from G1 Tert-/- mice. DC fibroblasts showed a variety of cellular phenotypes, including mitochondrial morphological aberration and dysfunction, reduced mitophagy, oxidative DNA damage, induced DNA damage foci, reduced proliferative capacity, and cellular senescence. The authors showed that treatment with 3mM NR significantly ameliorated all these cellular phenotypes in DC fibroblasts. Based on these findings, the authors argued that telomere dysfunction leads to increased CD38 expression and activity and causes impairments in mitochondrial function and a variety of cellular functions.

Although their findings are potentially interesting, the biggest weakness of this study is the extent of the CD38 contribution to NAD deficits in DC fibroblasts, as described precisely below. Additionally, all the results basically remain associations, and the underlying mechanism for CD38 upregulation in DC fibroblasts also remains unclear. Therefore, this study is premature as its current form to be considered for EMBO Journal.

Major comments:

1. The biggest logical problem in this study is that the contribution of CD38 to NAD+ decline is relatively very small. NAD+ levels in DC fibroblasts ranges ~0.5-1.5 nmol/mg protein, 50-90% decrease compared to fibroblasts from age-matched healthy individuals (~3-5 nmol/mg protein) (Figure 1a). However, the maximal levels of NAD+ that 78c, a specific CD38 inhibitor, was able to restore in DC fibroblasts were 1.2-1.6 nmol/mg protein, which are still far below the NAD+ levels in control fibroblasts. This immediately suggests that CD38 cannot be a major contributor to the observed NAD+ decrease in DC fibroblasts, which is consistent with the authors' observation that CD38 expression and activity are under detectable levels in DC fibroblasts. On the other hand, treatment with 3mM NR restored NAD+ levels to ~2.5-4.5 nmol/mg protein in DC fibroblasts, pretty close to the levels in control fibroblasts. This result strongly suggests that there must be a different major contributor, not CD38, to the NAD+ decrease in DC fibroblasts. Therefore, the authors should carefully reexamine what the major contributor would be for the observed NAD+ decline in DC fibroblasts.

2. Another possibility is that the use of the pharmacological method (78c) was inappropriate or insufficient in DC fibroblasts. The authors should try a genetic approach, such as CD38 knockdown,

to further assess a possible involvement of CD38 in their NAD+ decline.

3. The results from Tert-/- mice are simply correlations and relatively weak. If CD38 is a major contributor for the NAD+ decrease observed in G3 Tert-/- mice, administering 78c to them or crossing them to CD38-/- mice could restore NAD+ levels and ameliorates other cellular and tissue dysfunctions. The authors should obtain more direct evidence regarding whether CD38 is indeed a major contributor even in G3 Tert-/- mice.

4. Why did the authors require such a supraphysiological concentration of NR (3mM) to see its effect? It is now well known that NR is easily degraded into nicotinamide in plasma and in other conditions. This is probably the reason why the authors needed to change the media every 24 hrs. Even in this method, it would be inevitable to see an extremely high levels of nicotinamide in this culture condition. Therefore, the authors should monitor nicotinamide production and try to minimize the concentration of NR to avoid unnecessary confounding effects due to nicotinamide production.

Minor comments:

1. In the Discussion section, the authors have an interesting discussion regarding a recently published paper by Amano et al. The authors claim that their findings "directly revel the relevance of CD38 hyperactivity-mediated NAD dysregulation to the pathophysiology of human telomere-driven diseases and suggest a new approach for effective intervention of NAD dysregulation via CD38 inhibition." Because the authors do not have any direct in vivo evidence for a potential involvement of CD38, this is an inappropriate statement. The authors should either provide direct in vivo evidence or eliminate this far-fetched statement.

2. Throughout this manuscript, the authors should carefully check references. Some of them are very old (more than 10 years ago), and some others are not well balanced.

Referee #3:

In "Elevated CD38 is associated with NAD dysregulation in a human disease model of telomere dysfunction" the authors aim at uncovering potential roles of NAD dysregulation and the role of CD38 in the telomere-dysfunction disease dyskeratosis congenita.

The authors make use of both human and mouse models of telomere dysfunction. On the human side, they use different fibroblasts obtained from DC patients. This is problematic on itself, as these fibroblasts typically senesce very quickly in culture (a point that I will discuss later on). Mice experiments were performed in TERT null mice (Generation 1 and 3), using brain tissue as a model. This is somewhat surprising, as central nervous system impairment is not traditionally seen as a major phenotype of DC.

Overall, the majority of data presented is not novel or specifically relevant for the disease, as it relies on well established consequences of using nicotinamide riboside (NR) and CD38 inhibition on their downstream targets. It is also extremely surprising that in a paper that focuses on DC and CD38, telomere lengths are not quantified and CD38 levels not analyzed. The only Figure which is relevant for DC and telomere biology is Figure 8. I'll describe my concerns:

Major issues:

It is difficult to understand how a paper that deals primarily with telomere shortening and CD38, no data is shown regarding telomere length and CD38 levels in any of the cells analyzed. This is a major flaw of this paper, that undermines any conclusions they authors might have.

Figure 1: Authors start by comparing NAD, SIRT1 and CD38 levels in DC fibroblasts and age-matched control fibroblasts. "Age matched control fibroblasts" is very vague and matters little (if at all) if different fibroblast lineages have been cultured for different population doublings, especially when referring to telomere biology. Therefore, the most important factor to take into account between the different fibroblast populations (in this study) is Population Doublings (PDL), and if the cells being analyzed begin to show early signs of senescence. Fibroblasts derived from DC patients senesce very quickly, due to telomere shortening. All the observed phenotypes in human cells in this paper could be caused by the fact that the authors are comparing normally proliferating cells (controls) against cells that are senescing (DC). Currently, as the manuscript is written, the authors seem to infer that DC fibroblasts display altered steady-state levels of NAD, SIRT1 and CD38 regardless their PDL/telomere length/senescence status, which is completely misleading. In other words: NAD, SIRT1 and CD38 levels vary simply because cells have entered senescence and/or have damaged telomeres, regardless if they are DC or control fibroblasts?

Figure 2: All data presented in this figure confirms well know biological phenomena: NR treatment increases NAD levels and modulates sequential downstream pathways. There is no reason to believe DC fibroblasts would act differently. Therefore, the fact that this is true also in DC fibroblasts is the absolutely expected observation.

Figure 3: Similar to figure 2. All data presented in this figure confirms well know biological phenomena: inhibition of CD38 increases NAD levels and modulates sequential downstream pathways. The fact that this is true also in DC fibroblasts is therefore absolutely expected and the authors show no relevance to the disease itself.

In conclusion, Figures 2-3 indicate no particular relevance of NAD or CD38 to DC, other than well established biochemical pathways acting in a similar manner to WT cells. The fact that the authors don't 1) show treatment of control cells 2) don't show telomere lengths, 3) don't show CD38 levels and 4) show no biological significance (will only do that in Figure 8) leads me to believe that Figures 2-3 are mostly uninformative and could be Supplemental.

Figure 4: The authors should compare telomere lengths in their G1 and G3 mice (in the same tissues analyzed for NAD concentration). Also please show beta-gal staining in these same tissues, so that we can be sure the figure is not comparing a normal tissue to a tissue that already shows signs of senescence. The conclusion (from the authors) of Figure 4 is "Collectively our results suggest that CD38 is a major NAD consuming enzyme that contributes to lower NAD levels and altered NAD signaling in human and mouse models with critically short telomeres". But again, the authors don't show any telomere length data, no CD38 levels and don't treat control groups (or analyze WT mice). Therefore, as is, this figure only confirms what is widely know about NAD biology, irrespective of telomere status.

Figure 5/6: why is it that data is shown only for DC1 and DC3 samples on Figure 5E-G? The differences seem minimal. Are there any physiological improvement with NR treatment in terms of mitochondria function? The authors should perform a Seahorse analysis. I am confused to what the authors consider "mitophagy dye? Figure 6 seems to show mitochondria and lysosome co-staining

most of the times, even in the control cells, which is most likely a methodological issue. This should be corrected. Please provide better images, with higher magnification, allowing for the specific visualization of the mitochondria and lysosome dyes.

Figure 7: The authors show no difference between oxidative base repair in WT vs DC cells after NR treatment, they both show improvement. Therefore the results are not specific to DC. However, this reviewer agrees this could be potentially relevant for DC, as minimal telomere improvement could lead to improved phenotypes (which is potentially shown in the following Figure. Figures 7B/C are of extremely poor quality. These images (TIFs need to be improved. I see at least two TIF events in NR treated cells that don't seem to have been quantified on Figure 7B.

