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Cellular senescence induction leads to progressive cell death via the INK4a-RB pathway in naked mole-rats

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1st Editorial Decision **5th May 2022**

Dear Dr. Miura,

Thank you for submitting your manuscript for consideration by the EMBO Journal. It has now been seen by two referees whose comments are shown below.

Given the referees' recommendations and our prediscussion regarding the inclusion of in vivo data, I would like to invite you to submit a revised version of the manuscript, addressing the comments of all reviewers. I should add that it is EMBO Journal policy to allow only a single round of revision, and acceptance of your manuscript will therefore depend on the completeness of your responses in this revised version.

When preparing your letter of response to the referees' comments, please bear in mind that this will form part of the Review Process File, and will therefore be available online to the community. For more details on our Transparent Editorial Process, please visit our website: https://www.embopress.org/page/journal/14602075/authorguide#transparentprocess

We generally allow three months as standard revision time, however in this case we have agreed to six months. As a matter of policy, competing manuscripts published during this period will not negatively impact our assessment of the conceptual advance presented by your study. However, we request that you contact the editor as soon as possible upon publication of any related work, to discuss how to proceed. Should you foresee a problem in meeting this six-month deadline, please let us know in advance and we may be able to grant an extension. I have attached a guide for revisions for your convenience.

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

Yours sincerely,

Kelly M Anderson, PhD
Editor
The EMBO Journal
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Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

We realize that it is difficult to revise to a specific deadline. In the interest of protecting the conceptual advance provided by the work, we recommend a revision within 3 months (3rd Aug 2022). Please discuss the revision progress ahead of this time with the editor if you require more time to complete the revisions. Use the link below to submit your revision:

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

In the study, Kawamura et al. found that the naked mole-rat (NMR) fibroblasts progressively undergo cell death through activation of the INK4a-Retinoblastoma protein (RB) pathway (INK4a-RB cell death). Mechanistic studies show that NMR fibroblasts accumulate serotonin and are vulnerable to hydrogen peroxide (H_2O_2). Their findings may serve as an evolutionary rationale for using senescent cell removal as an aging resisting strategy. Totally, they found a new phenomenon for explaining the unique phenotypes for the long-lived species, but I still have many concerns on their findings.

Major:

1. In figure 1G, the average and error bars for the p21 expression in mouse and NMR seems to be the exactly same, why? However, the statistical significance is different, e.g., data in day 6, 15, 18 and 21. Original data will be helpful to clarify this serious issue.
2. For the result that "Knockdown of INK4a significantly reduced the number of dead cells in NMR fibroblasts on day 21 after DXR", did the author knockdown INK4a and then treat the cells with DXR? Or knockdown INK4a after DXR? These two ways may produce different results. I would like to see the proliferation rate after knockdown of INK4a, knockdown of a cyclin-dependent kinase inhibitor may promote the rapid growth of cells, which may cause an appearance that cells are resistant to death. If they knocked down INK4a after cellular senescence with DXR, how did they achieve to screen out the INK4a downregulated cells since cells have become senescent. The detailed experimental methods should be provided in the Methods.
3. After DXR treatment, INK4a mRNA levels increased with an increased rate of apoptosis. Although the authors later tested the

cell death was independent of p53 in the INK4a upregulated cells in Figure 2. However, since DXR is a DNA-damage inducer, in Figure 1, the cell death can not be excluded from p53 and its downstream signaling activation. Testing the P53 protein expression as well as its transcriptional targets, such as the key apoptosis- or anti-apoptosis related genes, will help to clarify whether p53 was involved in the DXR induced senescence model.
4. In figure 2H-J, the authors tested the percentages of SA-beta-Gal-positive cells in the live and dead cells, and proposed that SA-beta-Gal-positive cells were significantly enriched in the floating dead cell population of INK4a-upregulated NMR fibroblasts. I am thinking that whether there is activity of SA-beta-Gal in dead cells. Are there any supporting references for testing the SA-beta-Gal in dead cells?
5. In figure 2, it is good to see that the author tested whether activation of RB or p53 proteins is required for activation of cell death in INK4a-upregulated NMR cells using the viral oncoprotein and its derivatives, p53 knockdown or KO will better validate the results.
6. Cell culture at high density usually cause a quiescent state, which share some hallmarks, with cellular senescence, such as upregulated p16 or p21, and even increased SA-beta-Gal activity. The authors should distinguish cell senescence from cell quiescence in their study (Supplementary Fig. 3), whether the mouse or NMR cells re-grow after passaging?
7. In Figure 4B, most of the enrichments may be associated with, or can be hallmarks of cellular senescence, rather than associated with cell death. A comparison of senescent NMR cells to INK4a upregulated senescent NMR cells may help to identify the death-associated factors instead of senesce-associated factors.
8. Likewise, the ROS levels are higher in senescent cells compared to young cells. In figure 5, the authors used INK4a upregulated or DXR treated NMR cells to compare the Mock or control cells, it is expective to see the MAO protein differences. To test whether INK4a did induce the death of senescent cells, I think there should be a senescent control, i.e., based on the status of senescence, further over expressing of INK4a to see whether the monoamine oxidase was activated. Doxycycline inducing system should work.
9. Most of the results are got in vitro. INK4a expression in NMR brain tissues hardly increase during aging, how about in other major organs? Whether it is possible to do some tests to see whether the INK4a are critical for the death of senescent cell in NMR?
10. Please carefully check the data and statistical analysis. For some figures, the SD is very small, and there is a notable difference among the mean values, but there is no significance. For example, in Fig 1F (left), the INK4A mRNA levels on day 6, it seems that the level is 2.5 times higher than the control, but ns.

Minor:
1. In figure 2G, please also provide the data of cell apoptosis after overexpressing INK4a.
2. There are some spelling or grammatical mistakes, please go through the manuscript and correct them. e.g., page 3, line 51; page 4, line 62; Figure 3c, SA-b-Gal should be SA-β-Gal...
3.Please keep consistent when using the error bars, upper and down.
4. In addition, different cells may depend on different nutrients or supplements in the medium for their survival. I am thinking that whether the medium is the optimal for the growth of NMR cells. If not, cell death may appear after long-term culturing.

Referee #3:

In this manuscript, the authors examine the potential resistance to cellular senescence in the long-lived naked mole rat (NMR). They demonstrate in vitro that upon induction of cellular senescence, NMR fibroblasts undergo cell death rather than remaining senescent and then causing tissue damage due to production of the SASP. They demonstrate that the cell death is due to the INK-4a-RB cell death pathway and does not require p53. A really novel finding of the study, arrived at through transcriptomic and metabolomic analyses, is that serotonin appears to be involved in the induction of cell death through its conversion to 5-HIAA, which generates peroxide.

Overall, these are interesting and important studies that provide, as the authors suggest, a "natural senolysis" model. The in vitro studies are done well and I have some minor comments on that for the authors to address.

The significant deficiency in the paper, however, is the lack of any in vivo data to support the authors conclusions. For example, does treatment of NMRs with an MAO inhibitor (either aged, or perhaps following injury) lead to an accumulation of senescent cells, and is this absent in a comparable mouse? This type of study would be a critical in vivo test of the model proposed in Figure 6 and should be provided.

Additional comments:

1. For the INK4a transductions, were species-specific sequences used? If not, is there evidence that mouse INK4a works in NMRs (or vice versa)?
2. Throughout, there is no mention of the sex of the animals used to derives cells. Did that make a difference in the results?

31 March 2023

Manuscript EMBOJ-2022-111133

Yoshimi Kawamura et al.

"Cellular senescence leads to progressive cell death via the INK4a-RB pathway in naked mole-rats"

Point-by-point responses to issues raised by the referees

REFEREES' COMMENTS:

Referee #1:

We appreciate the constructive comments by Referee #1. The comments have enabled us to perform additional experiments, the results of which supported and significantly improved the manuscript.

Major comments

1. In figure 1G, the average and error bars for the p21 expression in mouse and NMR seems to be the exactly same, why? However, the statistical significance is different, e.g., data in day 6, 15, 18 and 21. Original data will be helpful to clarify this serious issue.

(Response) We sincerely apologize for our error in misplacing the graph in Fig. 1G and thank Referee #1 for bringing it to our attention. The same graph for mice was mistakenly shown as NMR. We have replaced it with the correct one. The original data values for both mouse and NMR are shown below. Statistical analysis of these data was performed using Prism7 software with one-way ANOVA followed by Dunnett's multiple comparison test.

Mouse *p21*	Ctrl	3d	6d	9d	12d	15d	18d	21d
Mouse 1	1.000	5.363	3.412	4.861	4.978	5.636	5.009	5.259
Mouse 2	0.894	5.352	4.902	6.290	7.065	4.411	7.221	6.566
Mouse 3	1.030	4.679	3.632	4.882	6.112	4.073	5.869	5.303

NMR *p21*	Ctrl	3d	6d	9d	12d	15d	18d	21d
NMR 1	1.0000	7.0779	4.9270	5.7662	5.3560	5.3655	4.5618	4.2629
NMR 2	0.8719	5.3014	4.3052	7.5710	6.5380	5.9275	4.6699	4.7296
NMR 3	0.8861	5.5696	4.6530	5.1681	4.1793	4.4770	3.7698	3.5657

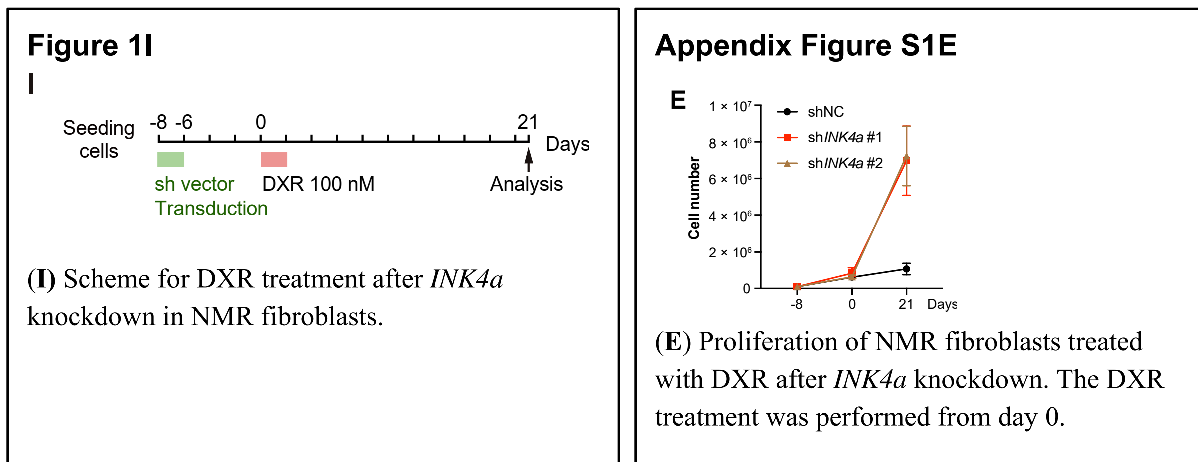
2. For the result that "Knockdown of INK4a significantly reduced the number of dead cells in NMR fibroblasts on day 21 after DXR", did the author knockdown INK4a and then treat the cells with DXR? Or knockdown INK4a after DXR? These two ways may produce different results. I would like to see the proliferation rate after knockdown of INK4a, knockdown of a cyclin-dependent kinase inhibitor may promote the rapid growth of cells, which may cause an appearance that cells are resistant to death. If they knocked down INK4a after cellular senescence with DXR, how did they achieve to screen out the INK4a downregulated cells since cells have become senescent. The detailed experimental methods should be provided in the Methods.

(Response) We apologize for the insufficient description in the original submission. In the previous manuscript, we performed *INK4a* knockdown and then treated NMR fibroblasts with DXR (Fig 1I). As noted by Referee #1, *INK4a* knockdown significantly decreased cell death and increased cell number (Appendix Fig S1E), suggesting that *INK4a* is required for cell cycle arrest and the resulting cell death after DXR treatment. In response to the referee's suggestion, we performed an additional experiment in which cellular senescence was induced in NMR fibroblasts by DXR treatment, after which the cells were transduced with the sh*INK4a* vector (Appendix Fig S1A). We confirmed a high transduction efficiency, as almost all cells were positive for GFP (Fig. R1 in this letter). We found that *INK4a* knockdown significantly reduced cell death and increased cell number (Appendix Fig S1B–D), suggesting that *INK4a* knockdown may allow cells in an early senescent state to re-enter the cell cycle (Childs *et al*, 2015), thereby evading cell death.

