

Ameliorating calcium homeostasis improves longevity and healthspan in progeroid and naturally aged mice

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This file contains all reviewer reports in order by version, followed by all author rebuttals in order by version.

Version 0:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

The manuscript "Ameliorating calcium homeostasis improves longevity and healthspan in 1 progeroid and naturally aged mice" describes how cytoplasmic accumulation of the protein S100A6 accelerates aging. Progeria patients show elevated levels, as do LMNA progeroid mice. S100A6 was found to bind PARP1, causing its ubiquitin-mediated degradation, thereby resulting in DNA damage.

In general, the data are strong and back up the conclusions as drawn. I recommend publication, provided a few questions regarding senescence are addressed.

1) The authors argue that knockdown of S100A6 reduces SABGAL (Fig. 1k) and restores proliferation. However, a change from 60% to ~45% is of course not very likely to have a major impact. These cells should be followed (much) longer over time to see if the population fully gets into cell cycle progression, or whether they fall back into becoming largely senescent. Something similar holds true for 2ABP (Fig. 1y). How significant is the restoration of Lamin B1 (Fig. 1v) and a ~20% reduction in SABGAL?

2) It is not particularly common to see expression of IL6 and IL8 measured through western blot, whereas QPCR works well. To make sure the bands measured here are indeed IL6/8, at least for the most crucial figures a QPCR should be added.

3) The in vivo experiment with 2ABP and MIA is strong, in principle. However, what is the expression in these tissues (Heart, Lung, Muscle) of SABGAL, Lamin B1, PARP1, SASP etc? In other words; are the reduced drop in body weight, Overall Survival benefit etc linked to DDR, senescence/SASP, or might this be due to something else? PARP1 is measured in Fig. 8, adding credibility, but why not in Fig. 4 and 6? At least the obvious markers (SABGAL, PARP1, S100A6 and Lamin B1) should be added to the most crucial panels of these figures.

4) The connection to cGAS/STING is not unlikely, but also not exactly proven by this study. Either this pathway and expression of these proteins themselves should be measured, or the claims should be made weaker in that this is a hypothesis, but not proven.

Minor:

- Most figures addressing Ca²⁺ signaling show a measurement of its concentration in a sample. It would be helpful to examine this with a reporter system, or Calcein-AM as well.

- In Fig. 3a (and less so in 5o) it is unclear whether this was stable expression of FLAG-Progerin, or a transient expression. The latter is notoriously challenging in IMR90 and may well result in susceptibility to DNA damage per se, especially in case of electroporation. An empty vector control would not to a similar response. A FLAG-GFP may transiently do something similar.

- The figure order is off here and there (Fig. 1, l m k).

Reviewer #2

(Remarks to the Author)

In this study, Xiang et.al. sought to link cytoplasmic calcium homeostasis to aging and Hutchinson-Gilford progeria syndrome (HGPS), and claimed a mechanism involving S100A6-mediated PARP1 degradation, DNA damage and cGAS-STING pathway activation. The authors further demonstrated that mianserin (MIA), a tetracyclic antidepressant, ameliorated senescence phenotypes in HGPS and naturally aged models by restoring calcium homeostasis.

The major problem of this work is that the authors used wrong methods to measure cytoplasmic and ER Ca^{2+} . No reliable data related to cytoplasmic Ca^{2+} homeostasis was presented.

1. The authors measured the total $[\text{Ca}^{2+}]$ in various tissues using a kit. However, as extracellular $[\text{Ca}^{2+}]$ is 4 orders of magnitude higher than cytoplasmic $[\text{Ca}^{2+}]$, the variations in extracellular fluid (including blood) precludes a reliable estimation of intracellular Ca^{2+} . Total Ca^{2+} measurement of tissues should not be used to support any conclusion related to cytoplasmic Ca^{2+} .
2. The authors used Fluo-4 AM and ER-tracker to stain Ca^{2+} and the ER and stated that concentration of Ca^{2+} in the ER decreased while that in the cytoplasm increased. Fluo-4 is an indicator used to measure $[\text{Ca}^{2+}]$ lower than 1 micromolar, and is not able to detect ER Ca^{2+} which was much higher. Moreover, ER of many cell types lacks the enzyme to digest the AM ester, keeping Fluo-4 AM inactive in ER.
3. The measurement of downstream signals of cGAS-STING, such as IL-6 and IL-8 by the whole-cell lysate is unreliable. Since these cytokines are secreted, the detected signal represents, to unknown extent, their inactive precursors, rather than functional secreted proteins.
4. 2-APB is a “dirty” (without specificity) inhibitor of some Ca^{2+} permeable channels, not a “calcium inhibitor” as the authors said. Specific calcium signaling pathways (e.g., SOCE, IP3R or TRP channels), should be determined by reliable methods.
5. Statistical issues. For example, many biochemical data lack statistical analysis. What statistical test was used for Kaplan-Meier survival curves? Why the control group has a high baseline SA- β -gal signal? SA- β -gal staining images should be provided.