Figure 8: This is the only figure that indeed is specific to DC and seems to indicate phenotypical improvement. But these results (proliferation, BRDU, beta-gal need also to be performed with the CD38 inhibitor (78c used in the initial figures to show, as the authors claim, that these phenotypes are caused by CD38. It would also be more convincing if the authors could show improvement in more samples (only two for some experiments. Another interesting addition would be treatment of G5 mice with both compounds and analysis of tissue viability (G3 mice are usually "normal", G5 show severe deficit, particularly hematopoietic).

My sincerest thanks for the opportunity to have our manuscript considered at EMBO J. The reviewers' constructive comments are very helpful and after carefully considering them, I am confident that we can address their major critiques and other non-experimental suggestions. After reading our plans for revision below, I'd be grateful to hear your thoughts on whether EMBO J. would consider a revised manuscript, or a new submission. In brief, we plan to address the major concerns raised by the reviewers as follows:

Reviewers 1 and 2

The reviewers requested further evidence that CD38 is elevated in DC fibroblasts; indeed we do have this data, and will include it. As requested, we also tested whether PARP or sirtuin inhibition affected NAD levels in DC fibroblasts and found little impact. This data will also be included. Thus, we feel confident in our hypothesis that CD38 is a major contributor to NAD decline in DC.

The reviewers requested CD38 knockdown in DC cells, to strengthen the direct role of CD38 hyperactivity in NAD dysregulation in DC cells, and to potentially mitigate any inefficiency of the CD38 inhibitor in normalizing NAD level and other cellular phenotypes in DC cells. We agree, and will address it using CRISPR-Cas9 mediated CD38 disruption in DC cells.

Reviewer 2 remarked on the concentration (3mM) of NR used in this study. We will reference other studies that have employed the same dose and, as requested, we will also include experiments at other dosages, e.g. 1 mM NR, which was also effective in normalizing NAD levels in DC cells.

Reviewer 3

We appreciate this reviewer's concern that the DC fibroblasts may already be senescent, which would impact our conclusions. We too shared this concern, which is why we went to great lengths to use early passage (P1 or P2) DC fibroblasts that do not show proliferative defects (Figure 8a, days 1-12). Indeed, only after prolonged culture do they enter replicative senescence (Fig 8a, >13 days). We also took care to assess this issue when evaluating the effectiveness of NR supplementation, in which we compared the effects of NR addition (or vehicle control) before senescence onset and upon continuous passaging until the cells entered senescence. Thus, it is important to stress that our kinetic studies show that NR-treated DC cells undergo a delayed entry into senescence (e.g. Fig. 8).

This reviewer requested telomere length measurements in DC and G1/G3 mice. We have published extensive telomere length characterization of these late generation mTERT KO animals, and showed that end-to-end fusions arise concomitantly with infertility (e.g. Erdmann et al., 2004). The G3 animals in this study are also infertile, however we can include telomere q-FISH for both the mice and DC cells.

This reviewer pointed out that we did not provide CD38 expression data in the manuscript. However, we did include it for the TERT KO tissues (Figure 4), and as stated above we also had data for the DC fibroblasts which we will now include. We will also attempt to clarify Figures 2 and 3, in which the intent was to further confirm NAD dysfunction in DC cells, and that the primary mechanism is via CD38. The CRISPR-Cas9 experiment mentioned above would also help to address this potential concern.

Lastly, although we don't plan to include such data in a revised manuscript, I would like to share that we have begun to generate double knockout mice for mTert and CD38, which will allow us to further investigate the impact of CD38 on aging and disease-related phenotypes due to telomere dysfunction. As you can appreciate, the breeding will
require another 1-2 years to achieve critically short telomere lengths in the CD38 background. We hope you will appreciate these are long-term experiments, and as such we feel they are outside the scope of what could be accomplished within a timely revision period.
We are grateful for thoughtful comments and input throughout the review process, and I am looking forward to hearing your suggestions after you have considered our response.

Thank you for your response to the reviews and outlining how you may address them. I do realize that the paper could become a more compelling EMBO Journal candidate if you should be able to satisfactorily address the reviewers' concerns, and would therefore in principle not be opposed to considering a resubmitted version once more - should the planned experiments indeed result in strengthening of the key conclusions. I agree that most revision proposals seem to go into the right direction, but I also note some apparent omissions. In addition to the crucial analyses of CD38 expression and genetic/knockdown analyses of CD38 roles, it seems the NAD flux analyses (ref 1) and the concern regarding only partial rescue by 78c (as compared to NR) need to be taken on board too, as well as the issue that the phenotypic rescue by NR in the latter part (esp. Fig 8) needs to be recapitulated also by CD38 inhibition specifically (requested specifically by both referees 1 and 3). With regard to telomere length measurements, I guess your previous studies did not compare them with regard to differential NAD status, so it may be important to include such comparative analyses here.

Given the extent of these referee concerns, I hope you understand that I am currently not in the position to commit to a revised version, but I would suggest that you get in touch with me once you are preparing to resubmit, sending me a point-by-point response letter indicating how each issue may have been addressed - based on this more concrete information, I am sure I should then be able to give you a more definitive answer on whether we could send the study once more to the original referees.

I hope you find these considerations helpful for now.

Response to Reviewers' Comments

We thank all reviewers for their thoughtful evaluation of our manuscript. In light of the reviewers' and the editor's comments we have modified the title to reflect the fact that this manuscript deciphers NAD metabolism and intervention in a human disease model with telomere dysfunction. Hereunder we would be explicit about the NEW experiments we have included in the manuscript.

1) We have added evidence that CD38 expression is elevated in DC fibroblasts, in comparison to age-matched healthy controls (Fig. 2A). These results are consistent with our early observation that late generation telomerase null mice display elevated CD38 levels and NADase activity (Fig. 3).
2) To strengthen the direct role of CD38 overexpression in NAD dysregulation in DC, we generated CD38 knockdown DC fibroblasts using the lentiviral CD38 shRNAs (and the scrambled shRNA control) (Fig. 2C) and examined basal levels of NAD, or upon treatment with PARP1 and SIRT1 inhibitors (Fig. 2D and E).
3) To further corroborate the role of NAD biosynthesis and other NAD consuming enzymes in NAD dysregulation in DC cells, we performed the following experiments:
   a) we examined the impact of the PARP and sirtuin inhibitors on intracellular NAD levels in DC fibroblasts and found that PARP and sirtuin inhibition had little impact on intracellular NAD levels (Appendix Fig. S2A and S2C). This result further strengthens our conclusion that low PARylation and SIRT1-deacetylation activities unlikely contribute to NAD decline in DC fibroblasts.
   b) We showed that the expression of another NADase, SARM1 is unaltered in DC fibroblasts (Fig. 2A).
   c) We showed that DC fibroblasts efficiently convert NAM (a substrate for NAD biosynthesis) to NAD, supporting that NAD biosynthesis is intact in DC cells (Fig. 1F).
4) To understand the biological consequence of CD38 overexpression in DC, we examined the replicative potential of DC fibroblasts in which CD38 was knocked down via lentiviral shRNAs. We found that reduced CD38 leads to a delay in the onset of replicative senescence of DC fibroblasts (Fig. 7A and Appendix Fig. S6). In addition, CD38 knockdown led to a reduction in mitochondrial ROS in DC fibroblasts (Fig. 4D).
5) We have provided evidence that NR supplementation, at lower doses, effectively improves the NAD levels (Fig. 1F) and mitochondria quality (Appendix Fig. S5D) and delays the replicative senescence of DC fibroblasts (Appendix Fig. S6).
6) We included telomere length measurement in DC patients' PBMCs (Appendix Table S1) and in DC fibroblasts with mock and NR supplementation (Fig. 6A).
7) We have shown that ATM inhibition led to a decline in CD38 levels in DC fibroblasts (Fig. 2B), suggesting the involvement of a telomere dysfunction-induced DNA damage response in CD38 overexpression.

We have addressed the specific concerns by the reviewers. Together, these experimental additions support our discovery of a direct, underlying defect in NAD regulation when telomeres are short, and that this dysregulation is functionally relevant to the pathophysiology of human telomere-driven diseases. We provide a point-by-point response to each reviewer as follows:

Reviewer #1:

In the first part of the manuscript the authors demonstrate that there is an NAD decline in cells derived from DC patients and that the expression of enzymes involved on the synthesis of NAD do not decline. Furthermore, they propose that two NAD consuming enzymes PARP1 and SIRT1 decrease. However, changes in CD38 expression are not demonstrated. These would have to be documented. Imaging or flow may increase the sensitivity. Furthermore, PCR for the enzymes of the synthesis and degradation of NAD would help including CD38. In addition, flux studies are necessary to demonstrate that these cells have increased turnover of NAD via its degradation. Use of inhibitor of PARP and SIRT1 could help defining their contributions. But the most important is that one should be able to show increase in CD38 if that is the case. The CD38 NADase activity could also be detected over several hours. Finally, the use of molecular biology approaches to decrease CD38 expression should increase NAD levels and promote similar effects as the small molecule CD38 inhibitor and also NR.