In NMR fibroblasts treated with DXR, cell cycle arrest was observed on day 3, along with an increased trend of *INK4a* expression and SA-β-Gal activity (Fig 1D–F). However, cell death was not activated at this time point. Delayed and progressive cell death was subsequently observed and became significant at day 12, together with *INK4a* upregulation (Fig 1H). On the other hand, experimentally increasing *INK4a* expression in senescent cells by transducing cells with *INK4a* after DXR treatment or by using different vectors with different expression levels of *INK4a* did not significantly affect the rate of cell death (Appendix Fig S6A–C, F–I). Thus, we

conclude that delayed and progressive cell death is not solely dependent on *INK4a* levels but rather on changes that occur after INK4a-RB activation, the subsequent cell cycle arrest, and the resulting cellular senescence.

We have added the new data and the description in the Results, Discussion, and Methods sections (Fig. 1I, Appendix Fig S1A–E, Appendix Fig S6A–C, F–I, lines 132–143 and 285–292 in Results, lines 347–352 in Discussion, and lines 548–556 in Methods).



(**I**) Scheme for DXR treatment after *INK4a* knockdown in NMR fibroblasts.

(**E**) Proliferation of NMR fibroblasts treated with DXR after *INK4a* knockdown. The DXR treatment was performed from day 0.

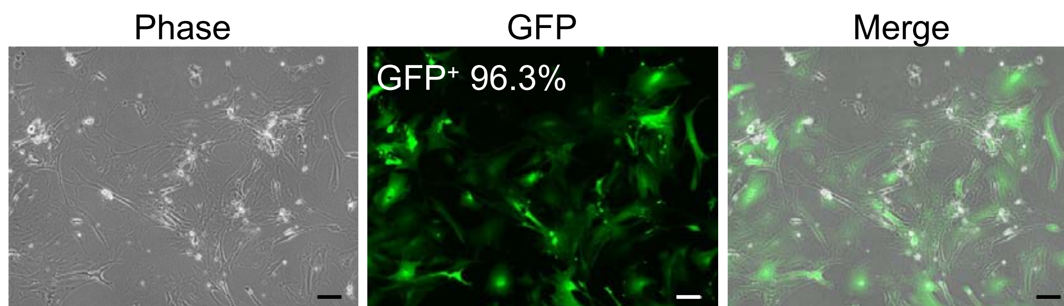
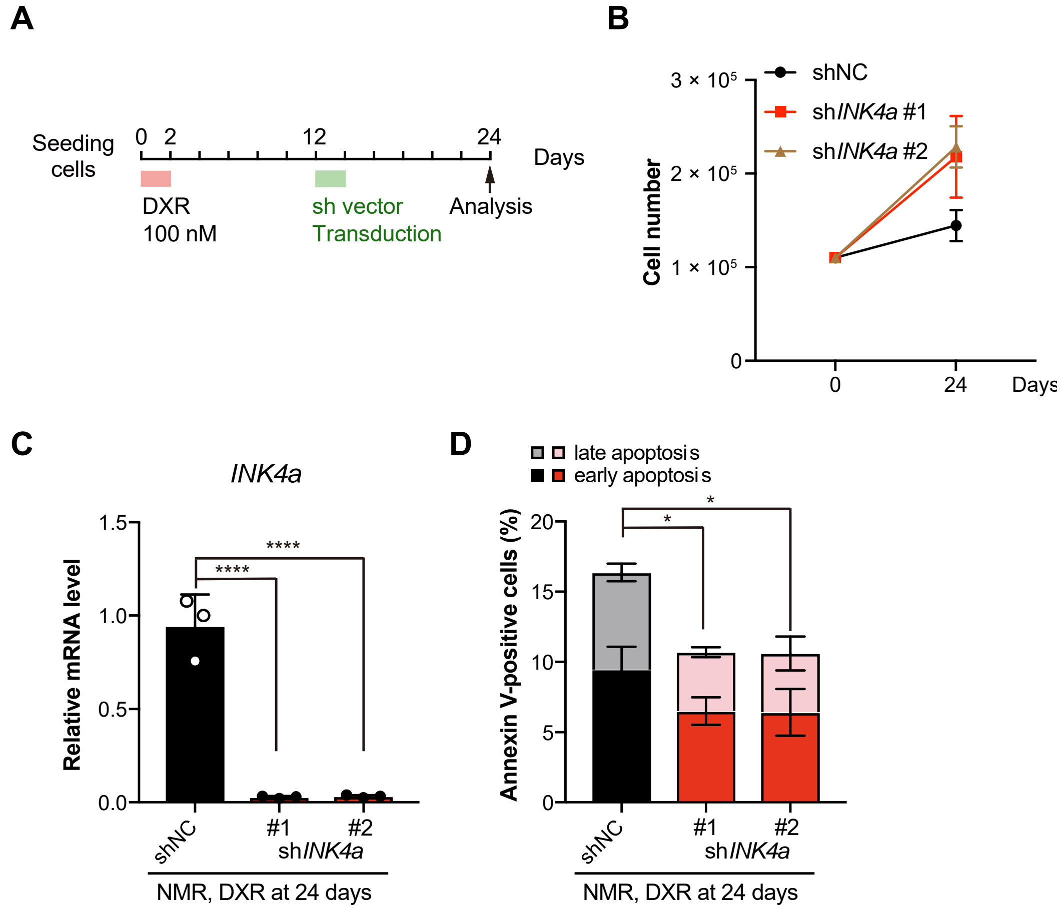


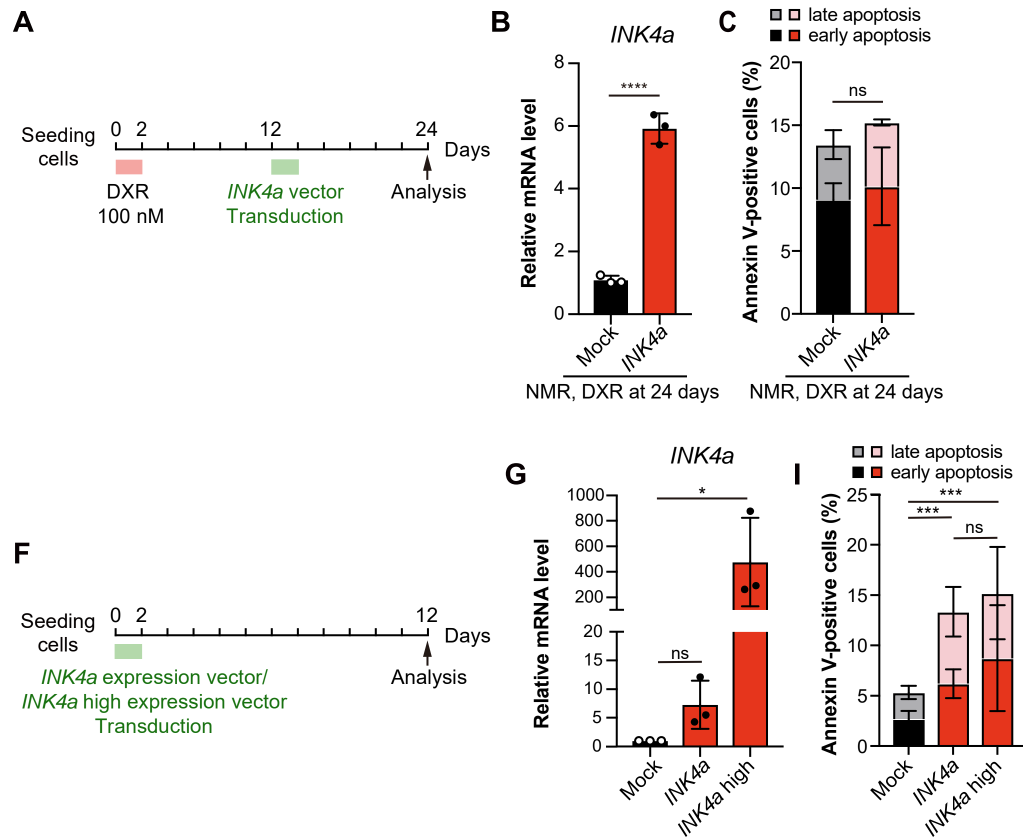
Fig R1. Transduction efficiency of sh*INK4a*-GFP vectors into NMR cells (n = 3). Scale bar, 100 μm.

Appendix Figure S1A–D



(**A**) Scheme for doxorubicin (DXR) treatment before *INK4a* knockdown.
(**B**) Proliferation of NMR fibroblasts treated with DXR before *INK4a* knockdown.
(**C**, **D**) qRT-PCR analysis of the expression of *INK4a* normalized to *ACTB* mRNA levels (**C**) and quantification of Annexin V-positive cells (%) (Annexin V+/PI– as early apoptotic and Annexin V+/PI+ double-positive as late apoptotic) (**D**) in NMR fibroblasts treated with DXR before *INK4a* knockdown.

Appendix Figure S6A–C, F, G, I



(**A**) Scheme for *INK4a* overexpression after doxorubicin (DXR) treatment in NMR fibroblasts.
(**B**–**C**) qRT-PCR analysis of the expression of *INK4a* normalized to *ACTB* mRNA levels (**B**), quantification of Annexin V-positive cells (%) (Annexin V+/PI– as early apoptotic and Annexin V+/PI+ double-positive as late apoptotic) (**C**) in DXR-treated NMR fibroblasts transduced with *INK4a*.
(**F**) Scheme for *INK4a* overexpression in NMR fibroblasts using vectors with different expression levels.
(**G**) qRT-PCR analysis of the expression of *INK4a* normalized to *ACTB* mRNA levels at 12 days after *INK4a* transduction.
(**I**) Quantification of Annexin V-positive cells (%) (Annexin V+/PI– as early apoptotic and Annexin V+/PI+ double-positive as late apoptotic) at 12 days after *INK4a* transduction.
* $P < 0.05$; *** $P < 0.001$; **** $P < 0.0001$; ns, not significant. Unpaired t-test versus control for (**B**, **C** and **D**). Data are expressed as the mean ± SD from $n = 3$ biological replicates. One-way ANOVA followed by Dunnett's multiple comparison test for (**G**).One-way ANOVA followed by Sidak's multiple comparisons test for (**I**).

3. After DXR treatment, INK4a mRNA levels increased with an increased rate of apoptosis. Although the authors later tested the cell death was independent of p53 in the INK4a upregulated cells in Figure 2. However, since DXR is a DNA-damage inducer, in Figure 1, the cell death can not be excluded from p53 and its downstream signaling activation. Testing the P53 protein expression as well as its transcriptional targets, such as the key apoptosis- or anti-apoptosis related genes, will help to clarify whether p53 was involved in the DXR induced senescence model.

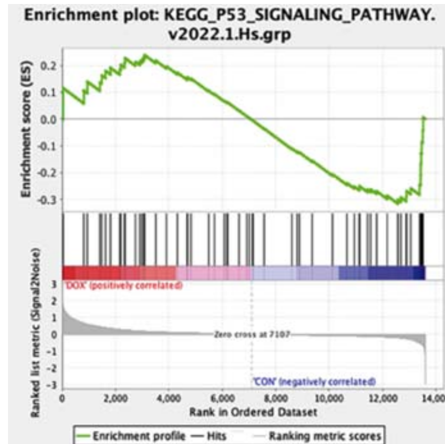
(Response) Thank you for your valuable comment. We performed mRNA sequencing and analyzed p53 and its target genes to determine whether p53 is activated in DXR-treated NMR fibroblasts that exhibit delayed and progressive cell death. The results showed that genes related to the KEGG pathway "p53 signaling pathway" were not enriched among the differentially expressed genes in DXR-treated NMR cells on day 21 (Appendix Fig S3A). Furthermore, the expression of p53 target genes related to apoptosis (Fischer, 2017) did not show consistent changes in DXR-treated NMR cells (Appendix Fig S3B).