Reviewer #3

(Remarks to the Author)

This is a very well-designed study identifies calcium (Ca^{2+}) homeostasis disruption as a key driver of aging and progeria. Specifically, the Authors revealed that elevated cytoplasmic calcium levels lead to accumulation of the Ca^{2+} -binding protein S100A6, which promotes aging by degrading PARP1, causing DNA damage, chromatin fragmentation, and secretion of SASP. With treatment of the antidepressant compound mianserin, the Authors measured restored calcium balance, decreased senescence, and extended lifespan in both progeric and naturally aging mice, highlighting the potential of MIA as an anti-aging therapeutic. Overall, the whole study appears to have been rigorously conducted and presented, and the manuscript is largely well-written. However, I still have some major concerns about this study that need to be addressed to better clarify the role of calcium homeostasis and its impact on cellular senescence.

Major concerns:

1. My primary concern lies in the use of the term “intracellular calcium” and the measurement of Ca^{2+} concentrations. Intracellular calcium encompasses both cytosolic Ca^{2+} and calcium stored in organelles such as the endoplasmic/sarcoplasmic reticulum (ER/SR). Based on the introduction and the reported localization of S100A6 in this study, it appears that the changes in “intracellular Ca^{2+} ” concentrations are actually referring specifically to cytosolic Ca^{2+} . However, cytosolic Ca^{2+} concentrations typically range in the nanomolar level (approximately 100–200 nM), whereas all the reported Ca^{2+} concentrations in this study are in the micromolar range (given muscle Ca^{2+} concentration in this study is from ~0.5–2 $\mu\text{g}/\text{ml}$, which is about 12–50 μM), which is inconsistent with established values for cytosolic Ca^{2+} in any cell type. I also noted that the authors used a calcium measurement kit from Abcam, which, according to the manufacturer’s instruction, is only capable of detecting calcium at micromolar levels. Therefore, the authors need to clarify both their method of Ca^{2+} measurement and the specific intracellular compartment to which the measured Ca^{2+} refers.
2. This study claims that S100A6 levels are closely associated with cytosolic Ca^{2+} concentrations, and the Authors report that deletion of S100A6 leads to a reduction in cytosolic Ca^{2+} levels. However, the regulatory relationship between S100A6 and cytosolic Ca^{2+} is not clearly explained. Specifically, it remains unclear how the absence of S100A6 results in decreased cytosolic Ca^{2+} —does S100A6 actively regulate calcium levels, or is its expression simply a response to elevated cytosolic Ca^{2+} ? In other words, is the increase in S100A6 a cause or a consequence of disrupted calcium homeostasis? A more detailed discussion of the mechanistic link between S100A6 and cytosolic Ca^{2+} regulation is needed to clarify this point.
3. Regarding the muscle force measurements, the authors state that force was measured as specific force, which refers to force production normalized to muscle size. The standard unit for specific force is typically mN/mm^2 or N/cm^2 . However, in this study, the reported units are in mN, indicating that only absolute force was measured. This needs to be clarified. Furthermore, the force values should be normalized to muscle size, especially given that muscle size varies across the experimental groups. Normalizing force to muscle cross-sectional area would provide a more accurate representation of muscle quality and function in this study.
4. Following up on the previous point, the tetanic force in this study was measured using a 100 Hz tetanic stimulus. However, according to prior studies, tetanic force is typically elicited using stimulation frequencies of around 150–200 Hz to

ensure maximal force production. The authors should provide a justification for using 100 Hz in this context and clarify whether this frequency is sufficient to induce maximal tetanic force in the muscle type studied. Without this clarification, it remains uncertain whether the reported force measurements truly reflect the muscle's maximum contractile capacity.

5. Beta-actin is commonly considered a housekeeping protein; however, its suitability as a loading control in aging tissues is questionable for several reasons. Studies have shown that ACTB expression can vary with age, and aging-related changes in cytoskeletal dynamics may further affect actin levels. In fact, beta-actin levels appear to vary in several panels in this study, such as Figures 1d–1g. Therefore, in aging tissues, a more appropriate approach for quantifying target protein levels would be to use total protein staining as a loading control. If the authors choose to use beta-actin, they should first validate its expression stability across different experimental groups and tissue types.