Response: We thank the reviewer for these valuable comments. As requested, we have now provided the following experimental support:
1) CD38 expression is elevated in DC fibroblasts, in comparison to age-matched healthy controls (Fig. 2A). These results are consistent with our observation that late generation telomerase null mice display elevated CD38 levels and NADase activity (Fig. 3).

2) To strengthen the direct role of CD38 overexpression in NAD dysregulation in DC, we generated CD38 knockdown DC fibroblasts using the lentiviral CD38 shRNAs (and the scrambled shRNA control) (Fig. 2C). Although CD38 knockdown led to a moderate increase in intracellular NAD levels in DC fibroblasts (Fig. 2D), it significantly boosted the NAD consumption activities of PARPs (Fig. 2E). A slight increase in SIRT1 deacetylation of p53 was also observed in the CD38 knockdown DC fibroblast (Fig. 2E). Thus, accumulated NAD upon CD38 inhibition is likely consumed by PARPs and SIRT1, since PARylation and SIRT1 deacetylation activities were particularly low in DC fibroblasts (Fig. 1D). In support of this notion, intracellular NAD levels were significantly elevated in the CD38 knockdown DC fibroblasts when PARP1 and SIRT1 NAD consuming activities were inhibited (Fig. 2D). It is noteworthy that PARP1 and SIRT1 inhibition had little impact on NAD levels in DC fibroblasts (Appendix Fig. S2C), which is likely due to reduced expression and activities of these two NAD enzymes in DC fibroblasts (Fig. 1D). Similarly, 78c treatment moderately increased intracellular

NAD levels in DC fibroblasts, but led to significantly elevated NAD levels, when combined with PARP1 and SIRT1 inhibitors or with low dose NMN (a NAD precursor) (Appendix Fig. S4).

Our many attempts to measure the NAD flux in DC fibroblasts was unsuccessful, in part because these cells grew poorly when switched to the culture medium with dialyzed FBS and isotopic NAM. However, we have added new evidence supporting the role of CD38 in NAD dysregulation in DC. First, we have shown that CD38 knockdown and inhibition by 78c contribute to NAD dysregulation (Fig. 2 and Appendix Fig. S4). Second, we found that PARP and sirtuin inhibition had little impact on the NAD levels in DC fibroblasts (Appendix Fig. S2B), which further strengthens our finding that low PARylation and SIRT1-deacetylation activities in DC fibroblasts unlikely contribute to NAD decline. Third, we found that the expression of another NADase, SARM1, is unaltered in DC fibroblasts by western blot analysis (Fig. 2A). Lastly, we demonstrated that DC fibroblasts efficiently convert NAM (a substrate for NAD biosynthesis) to NAD, supporting that NAD biosynthesis is intact in DC cells (Fig. 1F).

In figure 2 the gel data for some of the aspects described are not very convincing and need further validation. Positive and negative controls would help. For example, using PARP1 and SIRt1 inhibitors or stimulators.

In figure 3 a dose response of the inhibitor is necessary. In addition, comparison with another way to increase NAD levels should be done side by side. Why the 48 hrs. was done separate in not clear and it is difficult to compare the results.

Response:

We thank the reviewer for this suggestion. We have provided western blot analysis of PARylation and SIRT1-deacetylation activities in DC fibroblasts exposed to the PARP1 and/or SIRT1 inhibitors (Appendix Fig. S2A). We also showed that PARP1 and/or SIRT1 inhibition had little impact on the NAD levels in DC cells (Appendix Fig. S2C). Conversely, NR supplementation mitigated reduced PARylation and SIRT1-deacetylation activities in DC fibroblasts (Appendix Fig. S2D-F).

We have replaced the original Fig. 3. We now show that CD38 knockdown ameliorates NAD dysregulation in DC fibroblasts (Fig. 2C-E). We also added the data that ATM inhibition led to a decline in CD38 levels in DC cells (Fig. 2B). Interestingly, we noticed that intracellular NAD levels in DC fibroblasts are not affected by PARP1 and SIRT1 inhibition, but significantly increased when combined with CD38 knockdown (Fig. 2D) or 78c (Appendix Fig. S4). In addition, 78c in combination with low dose NMN (a NAD precursor) effectively promoted NAD levels in DC fibroblasts (Appendix Fig. S4). These results, together with the observation that PARylation and SIRT1 deacetylation activities were enhanced by CD38 knockdown, support the

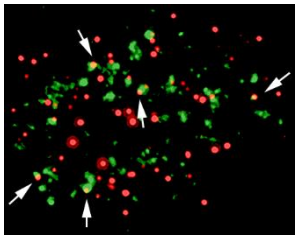
notion that low NAD levels limit PARylation and SIRT1 deacetylation activities, and that excessive NAD produced by CD38 knockdown or inhibition is consumed by PARPs and sirtuins. These results provide mechanistic insight into why CD38 knockdown or inhibition require PARPs and sirtuins inhibitors to effectively increase intracellular NAD levels in DC cells.

Collectively, our results support the notion that PARylation and SIRT1-deacetylation activities unlikely contribute to NAD decline; while CD38 levels impact NAD levels and/or PARylation and SIRT1-deacetylation activities in DC fibroblasts.

In figure 4. Imaging to determine the specific cellular compartment where CD38 is increased is necessary. The use of CD38 KO as a control for validate the antibody would be appreciated. expression of SARM1 (another NADase) should be explored.
The effect of the small molecule CD38 inhibitor or the use of a CD38 KO for in vivo functional studies is necessary to validate the hypothesis. Looking at least at mitochondrial function and telomere damage would be enough.

Response: We thank the reviewer for the suggestion. We agree with the reviewer's suggestion and confirmed the CD38 antibody specificity using the CD38 knockout mouse brain tissues (Fig. 3B). Toward addressing this question, we have begun to generate the double knockout mice for Tert and CD38, which will allow us to determine the specific cellular/tissue compartments where CD38 is increased and the impact of CD38 on aging and telomere-related phenotypes in mice. Because inbred mouse strains possess excessively long telomeres, the breeding will require another 1-2 years to achieve critically short telomere lengths in the CD38 background. We hope you will appreciate these are long-term experiments, and as such we feel they are outside the scope of what could be accomplished within a timely revision period. We have examined SAM1 expression (Fig. 2A) and the impact of CD38 inhibition on DC cell function (please see the response below).

Figures 5 and 6 are convincing. But if the goal is to demonstrate the role of CD38 one needs to show the effect of inhibitors or knockdown CD38 in the cells.
Figure 7. The same as above. In addition, figure b is not extremely easy to evaluate. It appears that there are co-stain areas on the NR treated that are not highlighted. The graph C could include only the data with the more than 3 TIF (it would be easier to read). The complete graph could go to supplementary data.
Figure 8 could also be repeated with CD38 inhibitor. Recently, it has also been demonstrated that CD38 can be induced by the SASP of senescent cells. This should be discussed as a potential cause for the proposed increase in CD38.



Response: We thank the reviewer for the suggestion. We highlighted TIFs in NR-treated DC fibroblasts and replaced the TIF image and the graph in Fig. 6B accordingly. To better illustrate TIFs, we enclosed an enlarged image at right and in Appendix Fig. EV1.

Mounting evidence supports that telomere repeat reserves determine cell proliferative capacity in culture. DC fibroblasts, due to less telomere repeat reserves, have fewer population doublings during prolonged culture. Thus, to understand the biological consequence of CD38 overexpression in DC, we focused on determining replicative potential of the scramble and CD38 shRNA DC fibroblasts. It is noteworthy that DC fibroblasts used in our experiments are capable of proliferating and enter senescent status only after prolonged culture (Fig. 7A); while decline in CD38 levels via shRNA treatment delays replicative senescence of DC fibroblasts (Fig. 7A and Appendix Fig. S6). Thus, our data support that elevated CD38 drives proliferating DC fibroblasts entering senescent status, a phenotype is amendable to CD38 knockdown. We also showed that the CD38 shRNA DC fibroblasts displayed reduced cellular and mitochondrial ROS (Fig. 4D), supporting that elevated CD38 contributes to mitochondrial abnormalities in DC cells.