Consistently, Western blot analysis showed that the p53 protein level was not significantly altered in DXR-treated NMR cells, in contrast to NMR cells that were induced to undergo acute cell death by high concentrations of etoposide (Appendix Fig S3C). These new data support that the activation of delayed and progressive cell death in NMR fibroblasts after senescence induction by low concentrations of DXR is independent of p53 activity.

We have added the new data (Appendix Fig S3A–C) and the description in the Results and Discussion sections (lines 189–200 in Results, lines 356–359 in Discussion).

Appendix Figure S3A–C

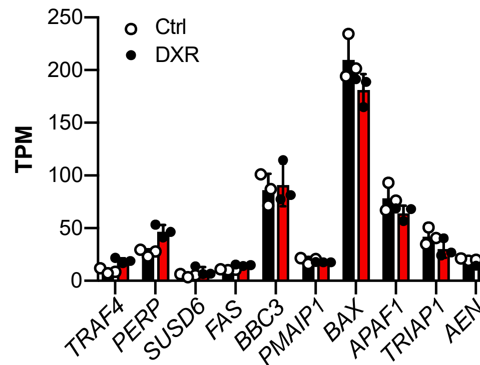
A



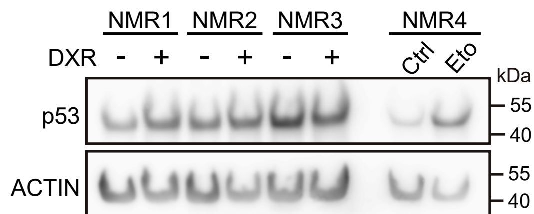
Enrichment Score = -0.316

Nominal p-value = 0.1875

B



C



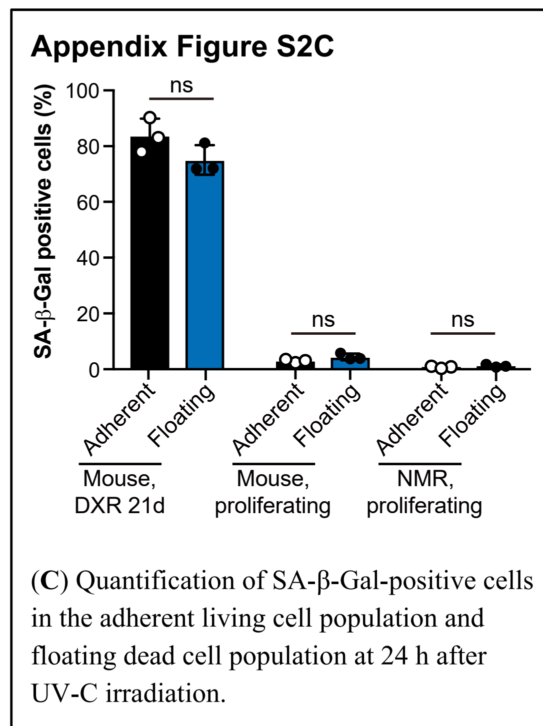
(**A**) Gene set enrichment analysis (GSEA) plot depicting p53 signaling pathway genes in control (Ctrl) versus NMR fibroblasts 21 days after doxorubicin (DXR) treatment.

(**B**) Expression levels of p53 target genes (transcripts per million, TPM) in Ctrl and NMR fibroblasts 21 days after DXR treatment.

(**C**) Western blot analysis of p53 in NMR fibroblasts 21 days after DXR treatment or 4 days after 200 µM etoposide (Eto) treatment. ACTIN was used as a loading control.

Data were obtained from n = 3 biological replicates.

4. In figure 2H-J, the authors tested the percentages of SA-beta-Gal-positive cells in the live and dead cells, and proposed that SA-beta-Gal-positive cells were significantly enriched in the floating dead cell population of INK4a-upregulated NMR fibroblasts. I am thinking that whether there is activity of SA-beta-Gal in dead cells. Are there any supporting references for testing the SA-beta-Gal in dead cells?



(**C**) Quantification of SA-β-Gal-positive cells in the adherent living cell population and floating dead cell population at 24 h after UV-C irradiation.

(Response) Because we could not find any evidence that SA-β-Gal activity is retained in dead cells, we performed additional experiments to determine whether killed mouse senescent cells retain SA-β-Gal activity by performing additional experiments. DXR-treated, mouse senescent cells were induced to undergo acute cell death by intense UV-C irradiation. The percentage of SA-β-Gal-positive cells in the resulting floating dead cell population at 24 hours after UV-C irradiation was comparable to that in the living senescent cells (Appendix Fig S2C). In contrast to the retention of SA-β-Gal activity in the floating dead cell population of *INK4a*-upregulated NMR fibroblasts (Fig 2J), non-senescent, proliferating NMR cells experimentally killed by intense UV-C did not show increased SA-β-Gal activity (Appendix Fig S2C). Similarly, proliferating mouse cells also did not show increased SA-β-Gal activity upon experimental killing by intense UV-C. These results suggest that dead cells retain SA-β-Gal activity according to their senescent state. We have added the new data and the description in the Results section (Appendix Fig S2C, lines 160–163).

5. In figure 2, it is good to see that the author tested whether activation of RB or p53 proteins is required for activation of cell death in INK4a-upregulated NMR cells using the viral oncoprotein and its derivatives, p53 knockdown or KO will better validate the results.

(Response) Based on the reviewer's suggestion, we constructed seven *p53* shRNA vectors to knock down *p53* in NMR fibroblasts; however, all vectors failed. In previous experiments, we successfully knocked down other genes using the same lentiviral knockdown system (Fig 1J, Appendix Fig S1C, Fig EV1H) (Miyawaki *et al*, 2016). Therefore, problems with the NMR *p53* sequence or a putative negative feedback mechanism may make knockdown difficult.

To induce cellular senescence, it is necessary to use primary fibroblasts at young passages. However, performing knockout with CRISPR/Cas9 requires long-term cell culture to select successfully knocked-out cells, which would alter the cellular characteristics and make them unsuitable for cellular senescence experiments. Therefore, we were unable to utilize the CRISPR/Cas9 system.

In previous studies, LTK1 is used to suppress p53 activity in NMR cells (Seluanov *et al*, 2009). In this study, we confirmed that the expression of the downstream gene *p21* was significantly suppressed in NMR cells transduced with *LTK1* (Fig. R2 in this letter), indicating adequate suppression of p53 activity. *LTK1*-transduced NMR fibroblasts did not show any change in cell death after *INK4a* transduction or DXR treatment (Fig 3D and G). These results, together with the mRNA-seq and Western blot analyses performed in response to Referee #1's Major Comment 3 (Appendix Fig S3A–C), which showed no significant activation of the p53 protein and the p53 downstream genes in DXR-treated cells, indicate that activation of the INK4a-RB pathway contributes to the delayed, progressive cell death of NMR cells after senescence induction by DXR treatment or *INK4a* transduction and is independent of p53 activity.

We have included the new data and description in the Results and Discussion sections (Appendix Fig S3A–C, lines 189–200 in Results, lines 356–359 in Discussion).

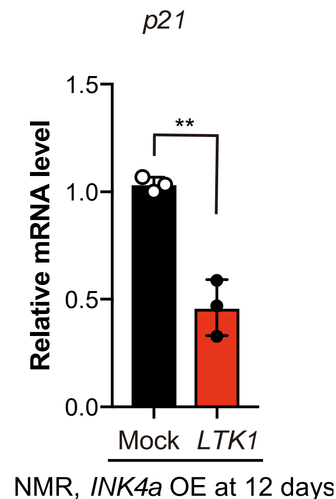


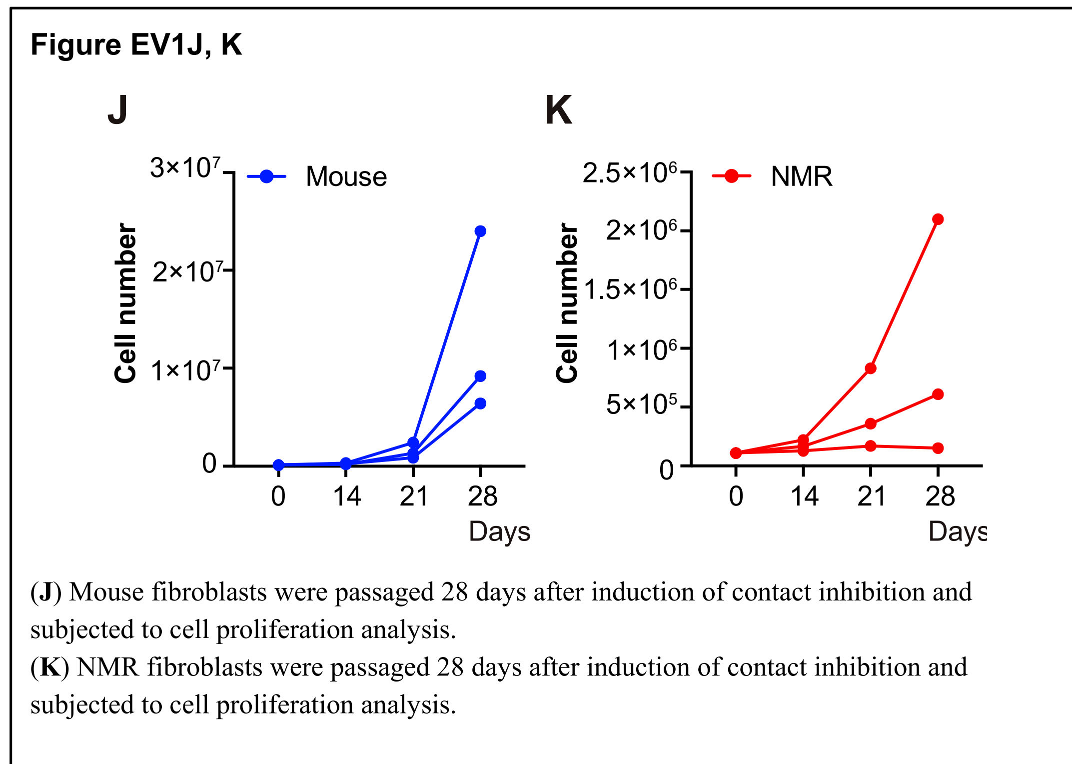
Fig. R2. qRT-PCR analysis of the expression of *p21* normalized to *ACTB* mRNA in NMR-fibroblasts transduced with *LTK1*- and *INK4a*-transduced NMR fibroblasts. ** $P < 0.01$; unpaired *t*-test versus control. Data are expressed as the mean ± SD of $n = 3$ biological replicates.

6. Cell culture at high density usually cause a quiescent state, which share some hallmarks, with cellular senescence, such as upregulated p16 or p21, and even increased SA-beta-Gal activity. The authors should distinguish cell senescence from cell quiescence in their study (Supplementary Fig. 3), whether the mouse or NMR cells re-grow after passaging?

(Response) Following the suggestion of Referee #1, we investigated whether mouse and NMR cells are in a quiescent or senescent state when cultured long-term after induction of contact inhibition. We passaged mouse and NMR fibroblasts at 28 days after induction of contact inhibition (Fig EV1A). Fig EV1J and K shows that the three primary mouse fibroblast cultures and two of three primary NMR fibroblast cultures resumed proliferation, whereas one NMR fibroblast culture did not. These results suggest that NMR fibroblasts contain both senescent and quiescent cells when cultured long-term after induction of contact inhibition. Taken together, the results of DXR treatment and long-term culture after contact inhibition suggest that NMR fibroblasts

activate INK4a-RB cell death in response to stimuli that upregulate *INK4a* and senescence and, in some cases, quiescence.

We have added the new data and the description in the Results and Discussion section (Fig EV1J and K, lines 214–224 in Results, and line 339–341 in Discussion).



(**J**) Mouse fibroblasts were passaged 28 days after induction of contact inhibition and subjected to cell proliferation analysis.
(**K**) NMR fibroblasts were passaged 28 days after induction of contact inhibition and subjected to cell proliferation analysis.