6. Two Ca^{2+} inhibitors are used in this study. For 2-APB, its mechanism as a Ca^{2+} inhibitor is briefly described in the manuscript as an IP_3 receptor blocker. However, for the other inhibitor, AML—although it is a commonly used antihypertensive drug—the authors should still provide a more detailed explanation of its mechanism of action and relevant signaling pathways.

Minor:

1. Line 59–61, Reference needed for the introduction of mutations in LMNA gene.

2. In Lines 63–65, the manuscript contains the only statement regarding Ca^{2+} concentration in HGPS patients. Since this study emphasizes calcium homeostasis and its role in HGPS, it would be beneficial to include additional background or evidence of calcium dysregulation in HGPS in this paragraph.

3. Line 119, definition needed for HEK-293T and CRL-1474 cells.

4. Line 148, the first appearance of shS100A6, need to define shS100A6 as short hairpin RNA (shRNA) designed to knock down the expression of the S100A6 gene.

5. The animal experimental data presented in the main results appear to be based solely on male mice. However, I noticed that both male and female mice were included in the extended data, and the outcomes seem comparable between both sexes. Please clarify why only male mice data are shown in the main results and provide a justification for this choice in the Methods section.

6. For all the Western blots, “kDa” is a standalone unit, so there should be a space between the numerical value and “kDa” (e.g., “25 kDa” instead of “25kDa”).

Reviewer #4

(Remarks to the Author)

In this study, Weifang Xiang et al. explore the role of S100A6-mediated calcium dysregulation in the pathogenesis of Hutchinson-Gilford progeria syndrome (HGPS) and propose MIA as a therapeutic intervention. While the topic is of substantial interest in aging biology, the manuscript suffers from significant mechanistic, methodological, and interpretational limitations that undermine the strength of its central claims and are not suitable for publication in its current form.

Main concerns:

1. The authors propose that disrupted calcium homeostasis leads to cytoplasmic S100A6 accumulation and its aberrant interaction with PARP1, contributing to PARP1 degradation. However, the causality of this pathway is not convincingly established. The manuscript lacks direct experimental evidence demonstrating that calcium fluxes actively drive S100A6 subcellular redistribution or binding to PARP1. No perturbation experiments (e.g., calcium chelation, ionophore treatment) were performed to dynamically modulate calcium levels and observe resulting changes in S100A6 localization or interaction strength with PARP1. Additionally, the involvement of the proposed chaperone CacyBP/SIP remains speculative, as no biochemical or genetic interrogation of this factor was conducted. The mechanistic link between calcium signaling and S100A6-PARP1 complex formation requires substantial reinforcement.

2. The study relies heavily on bulk colorimetric calcium assays, which are inherently limited in both spatial and temporal resolution. These measurements do not distinguish between cytosolic, ER, or mitochondrial calcium pools, nor do they provide dynamic insights into calcium signaling alterations in HGPS cells. More precise tools—such as fluorescent calcium indicators (e.g., Fluo-4, Fura-2) or genetically encoded calcium sensors (e.g., GCaMP)—should have been employed to monitor intracellular calcium fluxes in live cells. This represents a critical gap in validating the calcium dysregulation hypothesis.

3. The therapeutic effects of MIA are attributed to its modulation of serotonin receptors (HTR2B/HTR2C), leading to lowered intracellular calcium. However, this conclusion is weakly supported. The study lacks receptor-specific validation using genetic knockout models or receptor-selective antagonists. It is also unclear whether the observed calcium-lowering effect of MIA is specific to HTR2B/HTR2C inhibition or a consequence of broader, off-target actions. Additionally, no dose–response data or comparisons with known calcium modulators (e.g., BAPTA-AM, thapsigargin) are included, making it difficult to attribute MIA's anti-aging effects solely to calcium modulation. The therapeutic relevance of MIA in the context of HGPS, therefore, remains speculative.

4. Mouse studies are underpowered, especially for lifespan and endpoint analyses. Survival curves derived from small cohorts ($n < 30$) are not statistically reliable. Similarly, endpoint assays (e.g., histology, protein expression) conducted with fewer than 10 animals per group lack robustness. The authors must increase sample sizes in accordance with standard practices for aging and intervention studies.

5. Western blot data are presented without quantification or normalization controls, preventing proper evaluation of protein expression levels. Full-length blots, along with molecular weight markers and loading controls, should be included. The manuscript also lacks essential statistical annotations and confidence intervals for many datasets.