We agree with the reviewer's comments that SASP may contribute to increased CD38 expression in DC cells. As mentioned above, DC fibroblasts used in our experiments are capable of proliferating and enter senescent status only after prolonged culture, and CD38 knockdown significantly extends proliferative capacity of DC fibroblasts (Fig. 7A and Appendix Fig. S6). Thus, we believe that this mechanism may be activated after DC fibroblasts enter senescent status. We have now clarified this notion in Discussion. As emerging evidence suggests a crucial role for critically short telomere-induced DNA damage response (DDR) in cellular dysfunction in DC, we tested the role of DDR in driving CD38 overexpression in DC and found that ATM inhibition led to a decline in CD38 levels (Fig. 2B). Our results support that critically short telomere-induced ATM activation contributes to CD38 overexpression in DC cells.

Reviewer #2 also found the manuscript potentially representing interesting findings, but requested direct evidence of CD38 in NAD deficits and lower NR dosages in the experiments. We have addressed the reviewer's concerns as follows:

The biggest logical problem in this study is that the contribution of CD38 to NAD+ decline is relatively very small. NAD+ levels in DC fibroblasts ranges ~0.5-1.5 nmol/mg protein, 50-90% decrease compared to fibroblasts from age-matched healthy individuals (~3-5 nmol/mg protein) (Figure 1a). However, the maximal levels of NAD+ that 78c, a specific CD38 inhibitor, was able to restore in DC fibroblasts were 1.2-1.6 nmol/mg protein, which are still far below the NAD+ levels in control fibroblasts. This immediately suggests that CD38 cannot be a major contributor to the observed NAD+ decrease in DC fibroblasts, which is consistent with the authors' observation that CD38 expression and activity are under detectable levels in DC fibroblasts. On the other hand, treatment with 3mM NR restored NAD+ levels to ~2.5-4.5 nmol/mg protein in DC fibroblasts, pretty close to the levels in control fibroblasts. This result strongly suggests that there must be a different major contributor, not CD38, to the NAD+ decrease in DC fibroblasts. Therefore, the authors should carefully reexamine what the major contributor would be for the observed NAD+ decline in DC fibroblasts.

Another possibility is that the use of the pharmacological method (78c) was inappropriate or insufficient in DC fibroblasts. The authors should try a genetic approach, such as CD38 knockdown, to further assess a possible involvement of CD38 in their NAD+ decline.

Response: We thank the reviewer for this constructive input. To strengthen the role of CD38 overexpression in NAD dysregulation in DC cells, we have included the following new data:

1. CD38 expression is elevated in DC fibroblasts, in comparison to age-matched healthy controls (Fig. 2A). These results are in agreement with our observation that G3 *Tert* null mice (short telomere length) display elevated CD38 levels and NADase activity, in comparison to G1 *Tert* null mice (normal telomere length) (Fig. 3B-D). As emerging evidence suggests a crucial role for critically short telomere-induced DNA damage response (DDR) in cellular dysfunction in DC, we tested the role of DDR in driving CD38 overexpression in DC and found that ATM inhibition led to a decline in CD38 levels (Fig. 2B). Our results support that ATM activation contributes to CD38 overexpression in DC cells.

2. Per the reviewer's request, we have now generated CD38 knockdown DC fibroblasts using the lentiviral CD38 shRNAs (Fig. 2C) and added the data showing that CD38 knockdown ameliorates NAD dysregulation in DC fibroblasts (Fig. 2D). Although CD38 knockdown only led to a moderate increase in the NAD levels in DC cells (Fig. 2D), it significantly boosted the NAD consumption activities of PARPs (Fig. 2E). A slight increase in SIRT1 deacetylation of p53 was also observed in the CD38 knockdown DC fibroblast (Fig. 2E). However, intracellular NAD levels were significantly elevated in the CD38 knockdown DC fibroblasts when PARP1 and SIRT1 NAD consuming activities were inhibited (Fig. 2D). We also noticed that 78c treatment alone led to a moderate increase in the NAD levels in DC fibroblasts, but significantly enhanced the NAD levels when combined with the PARP1 and SIRT1 inhibitors (Appendix Fig. S4A), although the PARP1 and SIRT1 inhibitors had little impact on NAD levels in DC fibroblasts (Appendix Fig. S2C). Furthermore, 78c in combination with low dose NMN (a NAD precursor) effectively promoted NAD levels in DC fibroblasts (Appendix Fig. S4B). These results, together with the observation that PARylation and SIRT1 deacetylation activities were noticeably low in DC cells (Fig.1D-E), support the notion that low NAD levels limit PARylation and SIRT1 deacetylation activities and that NAD accumulation via CD38 knockdown or inhibition is consumed by PARPs and sirtuins. These results explain why CD38 knockdown or inhibition require PARP and sirtuin inhibition to effectively increase the NAD levels in DC cells.

3. We demonstrated that the inhibition of other NAD consuming enzymes PARPs or sirtuins had little impact on intracellular NAD levels in DC cells (Appendix Fig. S2C). These results further strengthen our observation that PARylation and SIRT1-deacetylation activities decline in DC cells (Fig. 1D) and therefore unlikely contribute to low NAD levels in DC fibroblasts.

5. We added the new evidence that expression of another NADase, SARM1 is unaltered in DC fibroblasts by western blot analysis (Fig. 2A).

The results from Tert-/- mice are simply correlations and relatively weak. If CD38 is a major contributor for the NAD+ decrease observed in G3 Tert-/- mice, administering 78c to them or crossing them to CD38-/- mice could restore NAD+ levels and ameliorates other cellular and tissue dysfunctions. The authors should obtain more direct evidence regarding whether CD38 is indeed a major contributor even in G3 Tert-/- mice.

Response: We thank the reviewer for the suggestion. This manuscript focuses on deciphering NAD metabolism and intervention in human telomere biology disease. In this study, the telomerase null mouse model was employed to verify the observation that increased CD38 is driven by short telomeres in human telomere biology disease. We agree with the reviewer's valid suggestion. We would like to share that we have begun to generate double knockout mice for Tert and CD38, which will allow us to investigate the impact of CD38 on aging and disease-related phenotypes due to telomere shortening. As the breeding will require another 1-2 years to achieve critically short telomere lengths in the CD38 background, we hope you will appreciate these are long-term experiments, and as such we feel they are outside the scope of what could be accomplished within a timely revision period.

Why did the authors require such a supraphysiological concentration of NR (3mM) to see its effect? It is now well known that NR is easily degraded into nicotinamide in plasma and in other conditions. This is probably the reason why the authors needed to change the media every 24 hrs. Even in this method, it would be inevitable to see an extremely high levels of nicotinamide in this culture condition. Therefore, the authors should monitor nicotinamide production and try to minimize the concentration of NR to avoid unnecessary confounding effects due to nicotinamide production.

Response: We thank the reviewer for the suggestion. Our results demonstrated that 3mM NR supplementation did not have any detrimental impact on age-matched healthy controls and DC fibroblasts; however, we agree with the reviewer's valid suggestion and tested a lower dose, 1 mM NR in DC cells. NR at low doses effectively normalized NAD levels in DC cells (Fig. 1F), which is comparable to age-matched controls (Fig 1A). Because telomere repeat reserves determine cell proliferative capacity, DC cells with less telomere repeats have limited replicative potential, which presents as the key feature of telomere biology disease. We therefore tested the efficacy of 1mM NR in ameliorating this key DC feature. Our new data showed that 1 mM NR efficiently delayed replicative senescence of DC fibroblasts (Appendix Fig. S6). In addition, 1 mM NR efficiently reduced damaged mitochondria in DC cells (Appendix Fig. S5D).

Minor comments:

1. In the Discussion section, the authors have an interesting discussion regarding a recently published paper by Amano et al. The authors claim that their findings "directly revel the relevance of CD38 hyperactivity-mediated NAD dysregulation to the pathophysiology of

human telomere-driven diseases and suggest a new approach for effective intervention of NAD dysregulation via CD38 inhibition." Because the authors do not have any direct in vivo evidence for a potential involvement of CD38, this is an inappropriate statement. The authors should either provide direct in vivo evidence or eliminate this far-fetched statement.

Response: We have provided additional evidence supporting the relevance of CD38 levels regulates NAD metabolism in DC cells (please see the response above). We have also reformatted the title and this sentence, which now read *"Our findings reveal the relevance of CD38 hyperactivity-mediated NAD dysregulation in human telomere-driven diseases and suggest a new approach for effective intervention of NAD dysregulation via CD38 inhibition"*.

2. Throughout this manuscript, the authors should carefully check references. Some of them are very old (more than 10 years ago), and some others are not well balanced.

Response: We thank the reviewer for this suggestion. We have updated the references accordingly. We kept some of the original references due to the impact of their unique work.