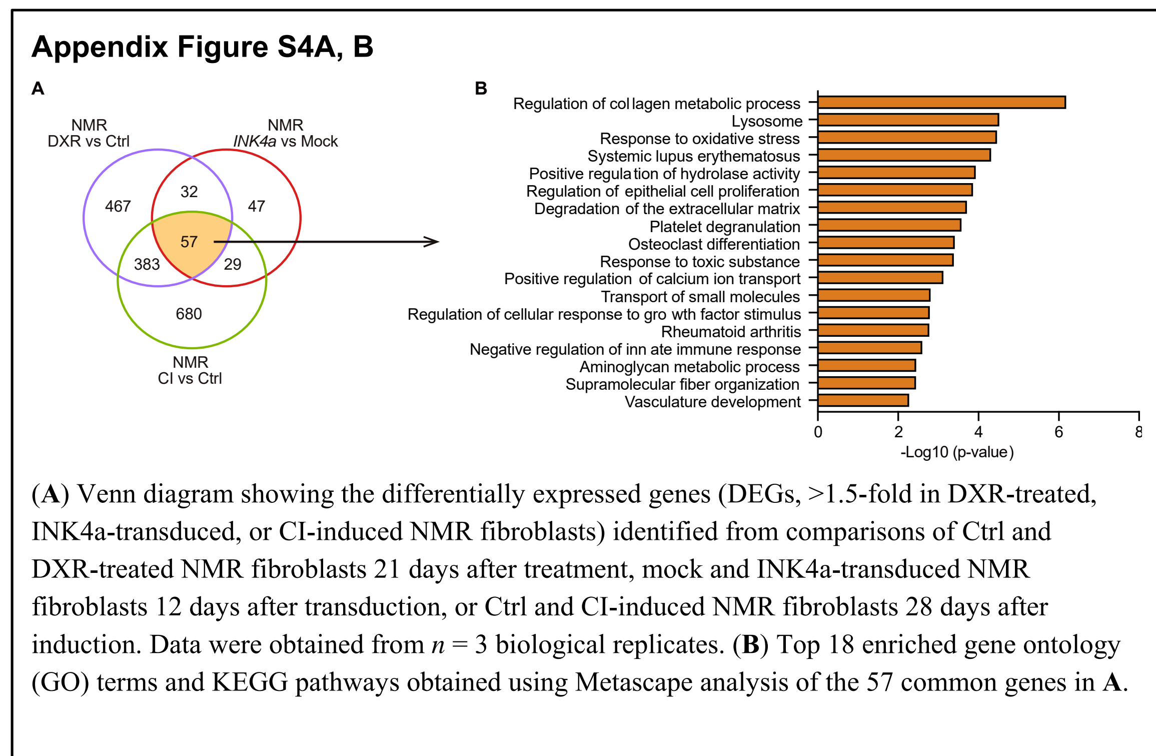
7. In Figure 4B, most of the enrichments may be associated with, or can be hallmarks of cellular senescence, rather than associated with cell death. A comparison of senescent NMR cells to INK4a upregulated senescent NMR cells may help to identify the death-associated factors instead of senesce-associated factors.

(Response) As mentioned by Referee #1, many senescence-related terms were observed in the RNA-seq data of *INK4a*-transduced NMR cells (Fig 4B). Although the term "positive regulation of cell death" was also enriched, we could not find any genes directly related to cell death including apoptosis, although we observed an increase in annexin V-positive apoptotic cells after *INK4a* transduction (Table EV2, Fig 2G). Therefore, we focused on the "hydrogen peroxide metabolic process" because NMRs

are known to be highly vulnerable to hydrogen peroxide (Salmon *et al*, 2008), making this pathway a promising candidate as the cause of cell death.

As they become senescent, NMR cells exhibit activation of INK4a-RB cell death, although further increases in *INK4a* expression do not affect cell death (Appendix Fig S6C, I). Therefore, we anticipated that it would be difficult to isolate factors related to cell death by comparing senescent NMR cells to those with upregulated *INK4a* expression. Thus, we tried to identify cell death-related genes contributing to INK4a-RB cell death by comparing differentially expressed genes among 1) NMR fibroblasts treated with DXR, 2) NMR fibroblasts cultured long-term after induction of contact inhibition, and 3) NMR fibroblasts transduced with *INK4a* (Appendix Fig S4A) (all of these treatments upregulate *INK4a* and activate a delayed, progressive cell death). We focused on the differentially expressed genes common to these three groups. However, we did not observe a significant enrichment of cell death-related genes (Appendix Fig S4B, Table EV1). Therefore, further analysis is needed to identify death-inducing factors that contribute to INK4a-RB cell death in NMR cells, such as p53-independent apoptosis-related proteins.

We have added the new data and the description in the Results and Discussion sections (Appendix Fig S4, Table EV1, lines 228–235 in Results, and line 354–359 in Discussion).



(**A**) Venn diagram showing the differentially expressed genes (DEGs, >1.5-fold in DXR-treated, INK4a-transduced, or CI-induced NMR fibroblasts) identified from comparisons of Ctrl and DXR-treated NMR fibroblasts 21 days after treatment, mock and INK4a-transduced NMR fibroblasts 12 days after transduction, or Ctrl and CI-induced NMR fibroblasts 28 days after induction. Data were obtained from $n = 3$ biological replicates. (**B**) Top 18 enriched gene ontology (GO) terms and KEGG pathways obtained using Metascape analysis of the 57 common genes in **A**.

8. Likewise, the ROS levels are higher in senescent cells compared to young cells. In figure 5, the authors used INK4a upregulated or DXR treated NMR cells to compare the Mock or control cells, it is expective to see the MAO protein differences. To test whether INK4a did induce the death of senescent cells, I think there should be a senescent control, i.e., based on the status of senescence, further over expressing of INK4a to see whether the monoamine oxidase was activated. Doxycycline inducing system should work.

(Response) Based on the suggestion of Referee #1, we transduced *INK4a* overexpression into DXR-treated NMR cells (note that DXR-treated NMR cells upregulate endogenous *INK4a* and activate INK4a-RB cell death) to test whether additional INK4a overexpression would further increase MAO protein levels in senescent NMR cells (Appendix Fig S6A). Transduction of *INK4a* into DXR-treated NMR cells did not further increase MAO levels, ROS levels, or cell death (Appendix Fig S6B–E). These results support our conclusion that exceeding a certain threshold level of *INK4a* upregulation is sufficient to induce subsequent cellular changes that lead to progressive activation of INK4a-RB cell death. In contrast, we found that in *INK4a*-transduced mouse cells or DXR-treated mouse cells, MAO proteins were not increased (Fig 5D and G), indicating that the increase in MAO levels after INK4a-RB activation is likely unique to NMRs.

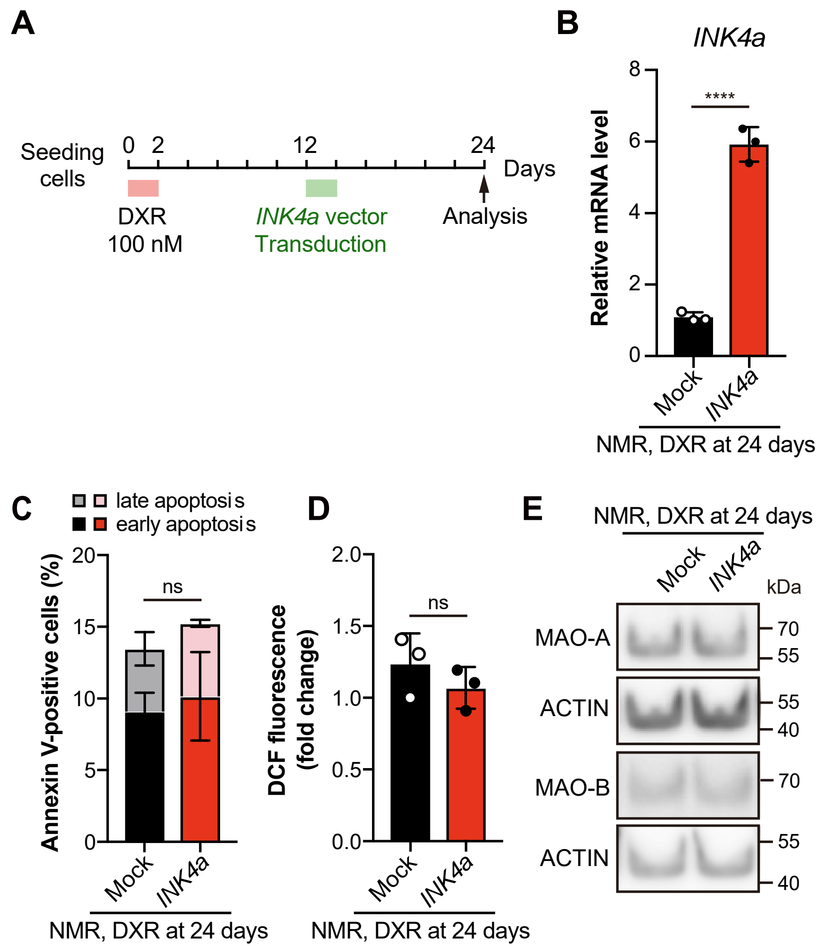
NMR cells have low mitochondrial activity, high mitochondrial ROS scavenging activity, and are susceptible to increased H_2O_2 (Lau *et al*, 2020; Munro *et al*, 2019; Salmon *et al*, 2008). In NMR cells, MAO protein levels are low in the proliferating state, while serotonin levels are high, suggesting that H_2O_2-generating serotonin metabolism is suppressed under normal conditions (Fig 4D, 5D and G). After *INK4a* upregulation and the resulting cellular changes, including senescence, NMR cells increased MAOs and activated serotonin metabolism. Thus, the serotonin metabolic switch that produces large amounts of H_2O_2 after INK4a-RB activation, is likely unique to NMR cells and induces INK4a-RB cell death in concert with the inherent vulnerability to H_2O_2.

As noted by Referee #1, senescent cells in humans and mice generally exhibit elevated levels of ROS, mostly due to mitochondrial dysfunction but do not activate cell

death (Childs *et al*, 2014). Although the role of MAOs in INK4a-RB cell death in NMR cells is evident, future analyses are needed to investigate how NMR cell senescence affects mitochondrial ROS dynamics.

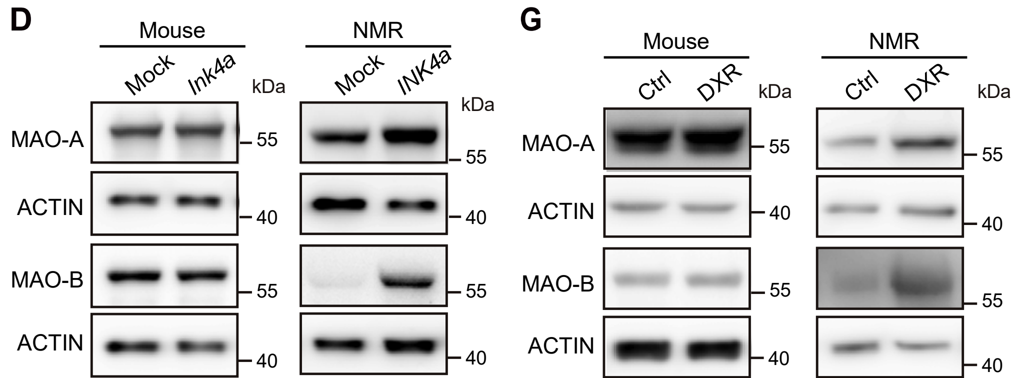
We appreciate the valuable feedback from Referee #1, which allowed us to include new data and deepen our discussion. We have added the new data and the description in the Results and Discussion sections (Fig 5D, G, Appendix Fig S6A–E, lines 266–268, 280–281, 285–292 in Results, 363–368, 381–388 in Discussion).