Version 1:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Comments to the Point-by-Point Response to NCOMMS-25-10890-T

Title: Ameliorating calcium homeostasis improves longevity and healthspan in progeroid and naturally aged mice

Reviewer comments in between [].

Reviewer #1 (Remarks to the Author): The manuscript "Ameliorating calcium homeostasis improves longevity and healthspan in progeroid and naturally aged mice" describes how cytoplasmic accumulation of the protein S100A6 accelerates aging. Progeria patients show elevated levels, as do LMNA progeroid mice. S100A6 was found to bind PARP1, causing its ubiquitin-mediated degradation, thereby resulting in DNA damage. In general, the data are strong and back up the conclusions as drawn. I recommend publication, provided a few questions regarding senescence are addressed.

Response:

We gratefully acknowledge the reviewer for the positive assessment of our work, as well as for the insightful criticisms and constructive suggestions, which have been invaluable in improving the quality of the manuscript. We have carefully considered all the comments and have addressed each point in detail below, with corresponding revisions made to the manuscript accordingly.

Specific comments:

Q1: The authors argue that knockdown of S100A6 reduces SA- β -gal (Fig. 1k) and restores proliferation. However, a change from 60% to ~45% is of course not very likely to have a major impact. These cells should be followed (much) longer over time to see if the population fully gets into cell cycle progression, or whether they fall back into becoming largely senescent. Something similar holds true for 2ABP (Fig. 1y). How significant is the restoration of Lamin B1 (Fig. 1v) and a ~20% reduction in SA- β -gal?

Response: As suggested, after knocking down S100A6 in HGPS cells, we monitored the cells for 7 days to further clarify the role of S100A6 in senescence. We found that knocking down S100A6 for 4 or 7 days significantly reduced the proportion of SA- β -gal positive cells (Extended Data Fig. 2a, Fig. 1h and Fig. s1).

[These results are the same data s my comment was based on. In the new Fig. 1h, the reduction in SABGAL by shS100A6 is only some 15-20% (from ~60% to ~45%); this is not likely to be biologically meaningful. Hence I asked to do this over a longer period of time, which does not seem to have been done. This needs to be added.]

Furthermore, to assess the sustained effects on cell cycle re-entry, we performed Western blot analysis, which showed that the protein levels of Cyclin A2 and Lamin B1 were significantly restored at both 4 days and 7 days post S100A6 knockdown (Fig. 1f and Fig. s2).

Similarly, treatment with 2-APB for either 4 or 7 days significantly reduced the proportion of SA- β -gal positive HGPS patient cells (Fig. 4e, Fig. 4f and Fig. s4),

[Also in 4e and f, the difference in SABGL are minor. From ~60% to ~43% and ~38%; this is not likely to be biologically meaningful, raising the question as to whether the effects are on senescence or something else.]

accompanied by a marked recovery in the protein levels of Cyclin A2 and Lamin B1 (Fig. 4b and Fig. s3).

[The effects on Lamin B1 and Cyclin A2 in Fig. 4b are not particularly convincing. Other cell cycle and senescence markers should be examined to see if this effect is real]

Therefore, in subsequent experiments, we performed analyses at the 4-day time point to ensure timely and consistent evaluation while maintaining robust phenotypic changes.

We also provided detailed explanations in the revised manuscript (line 768-771, line 864-867 and line 1037-1038).

[See above; the data as presented right now are not convincing to claim a meaningful effect on (reducing) senescence.]

Extended Data Fig. 2 a, Fig. 1 h and Fig. s1. HGADFN127 cells were transfected with shS100A6 for 4 days (Extended Data Fig. 2a) and 7days (Fig. s1), followed by SA- β -gal staining. Scale bar: 100 μ m. The percentage of SA- β -gal positive cells was calculated in HGADFN127 cells transfected with shS100A6 for 4 days (Fig. 1h) and 7 days (Fig. s3).

Fig. 4 e-f and Fig. s4. HGADFN127 cells were treated with 2-APB for 4 days (Fig. 4e) and 7 days (Fig. s4), followed by SA- β -gal staining. Scale bar: 100 μ m. The percentage of SA- β -gal positive cells was calculated in HGADFN127 cells treated with 2-APB for 4 days (Fig. 4f) and 7 days (Fig. s4).

Fig. 1 f and Fig. s2. Western blot analysis of the protein levels of cyclin A2 and lamin B1 was performed in HGADFN127 cells that had been transfected with shS100A6 for 4 days (Fig. 1 f) and 7 days (Fig. s2).