Referee #3 pointed out potential concerns about the relevance of the data to human disease. This reviewer also requested additional data related to telomere lengths and CD38 levels.

Major issues:

It is difficult to understand how a paper that deals primarily with telomere shortening and CD38, no data is shown regarding telomere length and CD38 levels in any of the cells analyzed. This is a major flaw of this paper, that undermines any conclusions they authors might have.

Response: We thank the reviewer for the valuable comments and have addressed his or her concerns as follows:

1. We have added evidence that CD38 expression is elevated in DC fibroblasts, in comparison to aged-matched healthy controls (Fig. 2A). These results are in agreement with our observation that G3, but not G1 telomerase null mice display an elevated CD38 expression and NADase activity (Fig. 3). To strengthen the direct role of elevated CD38 in NAD dysregulation in DC cells, we generated the CD38 knockdown DC fibroblasts using the lentiviral CD38 shRNAs (Fig. 2C), and added the data showing that CD38 knockdown ameliorates NAD dysregulation in DC cells (Fig. 2 D-E and please see the comments below and to Reviewers 1 and 2). In addition, we observed that PARylation and SIRT1-deacetylation activities decline (Fig. 1D), and inhibition of PARylation and SIRT1 deacetylation activities had little impact on the NAD levels (Appendix Fig. 2C) in DC fibroblasts. Furthermore, another NADase, SARM1 level is unaltered (Fig. 2A) in DC fibroblasts. Thus, these data support that elevated CD38 NADase, but not PARPs and Sirtuins contribute to NAD dysregulation in DC cells.

2. This reviewer requested telomere length measurements in DC and G1/G3 mice. We have published extensive telomere length characterization of these Tert KO animals and showed that critically short telomeres (or signal free ends) arise concomitantly with infertility (e.g. Erdmann et al., PNAS. 2004. 101:6080-5). G3 Tert KO mice used in this study are infertile.

We have included the telomere length measurement in DC PBMCs by Flow-FISH (Appendix Table S1) and DC fibroblasts with and without NR treatment (Fig. 6A). Figure 6A shows the telomere length measurement in the age-matched healthy control and DC fibroblasts with mock or NR treatment, by quantitative PCR using telomere specific primers, as described by Cawthon RM. Nucleic Acids Res. 2002 30:e47. We apologize that we did not describe this feature in the original text and have added the following description in the revised text "We quantitatively assessed telomere length in DC and age-matched healthy control fibroblasts by quantitative telomere PCR (Cawthon, 2002, O'Callaghan, Baack et al., 2012), under conditions in which cells were mock-treated or supplemented with NR. Compared to the control fibroblasts, DC fibroblasts possessed a significantly reduced telomere signal (Fig 6A, lanes 1 versus 5 and 9). After NR supplementation, no significant difference in telomere PCR amplification was observed between mock and NR-treatment in either DC or control fibroblasts (Fig 6A, lanes 1 versus 3, lanes 5 versus 7, and lanes 9 versus 11). Thus, NR is unable to replenish telomere loss in DC fibroblasts. This observation is not unexpected, since DC cells used in this experiment are defective in the key telomerase components, TERT and DKC1 (Appendix Table S1)". We also added P values among these comparisons in the revised Fig. 6A. Our data suggests that NR does not replenish short telomere length, although it ameliorates telomeric oxidative base lesions and DSBs in DC cells. This observation is not unexpected, because dyskeratosis congenita patients have intrinsic defects in telomere maintenance, due to germline mutations of key telomere maintenance genes (Armanios, 2013, Bertuch, 2016, Savage, 2018). However, our results support a model in which short telomeres leads to NAD metabolism deficits (Appendix Fig. S7).

Figure 1: Authors start by comparing NAD, SIRT1 and CD38 levels in DC fibroblasts and age-matched control fibroblasts. "Age matched control fibroblasts" is very vague and matters little (if at all) if different fibroblast lineages have been cultured for different population doublings, especially when referring to telomere biology. Therefore, the most important factor to take into account between the different fibroblast populations (in this study) is Population Doublings (PDL), and if the cells being analyzed begin to show early signs of senescence. Fibroblasts derived from DC patients senesce very quickly, due to telomere shortening. All the observed phenotypes in human cells in this paper could be caused by the fact that the authors are comparing normally proliferating cells (controls) against cells that are senescing (DC). Currently, as the manuscript is written, the authors seem to infer that DC fibroblasts display altered steady-state levels of NAD, SIRT1 and CD38 regardless their PDL/telomere length/senescence status, which is completely misleading. In other words: NAD, SIRT1 and CD38 levels vary simply because cells have entered senescence and/or have damaged telomeres, regardless if they are DC or control fibroblasts?

Response: We appreciate this reviewer's concern that the DC fibroblasts may already be senescent, which would impact our conclusions. We too shared this concern, which is why we went to great lengths to use early passage (P1 or P2) DC fibroblasts that do not show

proliferative defects (Fig. 7A, days 1-24). Indeed, only after prolonged culture do they enter replicative senescence (Fig 7A, >24 days). Similar results were observed in additional DC fibroblast lines (Appendix Fig. S6). We also took care to assess this issue when evaluating the effectiveness of NR supplementation, in which we compared the effects of NR addition (or vehicle control) before senescence onset. Thus, it is important to stress that our kinetic studies show that NR treated DC cells undergo a delayed entry into senescence (e.g. Fig. 7 and Appendix Fig. S6). In addition, we have now shown that decline in CD38 level via shRNA also leads to extended cell proliferation and delays replicative senescence of DC fibroblasts (Fig. 7A and Appendix Fig. S6). We also clarified these points further in the Discussion.

Figure 2: All data presented in this figure confirms well know biological phenomena: NR treatment increases NAD levels and modulates sequential downstream pathways. There is no reason to believe DC fibroblasts would act differently. Therefore, the fact that this is true also in DC fibroblasts is the absolutely expected observation.

Figure 3: Similar to figure 2. All data presented in this figure confirms well know biological phenomena: inhibition of CD38 increases NAD levels and modulates sequential downstream pathways. The fact that this is true also in DC fibroblasts is therefore absolutely expected and the authors show no relevance to the disease itself.

In conclusion, Figures 2-3 indicate no particular relevance of NAD or CD38 to DC, other than well established biochemical pathways acting in a similar manner to WT cells. The fact that the authors don't 1) show treatment of control cells 2) don't show telomere lengths, 3) don't show CD38 levels and 4) show no biological significance (will only do that in Figure 8) leads me to believe that Figures 2-3 are mostly uninformative and could be Supplemental.

Response: We thank the reviewer for the suggestion. Per the reviewer's request, we have moved the original Figure 2 to Appendix (Appendix Fig S2). In the original Figure 2, our intent was to (a) demonstrate that DC is defective in PARylation and sirtuin deacetylation activities, (b) validate the role of PARPs and sirtuins in NAD decline in DC cells, and (c) test if NR is an efficacious reagent in normalizing the NAD levels and PARP and sirtuin activities in DC fibroblasts. It is noteworthy that telomere biology disorders, such as dyskeratosis congenita have no effective treatment. Thus, the NAD mechanism-based intervention strategies developed in this study may serve as a steppingstone for the future treatment of human telomere biology disorders such as DC.

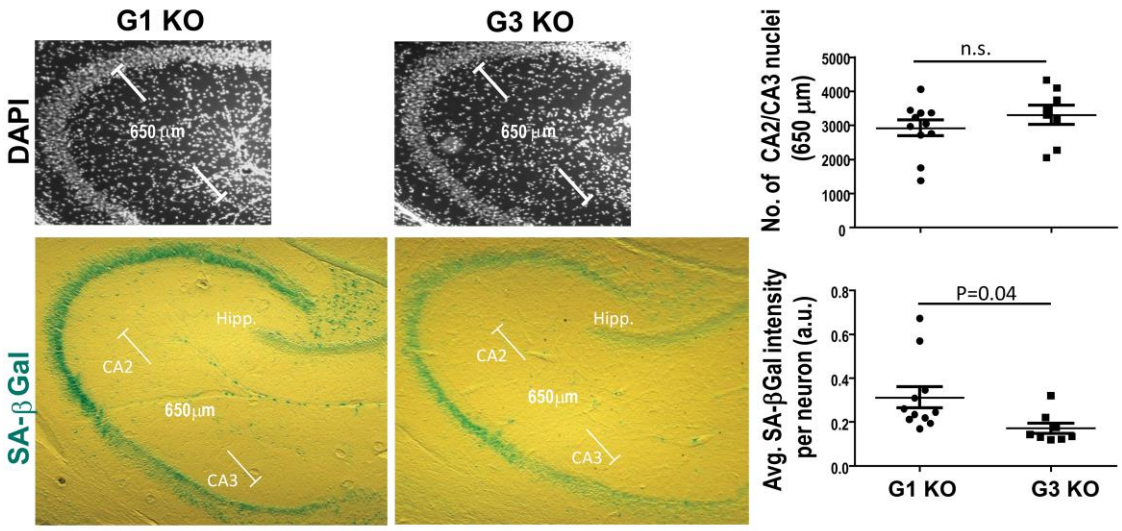
In the original Figure 3, we aimed to validate if CD38 contributes to reduced NAD levels in DC fibroblasts using a CD38 inhibitor. To strengthen the role of CD38 overexpression in NAD dysregulation and cellular dysfunction in DC cells, we have included new evidence that (a) CD38 expression is elevated in DC fibroblasts, in comparison to age-matched healthy controls (Fig. 2A), (b) decline in CD38 level via shRNA treatment delays replicative senescence (Fig. 7A and Appendix Fig S6) and cellular/mitochondrial ROS (Fig. 4D) of DC fibroblasts, and (c) CD38 knockdown ameliorates NAD dysregulation in DC cells (Fig. 2 and refer also to the response to Reviewers 1 and 2). In brief, CD38 knockdown led to a moderate increase in the