Appendix Figure S6A–E



(**A**) Scheme for *INK4a* overexpression after doxorubicin (DXR) treatment in NMR fibroblasts. (**B**–**D**) qRT-PCR analysis of the expression of *INK4a* normalized to *ACTB* mRNA levels (**B**), quantification of Annexin V-positive cells (%) (Annexin V+/PI– as early apoptotic and Annexin V+/PI+ double-positive as late apoptotic) (**C**), and quantification of reactive oxygen species (ROS) using 2',7'-dihydrodichlorofluorescin diacetate (DCFH-DA) (**D**) in DXR-treated NMR fibroblasts transduced with INK4a.
(**E**) Western blot analysis of monoamine oxidase (MAO)-A and MAO-B in NMR fibroblasts at 24 days after DXR treatment. ACTIN was used as a loading control. **** $P < 0.0001$; ns, not significant. Unpaired *t*-test versus control for (**B**, **C** and **D**). Data are expressed as the mean ± SD from $n = 3$ biological replicates.

Figure 5D, G

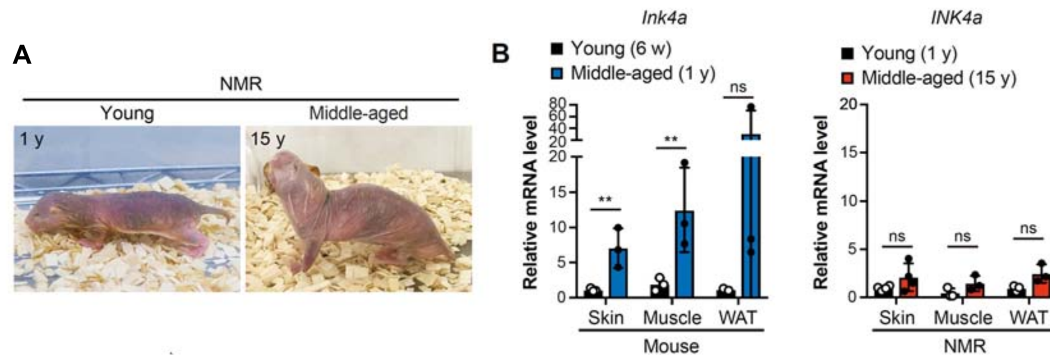


(**D**) Western blot of monoamine oxidase (MAO)-A and MAO-B in mouse or NMR fibroblasts at 20 days after *INK4a* transduction. ACTIN was used as a loading control.
(**G**) Western blot of MAO-A and MAO-B in mouse or NMR fibroblasts at 21 days after doxorubicin (DXR) treatment. ACTIN was used as a loading control.

9. Most of the results are got in vitro. INK4a expression in NMR brain tissues hardly increase during aging, how about in other major organs? Whether it is possible to do some tests to see whether the INK4a are critical for the death of senescent cell in NMR?

(Response) A previous study reported that *INK4a* expression in the NMR brain remains almost unchanged during aging, as described in lines 296–297 (Lee *et al*, 2020). In the first submission, we showed that the changes in *INK4a* expression in the NMR skin during aging are less pronounced than those in mice (Supplementary Figure 1 in the first submission paper). In response to the suggestion of Referee #1, we included the results of skin, abdominal muscle, and inguinal white adipose tissue biopsies obtained without sacrificing the animals (Fig 6A). These tissues were chosen because the number of middle-aged NMRs (15-year-old) in our laboratory is extremely limited, and we did not want to sacrifice them. The results showed that in these middle-aged NMR tissues, consistent with previous data from skin and brain, the upregulation of *INK4a* was lower than that in middle-aged mouse tissues (Fig. 6A and B).

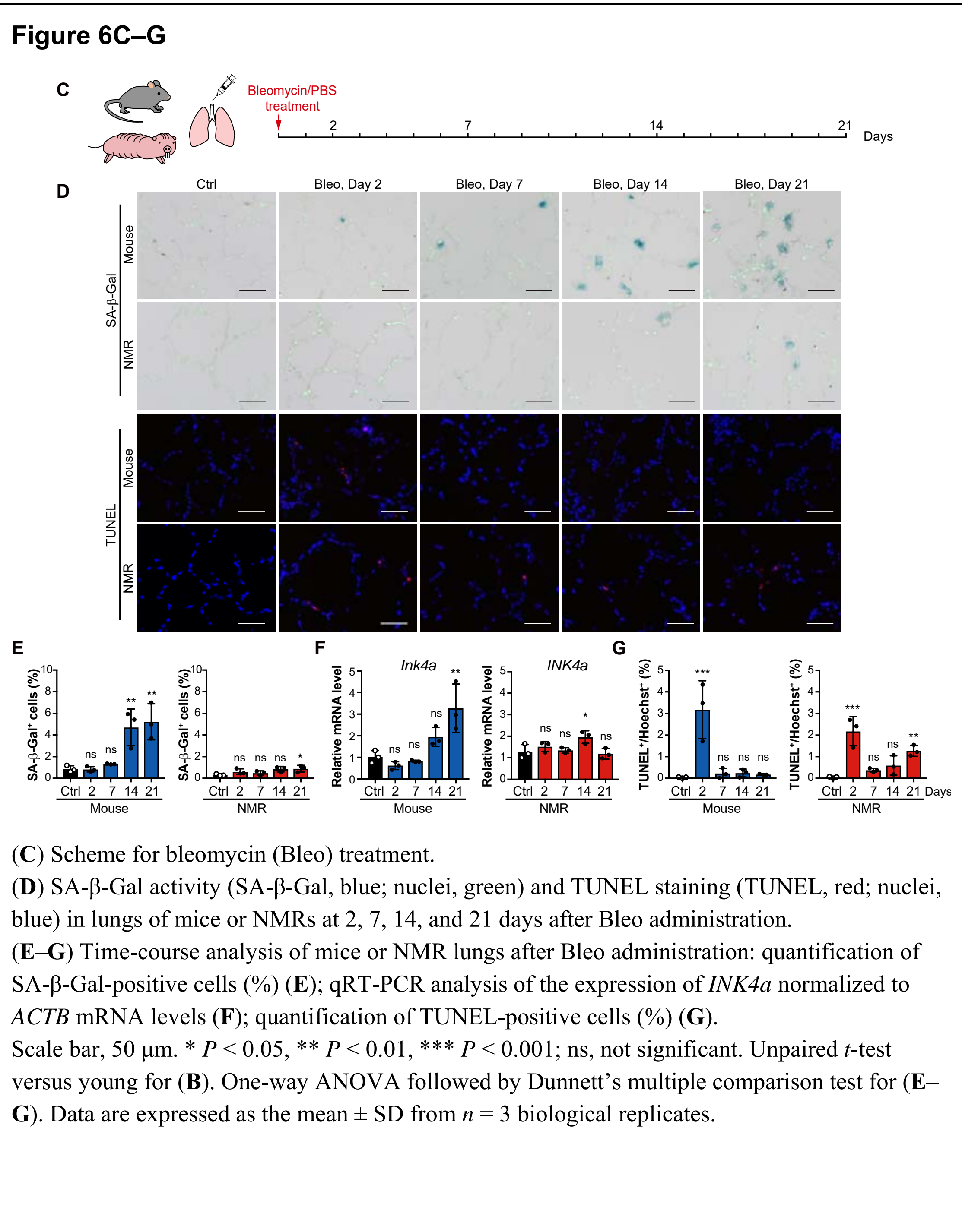
Figure 6A, B



(**A**) Left, young NMR (one-year-old). Right, middle-aged NMR (15-year-old).
(**B**) qRT-PCR analysis of INK4a expression in the skin, muscle, and white adipose tissue (WAT) of 6-week-old mice (young; 6 w), 1-year-old mice (middle-aged; 1 y), 1-year-old NMRs (young; 1 y), and 15-year-old NMRs (middle-aged; 15 y).

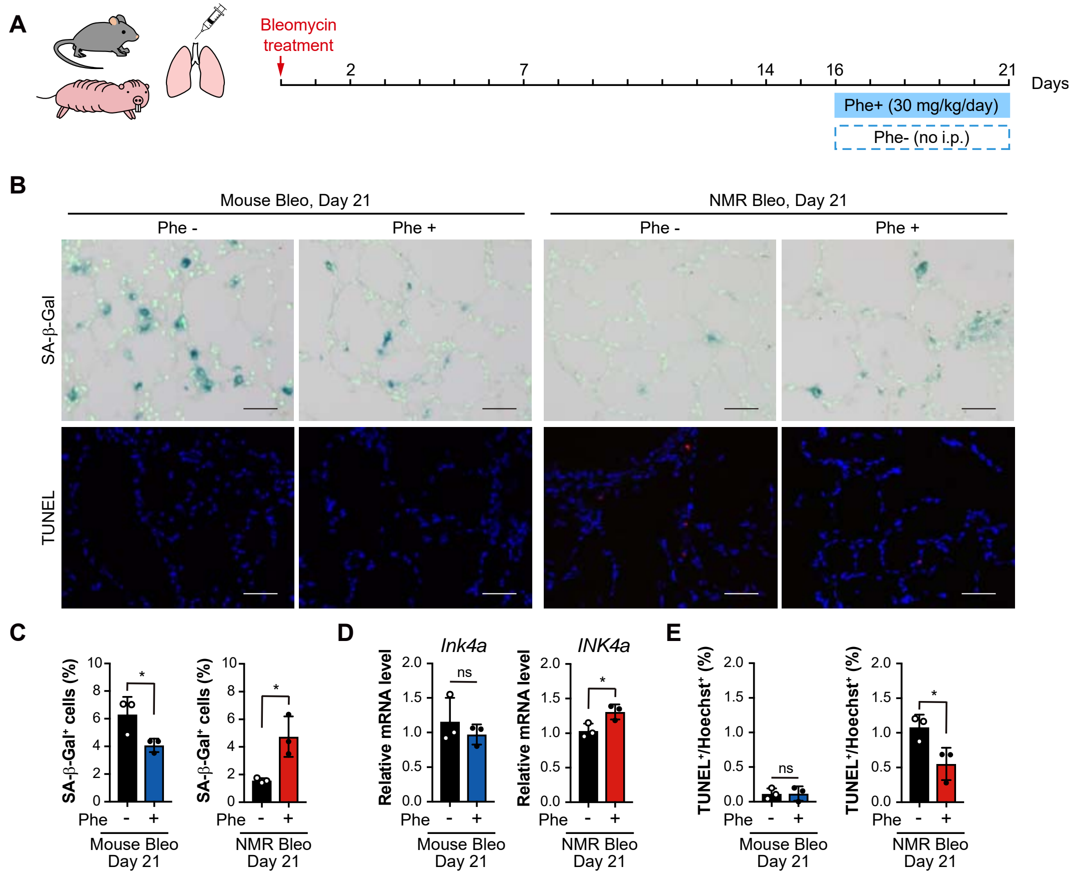
To determine whether senescence induction induces cell death in an MAO-dependent manner in NMR tissues *in vivo*, we induced cellular senescence in mouse and NMR lungs using bleomycin, a DNA-damaging agent (Fig 6C). Time course analysis showed that the number of SA-β-Gal positive cells and *INK4a* expression were lower in NMR lungs than in mouse lungs even on day 21 after bleomycin treatment (Fig 6D–F). These results suggest that NMR lungs are less likely to cause senescent cell accumulation in response to senescence-inducing stimuli. In both species, acute cell death was similarly increased on day 2. Notably, only NMR lungs exhibited delayed, progressive cell death that became significant on day 21 (Fig 6G), consistent with the *in vitro* findings. Furthermore, administration of phenelzine, a monoamine oxidase inhibitor, significantly increased the number of SA-β-Gal positive cells and *INK4a* expression and suppressed progressive cell death in bleomycin-treated NMR lungs (Fig 7A). The new results indicate that the *in vivo* induction of cellular senescence in NMR lungs leads to delayed, progressive cell death through MAO activation, which contributes to the suppression of senescent cell accumulation, consistent with our *in vitro* findings.

We have added the new data and the description in the Results section (Fig. 6, Fig. 7, lines 296–324).