Fig. 4 b and Fig. s3. Western blot analysis of the protein levels of cyclin A2, lamin B1, and S100A6 was performed in HGADFN127 cells treated with 2-APB for 4 days (Fig. 4 b) and 7 days (Fig. s3).

Q2: It is not particularly common to see expression of IL6 and IL8 measured through western blot, whereas QPCR works well. To make sure the bands measured here are indeed IL6/8, at least for the most crucial figures a QPCR should be added.

Response: Thank you for your valuable suggestions. As suggested, we added qPCR analysis to the most critical datasets (These data have been incorporated into Fig. 3g and k). Consistent with the finding from Western blot, both inhibition of NF- κ B signal and S100A6 knockdown significantly reduced the mRNA levels of IL6 and IL8 in HGPS cellular senescence. We also provided detailed explanations in the revised manuscript (line 840-841, and line 847-848). Fig. 3 (g-k). Quantitative RT-PCR analysis of IL6 and IL8 mRNA levels in HGADFN127 cells treated with p65 inhibitor RBM (10 μ M) for 24 h (g). Quantitative RT-PCR analysis of IL6 and IL8 mRNA levels in HGADFN127 cells with S100A6 deletion (k).

[Agreed]

Q3: The in vivo experiment with 2ABP and MIA is strong, in principle. However, what is the expression in these tissues (Heart, Lung, Muscle) of SA- β -gal, Lamin B1, PARP1, SASP etc? In other words; are the reduced drop in body weight, Overall Survival benefit etc linked to DDR, senescence/SASP, or might this be due to something else? PARP1 is measured in Fig. 8, adding credibility, but why not in Fig. 4 and 6? At least the obvious markers (SA- β -gal, PARP1, S100A6 and Lamin B1) should be added to the most crucial panels of these figures.

Response: Thank you for the valuable suggestions. As suggested, we supplemented immunohistochemical analysis of PARP1 in multiple tissue sections from LmnaG609G/G609G mice following 2-APB treatment and found that 2-APB increased PARP1 levels in multiple tissues such as the heart and lung (Fig. 4t-v).

[Agreed]

In addition, we examined the levels of PARP1, IL-6, IL-8, and Lamin B1 in multiple tissues from LmnaG609G/G609G mice after MIA treatment. MIA treatment increased PARP1 levels (Fig. 6u-w, Extended Data Fig. 6c-d), decreased IL-6 and IL-8 levels in multiple tissues of LmnaG609G/G609G mice (Fig. 6u, x, and y, Extended Data Fig. 6e-f). MIA treatment also increased the proportion of Lamin B1 positive cells in tissues of LmnaG609G/G609G mice, such as the lung and muscle (Extended Data Fig. 6g-h).

[IL6 (and IL8) staining is notoriously hard on (mouse) FFPE tissue sections. If this staining is real, it would be a major asset to the field. However, this also raises the bar as to whether it is real or not. Controls should be added. In all fairness, I think these should be moved to the supplementary figures as they will lead to discussion of an otherwise strong figure].

These findings indicate that MIA upregulates PARP1 and Lamin B1 protein levels and suppresses SASP secretion in multiple tissues of LmnaG609G/G609G mice. These results have been incorporated into Fig. 4, Fig. 6 and Extended Data Fig. 6.

We provided detailed explanations in the revision, as shown in line 886-887, line 965-967 and line 1100-1108.

Fig. 4 (t-v). IHC of PARP1 in the heart and lung of LmnaG609G/G609G mice (12 weeks), with or without 2-APB treatment (t, Scale bars, 50 μ m; n = 5). The quantification scores of PARP1 (u-v).

Fig. 6 (u-y). IHC was performed to detect S100A6, PARP1, IL6 and IL8 in the heart of LmnaG609G/G609G mice, with or without MIA treatment (u) (Scale bars, 50 μ m; n = 7). The quantification scores of S100A6 (v), PARP1 (w), IL6 (x) and IL8 (y) were also determined.

Extended Data Fig. 6 (c-h). IHC of PARP1 in lung and muscle of LmnaG609G/G609G mice (12 weeks), treated with or without 10 mg/kg MIA (c) (Scale bars, 100 μ m; n = 7). The quantification scores of S100A6 in lung and muscle (d). IHC of IL6 in muscle and IL8 in lung of LmnaG609G/G609G mice (12 weeks), treated with or without 10 mg/kg MIA (e) (Scale bars, 100 μ m; n = 7). The quantification scores of IL6 in muscle and IL8 in lung (f). IHC of Lamin B1 in lung and muscle of LmnaG609G/G609G mice (12 weeks), treated with or without 10 mg/kg MIA (g) (Scale bars, 50 μ m; n = 7). The quantification scores of Lamin B1 in lung and muscle (h).