NAD levels in DC cells, but it also boosted the NAD consumption activities of PARPs and SIRT1 (Fig. 2E). Thus, accumulated NAD upon CD38 inhibition is likely consumed by PARPs and SIRT1, since PARylation and SIRT1 deacetylation activities were particularly low in DC fibroblasts (Fig 1D). In support of this notion, the NAD levels were significantly elevated in the CD38 knockdown DC fibroblasts when PARP1 and SIRT1 NAD consuming activities were inhibited (Fig. 2D). It is noteworthy that PARP1 and SIRT1 inhibitors had little impact on NAD levels in DC fibroblasts (Appendix Fig. S2C). Similarly, 78c treatment alone led to a moderate increase in the NAD levels in DC fibroblasts, but significantly enhanced the NAD levels when combined with PARP1 and SIRT1 inhibitors (Appendix Fig. S4A). Thus, these data support that elevated CD38 NADase, but not PARPs and Sirtuins contribute to NAD dysregulation in DC cells.

Figure 4: The authors should compare telomere lengths in their G1 and G3 mice (in the same tissues analyzed for NAD concentration). Also please show beta-gal staining in these same tissues, so that we can be sure the figure is not comparing a normal tissue to a tissue that already shows signs of senescence. The conclusion (from the authors) of Figure 4 is "Collectively our results suggest that CD38 is a major NAD consuming enzyme that contributes to lower NAD levels and altered NAD signaling in human and mouse models with critically short telomeres". But again, the authors don't show any telomere length data, no CD38 levels and don't treat control groups (or analyze WT mice). Therefore, as is, this figure only confirms what is widely know about NAD biology, irrespective of telomere status.

Another interesting addition would be treatment of G5 mice with both compounds and analysis of tissue viability (G3 mice are usually "normal", G5 show severe deficit, particularly hematopoietic).

Response: We agree with the reviewer's suggestion and performed beta-gal staining in G1 and G3 *Tert* null mouse brain tissues. We did not find significant increase in beta-gal staining in G3 *Tert* null brain tissues, in comparison to G1 null brain tissues (see the results below).



SA-βGal activity (turquoise) and number of nuclei (DAPI) in hippocampal CA2-CA3 neurons in TERT G1 and G3 KO mice. The intensity of SA-βGal staining was analyzed by image J. n=3.

We have shown that late generation telomerase null mice display elevated CD38 levels and NADase activity (Fig. 3B-E). This reviewer requested telomere length measurements in G1/G3 mice. We have published extensive telomere length characterization of early and late generation Tert KO mice, and showed that telomeres shorten in successive generations and that critically short telomeres (signal free ends) arise concomitantly with infertility at the late generation (e.g. Liu et al, Curr Biol. 2000 10:1459-62; Erdmann et al., PNAS. 2004 101:6080-5). In brief, *Tert* heterozygous mice have been maintained via wild type and heterozygous mating and have an intermediate telomere length (Erdmann et al., PNAS 2004). *Tert* heterozygous mice were bred to produce *Tert*$^{-/-}$ mice. The *Tert*$^{-/-}$ mice were designated as generation 1 (G1), and the progeny of G1 cousins defined as G2, and so on. G1 *Tert*$^{-/-}$ mice have no telomerase activity but preserve telomere lengths that function in capping chromosome ends. Only in later generations (i.e. G3 in our mouse breeding colony), telomerase null mice have critically short telomeres that induce a DNA damage response in germline tissues, resulting in infertile (Liu et al Current Biology, 2000 and Erdmann et al., PNAS, 2004). Thus, to verify our observation that increased CD38 expression is driven by critically short telomeres in human telomere biology disease, we selectively compared G1 and G3 *Tert*$^{-/-}$ mice, i.e. an isogenic comparison, in which telomeres are long and functional (G1) or short and dysfunctional (G3). WT was not included in the comparison, because preserved telomerase activity may impact the studies.

This manuscript focuses on deciphering NAD metabolism and intervention in human telomere biology disease. We appreciate the reviewer's valuable suggestions. A recent report demonstrated that NR supplementation improves tissue function in late generation telomerase null mice (Amano H, *Cell Metab* 29: 1274-1290 e9, 2019). We therefore plan to focus on evaluation of functional interaction between telomeres and CD38 in mice. We would like to share that we have begun to generate double knockout mice for Tert and CD38, which will increase our confidence in determining the impact of CD38 on disease-related phenotypes, including hematopoietic failure, due to telomere dysfunction. The breeding will require another 1-2 years to achieve critically short telomere lengths in the CD38 background. We hope you will

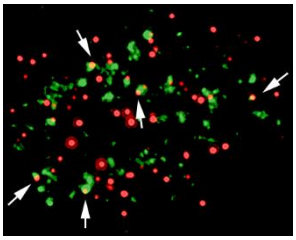
appreciate these are long-term experiments, and as such we feel they are outside the scope of what could be accomplished within a timely revision period.

Figure 5/6: why is it that data is shown only for DC1 and DC3 samples on Figure 5E-G? The differences seem minimal. Are there any physiological improvement with NR treatment in terms of mitochondria function? The authors should perform a Seahorse analysis. I am confused to what the authors consider "mitophagy dye? Figure 6 seems to show mitochondria and lysosome co-staining most of the times, even in the control cells, which is most likely a methodological issue. This should be corrected. Please provide better images, with higher magnification, allowing for the specific visualization of the mitochondria and lysosome dyes.

Response: We thank the reviewer for the suggestion. Per the reviewer's request, we added the ultra-thin sectioning and transmission electron microscopic analysis from additional DC lines (Appendix Fig. S5D). NR slightly improves mitochondria morphology. Nevertheless, NR significantly reduces mitochondrial and cellular ROS (Fig. 4B and C). In all experiments, DC cells are cultivated in 3% oxygen conditions. Because DC cells have increased ROS levels and grow poorly in normoxic conditions, the oxygen consumption rate in DC cells by Seahorse was inconsistent among experiments. We included this statement in the text.

Per the reviewer's request, we have revised the original Fig. 6 (now Fig. 5) with higher magnification. The colocalization of Mitophagy dye and Lyso dye is indicative of mitophagy events that normally occurs in age-matched healthy controls (vehicle), but significantly reduced in DC cells (vehicle). Only with NR treatment (NR), mitophagy events are significantly increased in DC cells. Thus, our results demonstrate that DC cells have reduced mitophagy, which is amendable by NR. We have clarified this in the text.

Figure 7: The authors show no difference between oxidative base repair in WT vs DC cells after NR treatment, they both show improvement. Therefore, the results are not specific to DC. However, this reviewer agrees this could be potentially relevant for DC, as minimal telomere improvement could lead to improved phenotypes (which is potentially shown in the following Figure). Figures 7B/C are of extremely poor quality. These images (TIFs) need to be improved. I see at least two TIF events in NR treated cells that don't seem to have been quantified on Figure 7B.



Response: We thank the reviewer for the suggestion. We highlighted TIFs in NR-treated DC fibroblasts and replaced the TIF image in Fig. 6B. To better illustrate TIFs, we enclosed an enlarged image at right and in Appendix Fig. EV1.

Figure 8: This is the only figure that indeed is specific to DC and seems to indicate phenotypical improvement. But these results (proliferation, BRDU, beta-gal) need also to be performed with the CD38 inhibitor (78c used in the initial figures) to show, as the authors claim, that these phenotypes are caused by CD38. It would also be more convincing if the authors could show improvement in more samples (only two for some experiments).