(**C**) Scheme for bleomycin (Bleo) treatment.
(**D**) SA-β-Gal activity (SA-β-Gal, blue; nuclei, green) and TUNEL staining (TUNEL, red; nuclei, blue) in lungs of mice or NMRs at 2, 7, 14, and 21 days after Bleo administration.
(**E**–**G**) Time-course analysis of mice or NMR lungs after Bleo administration: quantification of SA-β-Gal-positive cells (%) (**E**); qRT-PCR analysis of the expression of *INK4a* normalized to *ACTB* mRNA levels (**F**); quantification of TUNEL-positive cells (%) (**G**).
Scale bar, 50 µm. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; ns, not significant. Unpaired *t*-test versus young for (**B**). One-way ANOVA followed by Dunnett's multiple comparison test for (**E**–**G**). Data are expressed as the mean ± SD from $n = 3$ biological replicates.

Figure 7A–E



(**A**) Scheme for bleomycin (Bleo) treatment and additional phenelzine (Phe) treatment. Mouse or NMR lungs were treated with Phe for 5 days starting at 16 days after Bleo administration.
(**B**) SA-β-Gal activity (SA-β-Gal, blue; nuclei, green) and TUNEL staining (TUNEL, red; nuclei, blue) in lungs of mice or NMRs at 21 days after Bleo administration, with or without Phe are shown.
(**C**–**E**) Quantification of SA-β-Gal-positive cells (%) (**C**), qRT-PCR analysis of *INK4a* expression normalized to *ACTB* mRNA levels (**D**), quantification of TUNEL-positive cells (%) (**E**) in mouse or NMR lungs at 21 days after Bleo administration, with or without Phe.
Scale bar, 50 μm. * $P < 0.05$; ns, not significant. Unpaired *t*-test versus Phe- for (**C**–**E**).
Data are expressed as the mean ± SD from $n = 3$ biological replicates.

10. Please carefully check the data and statistical analysis. For some figures, the SD is very small, and there is a notable difference among the mean values, but there is no significance. For example, in Fig 1F (left), the INK4A mRNA levels on day 6, it seems that the level is 2.5 times higher than the control, but ns.

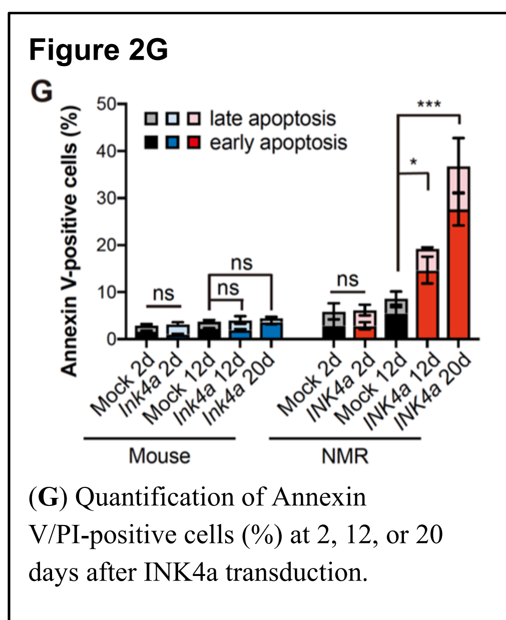
(Response) The original mouse data values from Fig. 1F are shown below. Statistical analysis of these data was performed using Prism7 software with one-way ANOVA followed by Dunnett's multiple comparison test against controls. As noted by Referee #1, there is a significant difference in the two-group test (unpaired *t*-test) between the ctrl and day 6 data. However, when comparing the ctrl and the seven groups from day 3 to day 21, a multiple comparison test needs to be used to compare more than three groups, and therefore, no statistically significant difference was found.

Mouse *Ink4a*	Ctrl	3d	6d	9d	12d	15d	18d	21d
Mouse 1	1.0000	2.4797	3.5390	5.5711	8.3567	11.0473	12.1235	15.1962
Mouse 2	0.8404	1.8143	3.3035	6.0209	9.2067	6.6265	14.1595	13.4254
Mouse 3	0.9388	1.6945	3.4624	6.4393	9.8333	9.9708	17.3243	12.4118

Minor comments

1. In figure 2G, please also provide the data of cell apoptosis after overexpressing INK4a.

(Response) We presented the data on apoptosis of mouse and NMR fibroblasts after *Ink4a*/*INK4a* transduction (Fig. 2G). Apoptosis did not increase immediately on day 2 after transduction in mouse and NMR fibroblasts. However, on day 12, only NMR cells, but not mouse cells, showed a significant increase in cell death, including apoptosis.



(**G**) Quantification of Annexin V/PI-positive cells (%) at 2, 12, or 20 days after INK4a transduction.

2. There are some spelling or grammatical mistakes, please go through the manuscript and correct them. e.g., page 3, line 51; page 4, line 62; Figure 3c, SA-b-Gal should be SA-β-Gal...

(Response) Thank you very much for pointing out our mistakes. We have made the necessary corrections.

3. Please keep consistent when using the error bars, upper and down.

(Response) We have corrected the error bars in the serotonin and 5-HIAA graphs in Fig 4D.

4. In addition, different cells may depend on different nutrients or supplements in the medium for their survival. I am thinking that whether the medium is the optimal for the growth of NMR cells. If not, cell death may appear after long-term culturing.

(Response) NMR fibroblasts are cultured in conventional fibroblast culture medium by us and other research groups (Miyawaki *et al*, 2016; Salmon *et al*, 2008; Seluanov *et al*, 2009). We also selected the appropriate serum after careful lot control. In response to Referee #1's comment, we cultured NMR fibroblasts for 21 days at appropriate cell densities and passages and assessed cell death rates with trypan blue staining after trypsinization. Fig. R3 in this letter shows that NMR cells can be maintained in culture without increased cell death, suggesting that our culture conditions are suitable for culturing NMR fibroblasts.

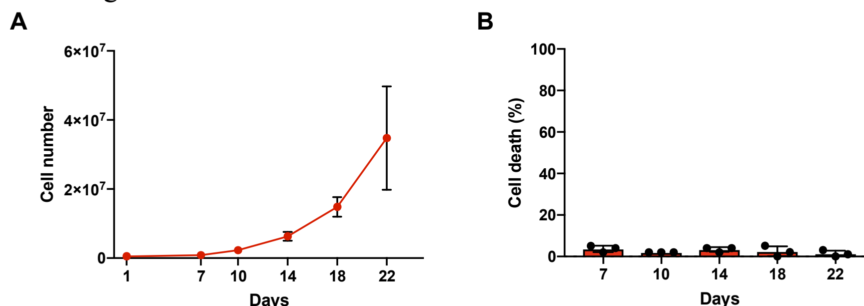


Fig R3. Cell growth (**A**) and cell death rates (**B**) of NMR fibroblasts (n = 3).

REFEREES' COMMENTS:

Referee #3:

We are grateful for the insightful feedback provided by Referee #3. The comments were very helpful in refining and improving the quality of our study.

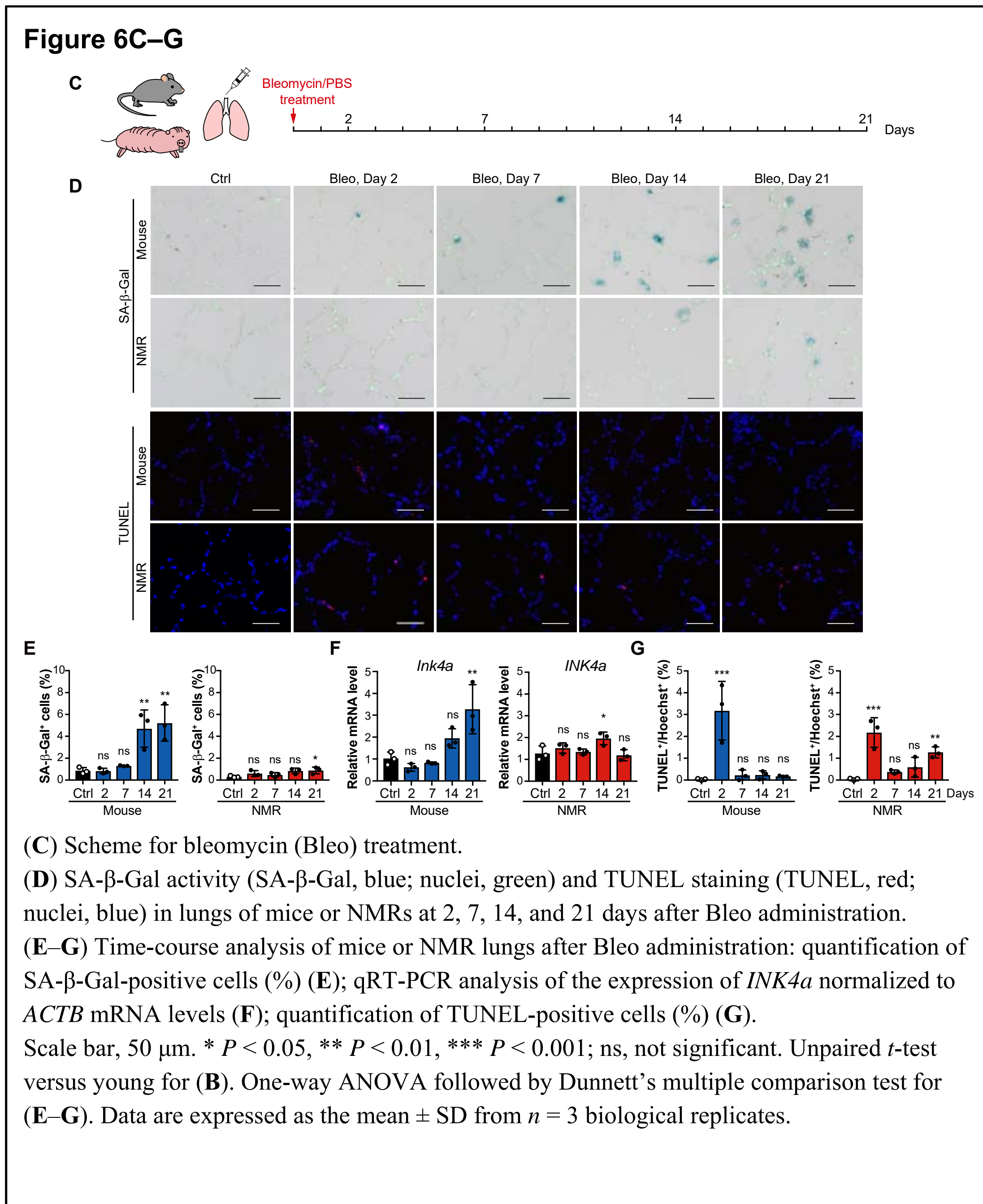
Major comments

The significant deficiency in the paper, however, is the lack of any in vivo data to support the authors conclusions. For example, does treatment of NMRs with an MAO inhibitor (either aged, or perhaps following injury) lead to an accumulation of senescent cells, and is this absent in a comparable mouse? This type of study would be a critical in vivo test of the model proposed in Figure 6 and should be provided.

(Response) Thank you very much for your suggestion regarding the *in vivo* experiment. To address this, we performed cellular senescence induction in mouse and NMR lungs using bleomycin, a DNA-damaging agent (Fig 6C). Time-course analysis revealed that the number of SA-β-Gal positive cells and *Ink4a* expression significantly increased on days 14 and 21 in mouse lungs, whereas the number of SA-β-Gal positive cells and *INK4a* expression remained lower in NMR lungs than in mouse lungs even on day 21 after bleomycin treatment (Fig 6E and F). Based on the results, it appears that NMR lungs are less likely to accumulating senescent cells in response to senescence-inducing stimuli. Both species showed an increase in acute cell death on day 2, and interestingly, only NMR lungs showed an increase in delayed and progressive cell death that became significant on day 21 (Fig 6G).