Q4: The connection to cGAS/STING is not unlikely, but also not exactly proven by this study. Either this pathway and expression of these proteins themselves should be measured, or the claims should be made weaker in that this is a hypothesis, but not proven.

Response: Thank you for the insightful suggestion. To more clearly validate the involvement of cGAS/STING pathway in the phenomena we observed, we detected the levels of cGAS, STING, p-STING and its downstream signaling molecules including IRF3, p-IRF3, p65 and p-p65. The results showed that cGAS protein level was unchanged, total STING level was reduced, and p-STING was elevated in HGPS patient cells. Consistently, downstream IRF3 and NF- κ B signaling pathways were activated (Fig. 3d).

[Agreed - though this western blot result remains descriptive. The wording should be more careful as to allow room for judgement]

The HGPS patient HGADFN127 cells were further treated with IRF3 inhibitor Cyclo (phe-pro) (cFP) and p65 inhibitor Rubiadin-1-methyl-ether (RBM) respectively. The results showed that the mRNA levels of IL-6 and IL-8 in HGADFN127 cells decreased after treatment with p65 inhibitor RBM (Fig. 3e-g). These results indicate that during HGPS cellular senescence, the cGAS-STING-NF- κ B signaling pathway is activated, leading to the promotion of SASP factor transcription. These results have been incorporated into Fig. 3d-g in the revised manuscript.

[3f+g are convincing, 3e is not; this should be removed or improved. Why no QPCR with cFP in that regard?]

We provided detailed explanations in the revision, as shown in line 834-841.

Fig.3 (d-g). Western blot analysis of the protein levels of S100A6, cyclin A2, lamin B1, cGAS, STING, p-STING, IRF3, p-IRF3, p65, and p-p65 in CRL-1474 and HGADFN127 cells (d). Western blot analysis of the protein levels of IRF3, p-IRF3, IL-6 and IL-8 in HGADFN127 cells treated with IRF3 inhibitor cFP (2.5 mM) for 24 h (e). Western blot analysis of the protein levels of p65, p-p65, IL-6 and IL-8 in HGADFN127 cells treated with p65 inhibitor RBM (10 μ M) for 24 h (f). Quantitative RT-PCR analysis of IL6 and IL8 mRNA levels in HGADFN127 cells treated with p65 inhibitor RBM (10 μ M) for 24 h (g).

Minor:

Q1: Most figures addressing Ca^{2+} signaling show a measurement of its concentration in a sample. It would be helpful to examine this with a reporter system, or Calcein-AM as well.

Response: Thank you for the valuable suggestion. As suggested, we isolated cells from multiple tissues of LmnaG609G/G609G progeroid mice and used Fluo-4 AM to measure cytoplasmic Ca^{2+} concentration. Ca^{2+} levels were significantly elevated in the cells from various tissues, including heart, lung, skin, (Fig. 1i-k), muscle and liver of LmnaG609G/G609G progeroid mice (Extended Data Fig. 2c-d). Elevated Ca^{2+} concentrations were also found in the cytoplasm of HGPS patient cells (HGADFN127, HGADFN155 and HGADFN167) (Fig. 1l). Furthermore, we performed immunofluorescence detection of Ca^{2+} in CRL-1474 and HGADFN127 cells using Fluo-4 AM, which also revealed elevated cytoplasmic Ca^{2+} levels in HGADFN127 cells (Fig. 1m). These results further demonstrate the dysregulation of Ca^{2+} homeostasis in HGPS senescence. The results have been incorporated into Fig. 1i-m and Extended Data Fig. 2c-d.

We also provided detailed explanations in the revision, as shown in line 772-778, and line 1041-1043.

[Agreed]

Fig. 1 (i-m). (i-k). Flow cytometry detection of Ca^{2+} concentration in cells isolated from multi-tissues was performed using Fluo-4 AM, including the lung (i), skin (j), and heart (k) of Lmna+/+ and LmnaG609G/G609G mice (n=7). (l). Flow cytometry detection of Ca^{2+} concentration in HGPS patient cells was performed using Fluo-4 AM (CRL-1474, HGADFN127, HGADFN155 and HGADFN167 cells). (m). Immunofluorescence detection of Ca^{2+} in HGADFN127 and CRL-1474 cells was performed using Fluo4-AM. Scale bars: 10 μ m.

Extended Data Fig. 2 (c-d). Flow cytometry detection of Ca^{2+} concentration in cells isolated from multi-tissues was performed using Fluo-4 AM, including the muscle (c) and liver (d) of Lmna+/+ and LmnaG609G/G609G mice (n=7).