Response: We thank the reviewer for the suggestion. Per the reviewer's request, we included population doubling analysis in DC cells treated with the CD38 shRNA and the scrambled shRNA control (Fig. 7A and Appendix Fig. S6) and included growth curves from additional DC lines (Appendix Fig. S6). We clarified these points further in the Discussion.

Thank you for your patience during our re-review of your previously submitted manuscript, which was slowed down due to both editors and referees being extremely busy in the current situation. I had sent your manuscript back to the original referees 1 and 2, as well as to a new arbitrating referee asked to evaluate the paper as well as to comment on the concerns previously raised by referee 3 and your response to them. As you will see from the reports copied below, the first two reviewers are by and large satisfied by the revisions in response to their original criticisms. However, referee 4 finds that the referee 3's concerns about telomere length measurements were not only well justified, and remain in his/her view valid despite your response in the current version.

As I find it difficult to take the paper further with this issue remaining open, I would at this point like to invite you to consider the points raised by referee 4, and come back with a tentative response letter describing how this might still be addressed/clarified. I appreciate the point made in your previous rebuttal that NR treatment would mainly alleviate downstream consequences of telomere shortening but not telomere attrition in itself, but it may help to expand on this some more, and also be important to understand why well-established standard assays such as those mentioned in the review have not been applied here, at least for particular key experiments. Finally, I would also appreciate some comments on the relevance of a study quoted by referee 1 for the present conclusions and as potential precedent.

Once you will have had time to consider the reviews and compile a response, I would be happy to schedule an informal (video) call with you to discuss possibilities for further consideration with you on the basis of your response letter.

REFEREE REPORTS

Referee #1:

The authors have responded experimentally most of my questions and the manuscript has been highly improved. The authors should include in their text and discussion reference from Tarrago cell Metabolism 2018, in the context of CD38 inhibition ameliorating Telomere associated damage. In this manuscript Tarrago demonstrating (TAFs) in aged mice can be ameliorated by treatment with the CD38i 78c.

Referee #2:

The authors adequately addressed this reviewer's comments and successfully added a new data set to their revised manuscript, providing more convincing evidence for the importance of CD38 in DC fibroblasts. As a result, this revised manuscript is now significantly improved and ready to be accepted.

Unfortunately, the authors were not able to provide in vivo evidence for the significance of CD38 in Tert-/- mice. Therefore, it would be important for the authors to add some statement regarding the limitation of their experimental approach for the in vivo significance of CD38 in telomere dysfunctional conditions, most likely to the very last paragraph in the Discussion section.

Referee #4:

In this manuscript, the authors claim that imbalance of NAD metabolism underlies phenotypes associated with telomere dysfunction. They use cells from patients suffering from dyskeratosis congenita as well as TERT-/- mice. I found this study to present interesting phenomenology, yet limited evidence for causative effect.

To me, the biggest issue is related to the lack of accurate measurement of telomere length throughout the study. PCR based analysis of telomere length is imperfect to say the least. Standard and robust assays used by the filed, including, Telomere blots, Q-FISH, and STELA, have not been exploited. This is critical simply because the authors claim that telomere dysfunction due to telomere shortening is at the core of the metabolic defect without actually showing telomere shortening. To that end, the authors need to report what is the telomere length at the time when they analyzed levels of NAD, ADP, etc. Importantly, it is critical to report the telomere length upon treatment with NR. Is the drug counteracting shortening or simply mitigating the outcome of telomere attrition?

With regards to the contribution of CD38 to the decline in NAD, it is relatively small. Knocking out CD38 will be necessary to firm up the conclusions.

Minor point:
Figure 1D and Figure 2A. are all samples run on the same gel? It is not accurate to compare levels if the controls and DC patient cells are not on the same gel.

This thoughtful and positive feedback is deeply appreciated. We can certainly address the reviewers' comments, and I've outlined below a brief summary below of our response:

Reviewer 1 requests that we further expand on the results we mentioned briefly from Tarrago et al., Cell Metabolism 2018 (on p. 11). We will do so, and we'll also further clarify that their findings do not impact the precedent for our work. Because telomere attrition during aging of wild-type inbred mice is negligible compared to the telomere erosion observed in DC human cells or Tert-/- mice, the age-associated effects they describe are unlikely to be influenced primarily by telomere dysfunction. Our results provide unique insights into the impact on NAD metabolic dysfunction in human cells with age and in other contexts were telomere integrity is compromised, and we will elaborate further on these points in the Discussion.

As suggested by Reviewer 2, we will add new text to the Discussion that stresses the important future questions regarding the precise role of CD38 in the pathology of Tert-/- animals.

Reviewer 4 requested that we perform telomere qFISH on different DC cell passages, with or without NR treatment. We agree: knowing whether NR supplementation counteracts shortening or simply mitigates the outcome of telomere attrition will be an informative result. We will conduct these experiments as soon as our Institute resumes on-site research activities, hopefully within the next 3-4 weeks. This reviewer also requested a CD38 knockout in DC cells, in addition to the chemical inhibition of CD38 and shRNA knockdown results we presented. It is a good suggestion, however I have some concerns about the feasibility and interpretation of such an experiment that I could discuss further in a follow-up call.

I look forward to hearing your thoughts on our above plans, and I would be glad to discuss them further via an informal video call at a date/time that is convenient for you.

2nd Editorial Decision 29th May 2020

Thank you again for submitting a new version of your manuscript on NAD metabolism links to telomere dysfunction to The EMBO Journal. In light of your tentative response to the re-reviews, we would be happy to consider the study further for publication, pending a final round of minor revision allowing you to particularly address the remaining concerns about telomere length measurements, and to clarify the referees' hesitations related to the significance of observed effects, as discussed.

In addition, there are also various editorial points I would ask you to incorporate at this point.

We much appreciate the thoughtful and positive feedback by all the reviewers. We feel the manuscript has been significantly strengthened by their input. We have detailed our response as follows:

Referee #1

The authors have responded experimentally to most of my questions and the manuscript has been highly improved. The authors should include in their text and discussion reference to Tarrago cell Metabolism 2018, in the context of CD38 inhibition ameliorating Telomere associated damage. In this manuscript Tarrago demonstrate (AFs in aged mice can be ameliorated by treatment with the CD38i 78c.

Response: We thank the reviewer for the suggestion. We have now included the following statement in the revised manuscript:

Page 19. “In addition, CD38 inhibition ameliorates age-related DNA damage, including telomeric loci in mice (Tarrago et al., 2018)”

Page 23. “Our finding is also in agreement with the reports showing that CD38 expression is elevated with age in mice, and is associated with an age-dependent decline of NAD, PARylation and SIRT deacetylation activities (Camacho-Pereira et al., 2016), while treatment of old mice with a CD38 inhibitor alleviates NAD levels and age-related DNA damage, including telomeric loci (Tarrago et al., 2018)”.

As a further point, although we do not discuss it in the manuscript, we note that Tarrago et al. measured TIFs in aged mice. TIFs are not a direct measurement of telomere length, but of persistently damaged/uncapped telomeres. Because telomere attrition during aging of wild-type mice is negligible compared to the telomere erosion observed in DC human cells or Tert-/- mice, the age-associated effects described by Tarrago et al. are unlikely influenced by telomere attrition. Our studies establish that the gradual telomere erosion is tightly linked to temporal changes in CD38 and NAD metabolism. Thus, it's possible that these two mechanisms additively contribute to age-associated NAD dysregulation.

Referee #2

The authors adequately addressed this reviewer's comments and successfully added a new data set to their revised manuscript, providing more convincing evidence for the importance of CD38 in DC fibroblasts. As a result, this revised manuscript is now significantly improved and ready to be accepted.

Unfortunately, the authors were not able to provide in vivo evidence for the significance of CD38 in Tert-/- mice. Therefore, it would be important for the authors to add some statement regarding the limitation of their experimental approach for the in vivo significance of CD38 in telomere dysfunctional conditions, most likely to the very last paragraph in the Discussion section.

Response: We thank the reviewer for the suggestion. We have added the following statement in the Discussion “In the future, it will be interesting to determine the *in vivo* significance of CD38 function under conditions of telomere dysfunction, for example in late generation telomerase null mice.” (pages 28)

Referee #3

In this manuscript, the authors claim that imbalance of NAD metabolism underlies phenotypes associated with telomere dysfunction. They use cells from patients suffering from dyskeratosis congenita as well as TERT-/- mice. I found this study to present interesting phenomenology, yet limited evidence for causative effect.