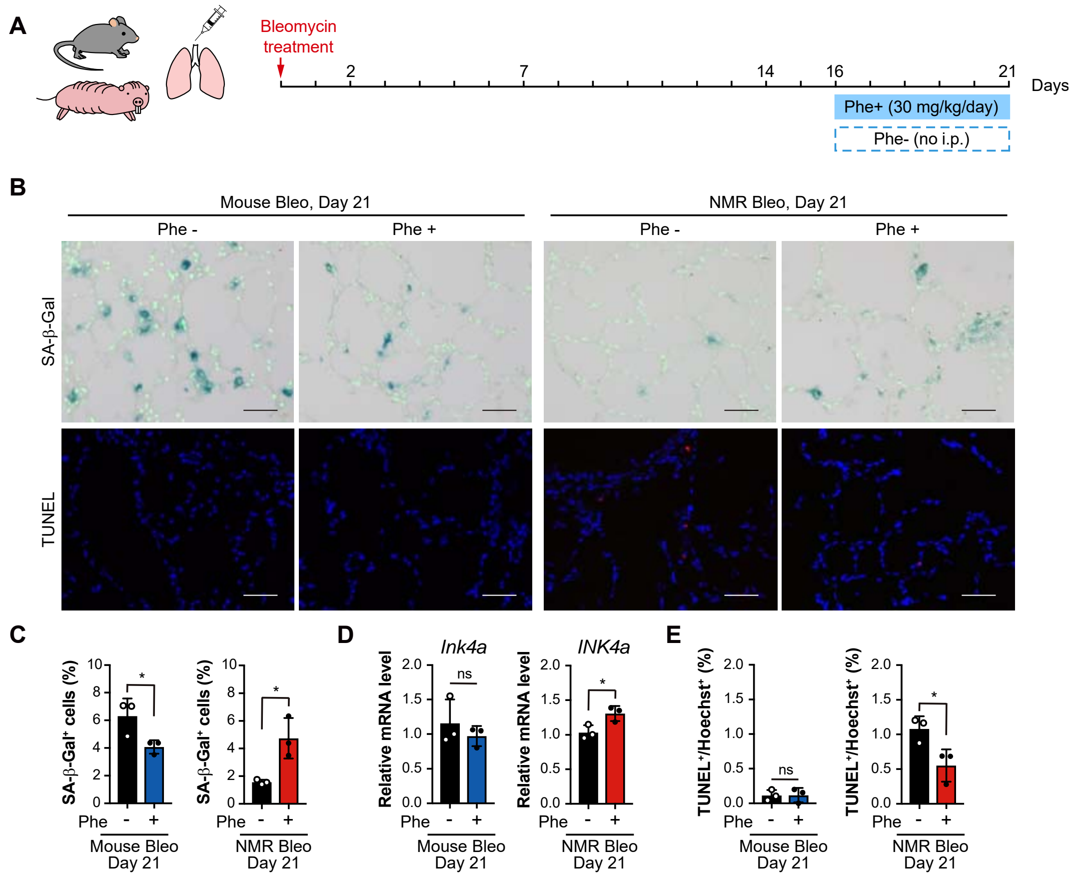
Importantly, administration of the monoamine oxidase inhibitor, phenelzine, significantly increased the number of SA-β-Gal positive cells and *INK4a* expression and suppressed progressive cell death (Fig 7A–E) in bleomycin-treated NMR lungs. Therefore, consistent with our *in vitro* findings, *in vivo* induction of cellular senescence leads to delayed, progressive cell death in NMR tissues through MAO activation, which contributes to the suppression of senescent cell accumulation. We are grateful to referee #3 for the experimental advice that allowed us to obtain these important data.

We have added the new data and the description in the Results section (Fig 6 and 7, lines 303–324).



(**C**) Scheme for bleomycin (Bleo) treatment.
(**D**) SA-β-Gal activity (SA-β-Gal, blue; nuclei, green) and TUNEL staining (TUNEL, red; nuclei, blue) in lungs of mice or NMRs at 2, 7, 14, and 21 days after Bleo administration.
(**E**–**G**) Time-course analysis of mice or NMR lungs after Bleo administration: quantification of SA-β-Gal-positive cells (%) (**E**); qRT-PCR analysis of the expression of *INK4a* normalized to *ACTB* mRNA levels (**F**); quantification of TUNEL-positive cells (%) (**G**).
Scale bar, 50 µm. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$; ns, not significant. Unpaired *t*-test versus young for (**B**). One-way ANOVA followed by Dunnett's multiple comparison test for (**E**–**G**). Data are expressed as the mean ± SD from $n = 3$ biological replicates.

Figure 7A–E



(**A**) Scheme for bleomycin (Bleo) treatment and additional phenelzine (Phe) treatment. Mouse or NMR lungs were treated with Phe for 5 days starting at 16 days after Bleo administration.
(**B**) SA-β-Gal activity (SA-β-Gal, blue; nuclei, green) and TUNEL staining (TUNEL, red; nuclei, blue) in lungs of mice or NMRs at 21 days after Bleo administration, with or without Phe are shown.
(**C**–**E**) Quantification of SA-β-Gal-positive cells (%) (**C**), qRT-PCR analysis of *INK4a* expression normalized to *ACTB* mRNA levels (**D**), quantification of TUNEL-positive cells (%) (**E**) in mouse lungs 21 days after Bleo administration, with or without Phe.
Scale bar, 50 µm. * $P < 0.05$; ns, not significant. Unpaired *t*-test versus Phe- for (**C**–**E**).
Data are expressed as the mean ± SD from $n = 3$ biological replicates.

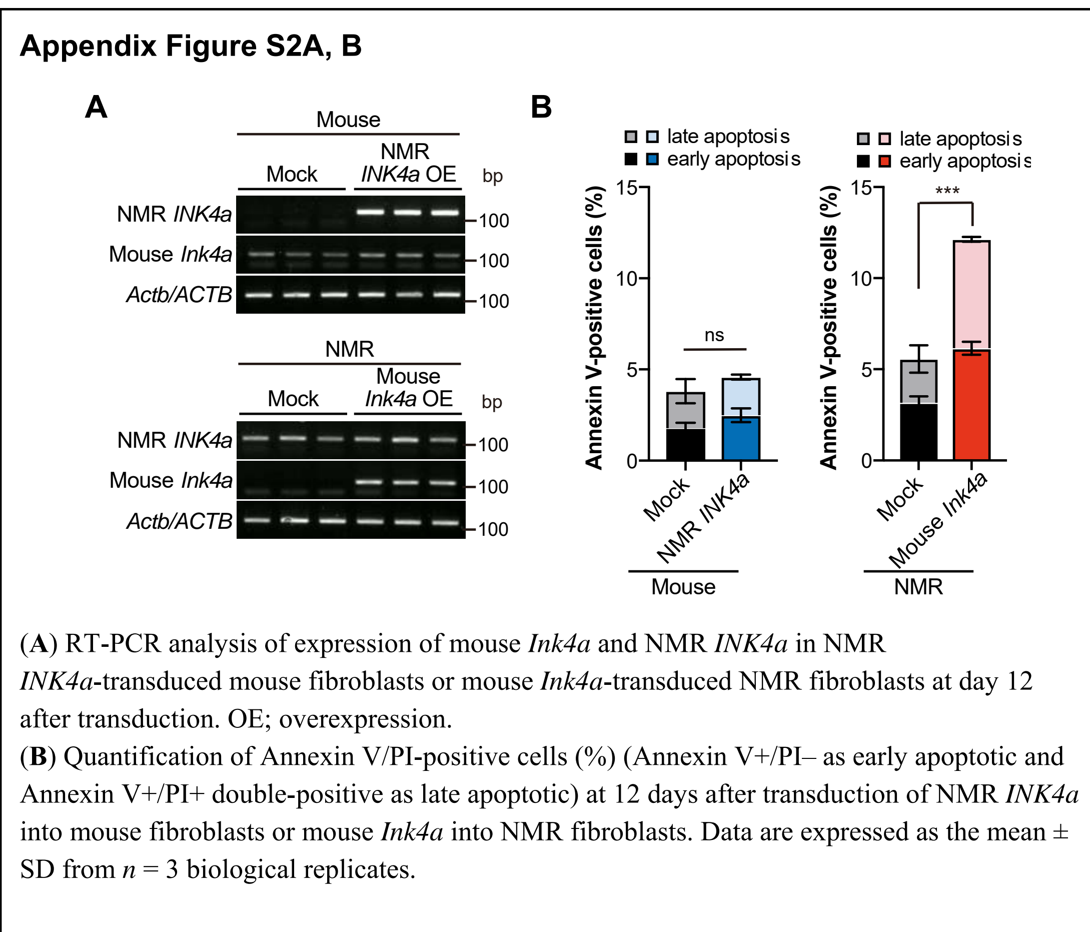
Other comments:

1. For the INK4a transductions, were species-specific sequences used? If not, is there evidence that mouse INK4a works in NMRs (or vice versa)?

(Response) We apologize for the lack of clarity in our previous description. We showed the data from the species-specific sequences of *Ink4a*/*INK4a* (mouse *Ink4a* was introduced into mouse cells, and NMR *INK4a* was introduced into NMR cells) (Fig 2). As a reference, we introduced NMR *INK4a* into mouse fibroblasts and mouse *Ink4a* into NMR fibroblasts, and cell death was increased only in NMR cells (Appendix Fig S2A and B). This indicates that the sequence difference in *Ink4a*/*INK4a* is not the cause of cell death in NMR.

We have included the new data and the description in the Results section (Appendix Fig. 2A and B, lines 155–157).

Appendix Figure S2A, B



(**A**) RT-PCR analysis of expression of mouse *Ink4a* and NMR *INK4a* in NMR *INK4a*-transduced mouse fibroblasts or mouse *Ink4a*-transduced NMR fibroblasts at day 12 after transduction. OE; overexpression.
(**B**) Quantification of Annexin V/PI-positive cells (%) (Annexin V+/PI– as early apoptotic and Annexin V+/PI+ double-positive as late apoptotic) at 12 days after transduction of NMR *INK4a* into mouse fibroblasts or mouse *Ink4a* into NMR fibroblasts. Data are expressed as the mean ± SD from n = 3 biological replicates.

2. Throughout, there is no mention of the sex of the animals used to derives cells. Did that make a difference in the results?

（**Response**）The sex of the NMRs used in our study is presented in Appendix Table S1.

We used both male and female NMRs for our *in vitro* experiments and found no significant differences in the results based on sex. Due to the limited availability of animals, we used mainly male NMRs for our *in vivo* experiments. Although we used male mice for the *in vitro* and *in vivo* experiments, except for biopsies, to the best of our knowledge, there are no reports of sex-based differences in the cellular senescence process in mice.

(Reference)

Childs BG, Baker DJ, Kirkland JL, Campisi J, van Deursen JM & Deursen JM Van (2014) Senescence and apoptosis: dueling or complementary cell fates? *EMBO Rep* 15: 1–15

Childs BG, Durik M, Baker DJ & van Deursen JM (2015) Cellular senescence in aging and age-related disease: from mechanisms to therapy. *Nat Med* 21: 1424–1435

Fischer M (2017) Census and evaluation of p53 target genes. *Oncogene* 36: 3943–3956

Lau GY, Milsom WK, Richards JG & Pamenter ME (2020) Heart mitochondria from naked mole-rats (Heterocephalus glaber) are more coupled, but similarly susceptible to anoxia-reoxygenation stress than in laboratory mice (Mus musculus). *Comp Biochem Physiol Part - B Biochem Mol Biol* 240: 110375

Lee BP, Smith M, Buffenstein R & Harries LW (2020) Negligible senescence in naked mole rats may be a consequence of well-maintained splicing regulation. *GeroScience* 42: 633–651

Miyawaki S, Kawamura Y, Oiwa Y, Shimizu A, Hachiya T, Bono H, Koya I, Okada Y, Kimura T, Tsuchiya Y, *et al* (2016) Tumour resistance in induced pluripotent stem cells derived from naked mole-rats. *Nat Commun* 7: 11471

Munro D, Baldy C, Pamenter ME & Treberg JR (2019) The exceptional longevity of the naked mole-rat may be explained by mitochondrial antioxidant defenses. *Aging Cell* 18: e12916

Salmon AB, Akha AAS, Buffenstein R & Miller RA (2008) Fibroblasts From Naked Mole-Rats Are Resistant to Multiple Forms of Cell Injury, But Sensitive to Peroxide, Ultraviolet

Light, and Endoplasmic Reticulum Stress. *Journals Gerontol Ser A Biol Sci Med Sci* 63: 232–241

Seluanov A, Hine C, Azpurua J, Feigenson M, Bozzella M, Mao Z, Catania KC & Gorbunova V (2009) Hypersensitivity to contact inhibition provides a clue to cancer resistance of naked mole-rat. *Proc Natl Acad Sci* 106: 19352–19357

1st Revision - Editorial Decision **29th Apr 2023**

Dear Prof. Miura,

Congratulations on a great revision! Overall, the referees are pleased with your revision. However, referee 1 has asked for some quantifications that we feel would greatly strengthen your important study.