Q2: In Fig. 3a (and less so in 5o) it is unclear whether this was stable expression of Flag-progerin, or a transient expression. The latter is notoriously challenging in IMR90 and may well result in susceptibility to DNA damage per se, especially in case of

electroporation. An empty vector control would not to a similar response. A FLAG-GFP may transiently do something similar.

Response: We agree that distinguishing between stable and transient expression, as well as the potential confounding effects of transfection methods, is critical for proper interpretation of the results. The stable expression of progerin promotes cellular senescence and cell cycle arrest, making it extremely difficult to obtain stable cell lines through conventional selection. Instead, we used lentiviral transduction to offer high infection efficiency and enable stable expression of progerin after infection. In this study, Flag-progerin was expressed in IMR90 and CRL1474 cells via a lentiviral packaging system. We confirmed the stable progerin expression and the induction of a senescent phenotype in the cells after transduction before proceeding with subsequent experiments.

[Please indicate this in the text. Lentiviral transduction of proteins (i.e. not merely a vector control) can also cause DNA damage on its own, so the fact this data was generated as such is relevant to note.

Q3: The figure order is off here and there (Fig. 1, l m k).

Response: Thank you for this kind reminder. Panels l, m, and k of Fig. 1 have been corrected.

[Agreed]

Reviewer #2

(Remarks to the Author)

I think the authors have answered my questions.

Reviewer #3

(Remarks to the Author)

I thank the authors for making thorough revisions in response to my initial comments. Most of my concerns have been addressed to my satisfaction, and I appreciate the authors' use of fluorescence intensity to demonstrate changes in calcium concentrations. However, there are still several areas where further clarification or revision is needed.

1. Specific Force Calculation (related to Q3)

In my third comment, I suggested reporting specific force instead of absolute force, and I'm glad to see that the authors have

made this change. However, in the Methods section (lines 572–573), the manuscript incorrectly states that specific force was calculated by normalizing to the TA muscle mass. Specific force should instead be normalized to the cross-sectional area (mm²) of the muscle, which is indeed the unit used in the figures, but introduced incorrectly in the method. Moreover, there is currently no description in the Methods of how the muscle cross-sectional area was determined. The authors should add this methodological detail. Additionally, in the figure legend (lines 953–954), it should be clearly stated that the forces presented are specific forces normalized to muscle cross-sectional area.

2. Force-Frequency Relationship (related to Q4)

In response to my fourth comment, the authors mentioned that their pilot study data showed that 100 Hz stimulation is sufficient to evoke maximal force production. However, to determine the optimal stimulation frequency for maximum force generation, a force-frequency curve should be established. I do not see such data presented in the current manuscript. The authors should either include this data within the main figures or at least provide it in the supplementary materials as supporting evidence.

3. Loading Control for Western Blot (related to Q5)

I appreciate the authors' revision in changing the loading control from β -actin to GAPDH. However, GAPDH is also not an ideal housekeeping protein under certain physiological conditions, particularly those involving altered redox balance. For example, during aging, oxidative stress can significantly affect GAPDH content. Ideally, total protein normalization remains the gold standard for controlling sample loading. That said, since the authors used two different loading controls and obtained consistent results, I consider the current data acceptable.

4. Minor Concerns

I still have several minor concerns from my initial review for which I did not see a formal point-by-point response. Although I noticed that some of these issues have been revised, others appear to remain unaddressed. I encourage the authors to list all the minor concerns, similar to how they addressed the major comments, and indicate how each has been revised or resolved.

Reviewer #4

(Remarks to the Author)

The authors have adequately addressed my previous comments, and the revised manuscript has been significantly improved.

Version 2:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

Most of my concerns have been addressed. I do propose to change the conclusions regarding the effects of the shS100A6 effects on senescence though. These results are simply weak.

Q1 Response: We sincerely apologize that our description of the experimental setup was insufficiently clear in the first revised manuscript. Following your previous suggestion, we extended the S100A6 knockdown duration in HGPS cells from 4 to 7 days and assessed SA- β -gal positive proportion. The results showed that both 4-day and 7-day S100A6 knockdown reduced the proportion of SA- β -gal-positive cells and the reduction is appropriate 18% (4 days) and 20% (7 days) (Fig.1h and Supplementary Fig. 2b-c).