To me, the biggest issue is related to the lack of accurate measurement of telomere length throughout the study. PCR based analysis of telomere length is imperfect to say the least. Standard and robust assays used by the field, including, Telomere blots, Q-FISH, and STELA, have not been exploited. This is critical simply because the authors claim that telomere dysfunction due to telomere shortening is at the core of the metabolic defect without actually showing telomere shortening. To that end, the authors need to report what is the telomere length at the time when they analyzed levels of NAD, ADP, etc. Importantly, it is critical to report the telomere length upon treatment with NR. Is the drug counteracting shortening or simply mitigating the outcome of telomere attrition?

Response: We thank the reviewer for the suggestion. We agree that knowing whether NR supplementation counteracts shortening or simply mitigates the outcome of telomere attrition will be an informative result. We have now included a new figure showing telomere length measurement of different DC and control cell passages, with or without NR treatment, by telomere restriction fragment analysis (Southern blot analysis). We found that DC fibroblasts possessed a significantly reduced telomere length, in comparison to the healthy control fibroblasts (Fig 6B, lanes 1 versus 4 and 7). After two week’s NR supplementation, no significant change in telomere length is observed in either DC or control fibroblasts (Fig 6B, lanes 3 versus 2, lanes 6 versus 5, and lanes 9 versus 8).This observation is in agreement with our quantitative-telomere PCR (Figure 6A) and telomere flow cytometry with fluorescence in situ hybridization analysis (Appendix Table S1). But NR supplementation clearly mitigated telomeric DNA damage, i.e. oxidative DNA lesions and TIFs in DC fibroblasts (Fig 6A, C and D), supporting that NR improves telomere integrity of DC cells.

With regards to the contribution of CD38 to the decline in NAD, it is relatively small. Knocking out CD38 will be necessary to firm up the conclusions.

Response: This reviewer requested a CD38 knockout in DC fibroblasts, in addition to the chemical inhibition of CD38 and shRNA knockdown results we presented. It is an excellent suggestion; however, limited proliferative capacity of DC fibroblasts impedes the feasibility of such an experiment. Although the limited proliferative capacity of DC fibroblasts precluded an ability to assess the impact of CD38 knockout on NAD levels, both chemical inhibition and shRNA-mediated knockdown of CD38 led to a similar effect

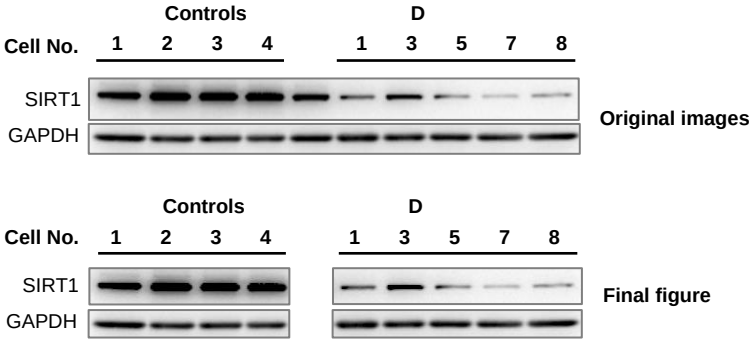
on NAD levels. We feel that the following experimental evidence supports the functional relationship between CD38 and NAD levels:

1) Although the CD38 knockdown led to only modest increases in NAD levels, this increase was greatly enhanced in CD38 knockdown cells when other NAD-consuming activities were inhibited (Fig 2D).
2) Chemical inhibition also showed the same results—i.e. a modest increase that was markedly elevated when the compensatory activities of PARP1 and SIRT1 were inhibited (Appendix Fig S4A).

Overall, the finding that two independent methods of CD38 inhibition led to similar results bolsters our conclusion that CD38 overexpression causes excessive NAD consumption, leading to a reduced NAD pool. In contrast, CD38 depletion/inhibition reduces NAD consumption; however, a portion of the “increased” NAD is likely utilized to boost NAD-dependent PARylation and SIRT1 deacetylation activities (Figure 2E), thereby resulting in the final moderate increase in NAD levels in DC cells.

Minor point:
Figure 1D and Figure 2A. are all samples run on the same gel? It is not accurate to compare levels if the controls and DC patient cells are not on the same gel.

Response: In these figures, controls and DC samples were run on the same gel, but an intervening sample was omitted as it was not relevant (see an example of original images below).



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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.

EMBO PRESS

YOU MUST COMPLETE ALL CELLS WITH A PINK BACKGROUND ↓

PLEASE NOTE THAT THIS CHECKLIST WILL BE PUBLISHED ALONGSIDE YOUR PAPER

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Journal Submitted to: EMBO J
Manuscript Number: EMBOJ-2019-103420R1

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 $\chi 2$ 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?	We did not compute statistical analysis to predetermine sample sizes prior to performing experiments. However, our sample sizes are similar to those published elsewhere. We describe sample sizes in greater detail in the figures and figure legends.
1.b. For animal studies, include a statement about sample size estimate even if no statistical methods were used.	We chose sample sizes that were comparable to the number of animals that we analyzed in previous studies on Tert-/- animals (Erdmann N, et al, Proc Natl Acad Sci U S A 101: 6080-5, 2004)
2. Describe inclusion/exclusion criteria if samples or animals were excluded from the analysis. Were the criteria pre-established?	Within each fixed sample, dead cells and mitotic cells (identified by DAPI staining) were excluded.
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.	We assured reliable and unbiased data quantification by having different investigators quantify experiments independently. Analysis were done on randomized samples and analyzed blinded. Recovery controls ensured similar recovery and replicates to ensure repeatability. Quantification was performed semi-autonomously using Quantity One (Bio-Rad) software with identical areas used to measure product formation intensities across conditions and assays.
For animal studies, include a statement about randomization even if no randomization was used.	The animals in each genotype group were randomly sampled.
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.	We assured reliable and unbiased data quantification by having different investigators quantify experiments independently. Analysis were done on randomized samples and analyzed blinded. Recovery controls ensured similar recovery and replicates to ensure repeatability. Quantification was performed semi-autonomously using Quantity One (Bio-Rad) software with identical areas used to measure product formation intensities across conditions and assays.
4.b. For animal studies, include a statement about blinding even if no blinding was done	Yes
5. For every figure, are statistical tests justified as appropriate?	Yes
Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it.	We did not assume equal variances between groups. Normal distributions were assumed for all t-tests and ANOVA.

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Is there an estimate of variation within each group of data?	Replicates were performed to ensure reproducibility wth standard deviation. Where present, bars represent standard deviartion of the mean (SD).
Is the variance similar between the groups that are being statistically compared?	For t-tests and ANOVA, we assume unequal variances between groups in general. Therefore, the similarity of variances was not assessed in these analyses.

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).	We described antibodies with required information in Appendix Supplement Methods.
7. Identify the source of cell lines and report if they were recently authenticated (e.g., by STR profiling) and tested for mycoplasma contamination.	Primary skin fibroblasts (the Coriell Cell Repository and the National Cancer Institute's Institutional Review Board approved study). A-549 (ATCC, male). Cell lines from ATCC or Coriell were authenticated by the vendor. Cells are routinely tested for mycoplasma contamination.

* 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.	Tert null mice (Liu et al., 2000, Curr Biol 10: 1459-62). CD38 null mice (Partida-Sanchez, Cockayne et al., 2001, Nat Med 7: 1209-16). Mouse tissues were collected from 6-month-old females. Mice were housed in the National Institute on Aging/NIH animal facility. Husbandry condition follows the "Guide for the Care and Use of Laboratory Animals" (National Academy Press, USA, 1996).
9. For experiments involving live vertebrates, include a statement of compliance with ethical regulations and identify the committee(s) approving the experiments.	Mouse experiments were approved by the Institutional Animal Care and Use Committee of the National Institute on Aging.
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.	Mouse experiments follows the "Guide for the Care and Use of Laboratory Animals" (National Academy Press, USA, 1996).

E- Human Subjects

11. Identify the committee(s) approving the study protocol.	National Cancer Institute's Institutional Review Board approved the study.
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.	Study participants or their legal guardian provided informed consent. The experiments conformed to the principles set out in the WMA Declaration of Helsinki and the Department of Health and Human Services Belmont Report
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.	De-identified data and samples from study participants are available after estabilishment of collaboration and material transfer agreements.
15. Report the clinical trial registration number (at ClinicalTrials.gov or equivalent), where applicable.	NCT00027274
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	The authors declare that all data supporting the findings of this study are available within the article and its Supplementary Information files are available from the authors upon request. All unique materials (e.g., Tert null mice described in this manuscript) are available upon request (contact: liuyie@mail.nih.gov).
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).	N/A
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.	No