When you submit your revised version, please also take care of the following editorial items and add this also to your point-by-point response:

1. Please remove red color from the manuscript text.

2. Below the abstract, please add up to five key words, which may or may not appear in the title, in alphabetical order, each separated by a slash (/).

3. Please remove the author contribution section from the main manuscript

4. For the Appendix File, please add a table of contents with page numbers. Appendix figure legends should be removed from the end of the Appendix file, and left only below the figures. Appendix Table S1-S2 should be included in Appendix file.

5. Please upload the source data checklist for the source data.

6. The synopsis image you provided is too large. It should be 550 pixels wide by 200-440 pixels high.

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

Kind regards,

Kelly

Kelly M Anderson, PhD
Editor
The EMBO Journal
k.anderson@embojournal.org

Further information is available in our Guide For Authors: https://www.embopress.org/page/journal/14602075/authorguide

Use the link below to submit your revision:

https://emboj.msubmit.net/cgi-bin/main.plex

Referee #1:

The authors have performed additional experiments to address most of my concerns. These new data have strengthened their conclusions. It is good to see that they performed in vivo experiments to provide support for the in vitro data. Some minor concerns are below:
1. In the long-term culture of NMR cells to observe senescence or quiescence, two out of 3 NMR cultures resumed proliferation and one did not. They concluded that "NMR fibroblasts cultured long-term with upregulated INK4a after induced CI contain both senescent and quiescent cells". It is a little not confirmative regarding statistical rigorousness, which however does not affect their major conclusions.
2. For some key evidence, e.g., p53 protein levels after stimuli treatment in NMR cells, it will be good if the authors can quantify it.

Referee #3:

The authors have addressed my concerns, including performing in vivo studies that greatly enhance the findings of this paper. Thank you.

4 May 2023

Manuscript EMBOJ-2022-111133

Yoshimi Kawamura et al.

"Cellular senescence leads to progressive cell death via the INK4a-RB pathway in naked mole-rats"

Point-by-point responses to the editorial comments and the referee's comments

EDITORIAL COMMENTS:

1. Please remove red color from the manuscript text.

(Response) We removed red color from our manuscript.

2. Below the abstract, please add up to five key words, which may or may not appear in the title, in alphabetical order, each separated by a slash (/).

(Response) We added the five key words in alphabetical order below the abstract.

3. Please remove the author contribution section from the main manuscript

(Response) We removed the author contribution section from the main manuscript.

4. For the Appendix File, please add a table of contents with page numbers. Appendix figure legends should be removed from the end of the Appendix file, and left only below the figures. Appendix Table S1-S2 should be included in Appendix file.

(Response) We added a table of contents and Appendix Tables S1-S2 and removed the figure legends from the end of the Appendix file.

5. Please upload the source data checklist for the source data.

(Response) Thankfully, Dr. Hannah Sonntag has already added the checklist to our manuscript. We filled out the checklist.

6. The synopsis image you provided is too large. It should be 550 pixels wide by 200-440 pixels high.

(Response) We reduced the file size of the synopsis image (550 pixels x 300 pixels).

REFEREES' COMMENTS:

Referee #1:

We appreciate the constructive comments by Referee #1.

1. In the long-term culture of NMR cells to observe senescence or quiescence, two out of 3 NMR cultures resumed proliferation and one did not. They concluded that "NMR fibroblasts cultured long-term with upregulated INK4a after induced CI contain both senescent and quiescent cells". It is a little not confirmative regarding statistical rigorousness, which however does not affect their major conclusions.

(Response) We agree with Referee 1 that Figure EV1K is not statistically rigorous to conclude whether the halt of cell division caused by contact inhibition was cellular senescence or quiescence. To increase the number of fibroblast cultures for statistical analysis, an experimental period of at least three months is required for the induction of contact inhibition and the regrowth assay after passages. However, even if we increase the number of fibroblast cultures in this experiment, it may not be sufficient to conclusively prove quiescence or senescence. From this perspective, we believe it is appropriate to weaken the wording of our result sentence and state them as follows (lines 223–225 in Results section): "This suggests that NMR fibroblasts cultured long-term with upregulated INK4a after induced CI **presumably** contain both quiescent cells and senescent cells."

2. For some key evidence, e.g., p53 protein levels after stimuli treatment in NMR cells, it will be good if the authors can quantify it.

(Response) Based on the referee's suggestion, key western blots (e.g., MAOs and p53) were quantified. We have added the quantification data in the Fig 5D and G, Appendix Fig S3C and S6E.

2nd Revision - Editorial Decision 12th May 2023

Dear Kyoko,

Congratulations on an excellent manuscript, I am pleased to inform you that your manuscript has been accepted for publication in the EMBO Journal. Thank you for your comprehensive response to the referee concerns and for providing detailed source data. It has been a pleasure to work with you to get this to the acceptance stage.

I will begin the final checks on your manuscript before submitting to the publisher next week. Once at the publisher, it will be about 3 weeks for your manuscript to be published online. As a reminder, the entire review process, including referee concerns and your point-by-point response, will be available to readers.

I will be in touch throughout the final editorial process until publication. In the meantime, I hope you find time to celebrate!

Kind regards,

Kelly

Kelly M Anderson, PhD
Editor
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EMBO Molecular Medicine - Author Guidelines

Reporting Checklist for Life Science Articles (updated January
This checklist is adapted from Materials Design Analysis Reporting (MDAR) Checklist for Authors. MDAR establishes a minimum set of requirements in transparent reporting in the life sciences (see Statement of Task: 10.31222/osf.io/9sm4x). Please follow the journal's guidelines in preparing your
Please note that a copy of this checklist will be published alongside your article.

Abridged guidelines for 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.
- ideally, figure panels should include only measurements that are directly comparable to each other and obtained with the same assay.
- plots include clearly labeled error bars for independent experiments and sample sizes. Unless justified, error bars should not be shown for technical
- if n<5, the individual data points from each experiment should be plotted. Any statistical test employed should be justified.
- Source Data should be included to report the data underlying figures according to the guidelines set out in the authorship guidelines on Data

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.

Please complete ALL of the questions below.
Select "Not Applicable" only when the requested information is not relevant for your study.

Materials

Newly Created Materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
New materials and reagents need to be available; do any restrictions apply?	Not Applicable	

Antibodies	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
For **antibodies** provide the following information: - Commercial antibodies: RRID (if possible) or supplier name, catalogue number and or/clone number - Non-commercial: RRID or citation	Yes	Materials and Methods section

DNA and RNA sequences	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Short novel DNA or RNA including primers, probes: provide the sequences.	Yes	Materials and Methods section, Appendix Table S2

Cell materials	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Cell lines: Provide species information, strain. Provide accession number in repository **OR** supplier name, catalog number, clone number, and/**OR** RRID.	Not Applicable	
Primary cultures: Provide species, strain, sex of origin, genetic modification status.	Yes	Materials and Methods section, Appendix Table S1
Report if the cell lines were recently **authenticated** (e.g., by STR profiling) and tested for mycoplasma contamination.	Yes	Materials and Methods section

Experimental animals	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Laboratory animals or Model organisms: Provide species, strain, sex, age, genetic modification status. Provide accession number in repository **OR** supplier name, catalog number, clone number, **OR** RRID.	Yes	Materials and Methods section, Appendix Table S1
Animal observed in or captured from the field: Provide species, sex, and age where possible.	Not Applicable	
Please detail **housing and husbandry conditions**.	Yes	Materials and Methods section

Plants and microbes	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Plants: provide species and strain, ecotype and cultivar where relevant, unique accession number if available, and source (including location for collected wild specimens).	Not Applicable	
Microbes: provide species and strain, unique accession number if available, and source.	Not Applicable	

Human research participants	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If collected and within the bounds of privacy constraints report on age, sex and gender or ethnicity for all study participants.	Not Applicable	

Core facilities	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If your work benefited from core facilities, was their service mentioned in the acknowledgments section?	Yes	Acknowledgments section

Design

Study protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
If study protocol has been **pre-registered, provide DOI in the manuscript**. For clinical trials, provide the trial registration number **OR** cite DOI.	Not Applicable	
Report the **clinical trial registration number** (at ClinicalTrials.gov or equivalent), where applicable.	Not Applicable	

Laboratory protocol	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Provide DOI OR other citation details if **external detailed step-by-step protocols** are available.	Not Applicable	

Experimental study design and statistics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Include a statement about **sample size** estimate even if no statistical methods were used.	Yes	Figure legends
Were any steps taken to minimize the effects of subjective bias when allocating animals/samples to treatment (e.g. **randomization procedure**)? If yes, have they been described?	Yes	Materials and Methods section
Include a statement about **blinding** even if no blinding was done.	Not Applicable	
Describe **inclusion/exclusion criteria** if samples or animals were excluded from the analysis. Were the criteria pre-established? If sample or data points were omitted from analysis, report if this was due to attrition or intentional exclusion and provide justification.	Not Applicable	
For every figure, are **statistical tests** justified as appropriate? Do the data meet the assumptions of the tests (e.g., normal distribution)? Describe any methods used to assess it. Is there an estimate of variation within each group of data? Is the variance similar between the groups that are being statistically compared?	Yes	Figure legends

Sample definition and in-laboratory replication	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
In the figure legends: state number of times the experiment was **replicated** in laboratory.	Yes	Figure legends
In the figure legends: define whether data describe **technical or biological replicates**.	Yes	Figure legends

Ethics

Ethics	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Studies involving **human participants**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval.	Not Applicable	
Studies involving **human participants**: 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.	Not Applicable	
Studies involving **human participants:** For publication of **patient photos**, include a statement confirming that consent to publish was obtained.	Not Applicable	
Studies involving experimental **animals**: State details of **authority granting ethics approval** (IRB or equivalent committee(s), provide reference number for approval. Include a statement of compliance with ethical regulations.	Yes	Materials and Methods section
Studies involving **specimen and field samples:** State if relevant **permits** obtained, provide details of authority approving study; if none were required, explain why.	Not Applicable	

Dual Use Research of Concern (DURC)	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Could your study fall under dual use research restrictions? Please check biosecurity documents and list of **select agents and toxins** (CDC): https://www.selectagents.gov/sat/list.htm	Not Applicable	
If you used a select agent, is the security level of the lab appropriate and reported in the manuscript?	Not Applicable	
If a study is subject to dual use research of concern regulations, is the name of the **authority granting approval and reference number** for the regulatory approval provided in the manuscript?	Not Applicable	

Reporting

The MDAR framework recommends adoption of discipline-specific guidelines, established and endorsed through community initiatives. Journals have their own policy about requiring specific guidelines and recommendations to complement MDAR.

Adherence to community standards	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
State if relevant guidelines or checklists (e.g., **ICMJE, MIBBI, ARRIVE, PRISMA**) have been followed or provided.	Not Applicable	
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.	Not Applicable	
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.	Not Applicable	

Data Availability

Data availability	Information included in the manuscript?	In which section is the information available? (Reagents and Tools Table, Materials and Methods, Figures, Data Availability Section)
Have **primary datasets** been deposited according to the journal's guidelines (see 'Data Deposition' section) and the respective accession numbers provided in the Data Availability Section?	Yes	Data Availability Section
Were **human clinical and genomic datasets** deposited in a public access-controlled repository in accordance to ethical obligations to the patients and to the applicable consent agreement?	Not Applicable	
Are **computational models** that are central and integral to a study available without restrictions in a machine-readable form? Were the relevant accession numbers or links provided?	Not Applicable	
If publicly available data were reused, provide the respective **data citations in the reference list**.	Not Applicable	