We think the modest change of SA- β -gal might be attributed to the fact that HGPS patient cells are already in a stable state of profound senescence, making it difficult to achieve a more substantial SA- β -gal reversal even after S100A6 knockdown. Actually, we observed that knockdown of S100A6 reduced the staining intensity of SA- β -gal-positive cells (Fig.1h and Supplementary Fig. 2b-c); however, in our quantification, these cells were still counted as positive, which may partly explain why SA- β -gal-positive proportion did not show a very significant decrease.

[The results in Fig. 1h, sup Fig 2b and Sup. Fig 2c are not convincing to argue that shS100A6 reduces senescence. This claim should therefore be removed from the manuscript]

To further confirm the role of S100A6 in HGPS senescence, we added more senescent markers in this version. Consistently, protein levels of Cyclin A2 and Lamin B1 were markedly restored, while IL-6, γ H2AX, p16, and p21, was downregulated after S100A6 knockdown for both 4 and 7 days (Fig.1f and Supplementary Fig. 2d). All these results showed that shS100A6 alleviated HGPS cellular senescence.

[Regarding this response “The results showed that both 4-day and 7-day S100A6 knockdown reduced the proportion of SA- β -gal-positive cells and the reduction is appropriate 18% (4 days) and 20% (7 days) (Fig.1h and Supplementary Fig. 2b-c).”: The results in Fig. 1h, sup Fig 2b and Sup. Fig 2c are not convincing to argue that shS100A6 reduces SABGAL/Senescence. This claim should therefore be removed from the manuscript.

[Regarding this response “We think the modest change of SA-β-gal might be attributed to the fact that HGPS patient cells are already in a stable state of profound senescence, making it difficult to achieve a more substantial SA-β-gal reversal even after S100A6 knockdown. Actually, we observed that knockdown of S100A6 reduced the staining intensity of SA-β-gal–positive cells (Fig.1h and Supplementary Fig. 2b-c); however, in our quantification, these cells were still counted as positive, which may partly explain why SA-β-gal–positive proportion did not show a very significant decrease.”

The last sentence here confirms there is not a very significant effect. Therefore the claim is an overstatement. If cells were counted as positive that are not, the quantification should be performed differently. With these figures, the statement that shS100A6 reduces SABGAL/Senescence is simply not correct.]

[Regarding this response “To further confirm the role of S100A6 in HGPS senescence, we added more senescent markers in this version. Consistently, protein levels of Cyclin A2 and Lamin B1 were markedly restored, while IL-6, γH2AX, p16, and p21, was downregulated after S100A6 knockdown for both 4 and 7 days (Fig.1f and Supplementary Fig. 2d). All these results showed that shS100A6 alleviated HGPS cellular senescence.”

These western blot results also show marginal effects (one would like to see the full blots to exclude exposure artefacts and a positive control lane next to them). Also, IL-6 is not something the field shows through western blot. All in all, the data to support the argument that shS100A6 reduces senescence is not convincing.]

I find the other questions to have been addressed.

Reviewer #3

(Remarks to the Author)

I have carefully reviewed the revised manuscript and the authors' responses to the previous comments. I am satisfied that all of my concerns have been thoroughly and appropriately addressed. The authors have revised the manuscript accordingly, and the changes have improved the clarity, rigor, and overall quality of the work.

In particular, the revised analyses, clarifications in the text, and additional data where provided have strengthened the conclusions and resolved the issues raised in earlier rounds of review. I have no further concerns or requests for revision and believe the manuscript is now suitable for publication.

Version 3:

Reviewer comments:

Reviewer #1

(Remarks to the Author)

These modifications improved the credibility of the data. The work is attractive and by focusing on the strongest results, this should make for an elegant piece of work. I am ok with publication in this form.

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Point-by-Point Response to NCOMMS-25-10890-T

Title: Ameliorating calcium homeostasis improves longevity and healthspan in progeroid and naturally aged mice

Reviewer #1 (Remarks to the Author): The manuscript “Ameliorating calcium homeostasis improves longevity and healthspan in progeroid and naturally aged mice” describes how cytoplasmic accumulation of the protein S100A6 accelerates aging. Progeria patients show elevated levels, as do LMNA progeroid mice. S100A6 was found to bind PARP1, causing its ubiquitin-mediated degradation, thereby resulting in DNA damage. In general, the data are strong and back up the conclusions as drawn. I recommend publication, provided a few questions regarding senescence are addressed.

Response:

We gratefully acknowledge the reviewer for the positive assessment of our work, as well as for the insightful criticisms and constructive suggestions, which have been invaluable in improving the quality of the manuscript. We have carefully considered all the comments and have addressed each point in detail below, with corresponding revisions made to the manuscript accordingly.

Specific comments:

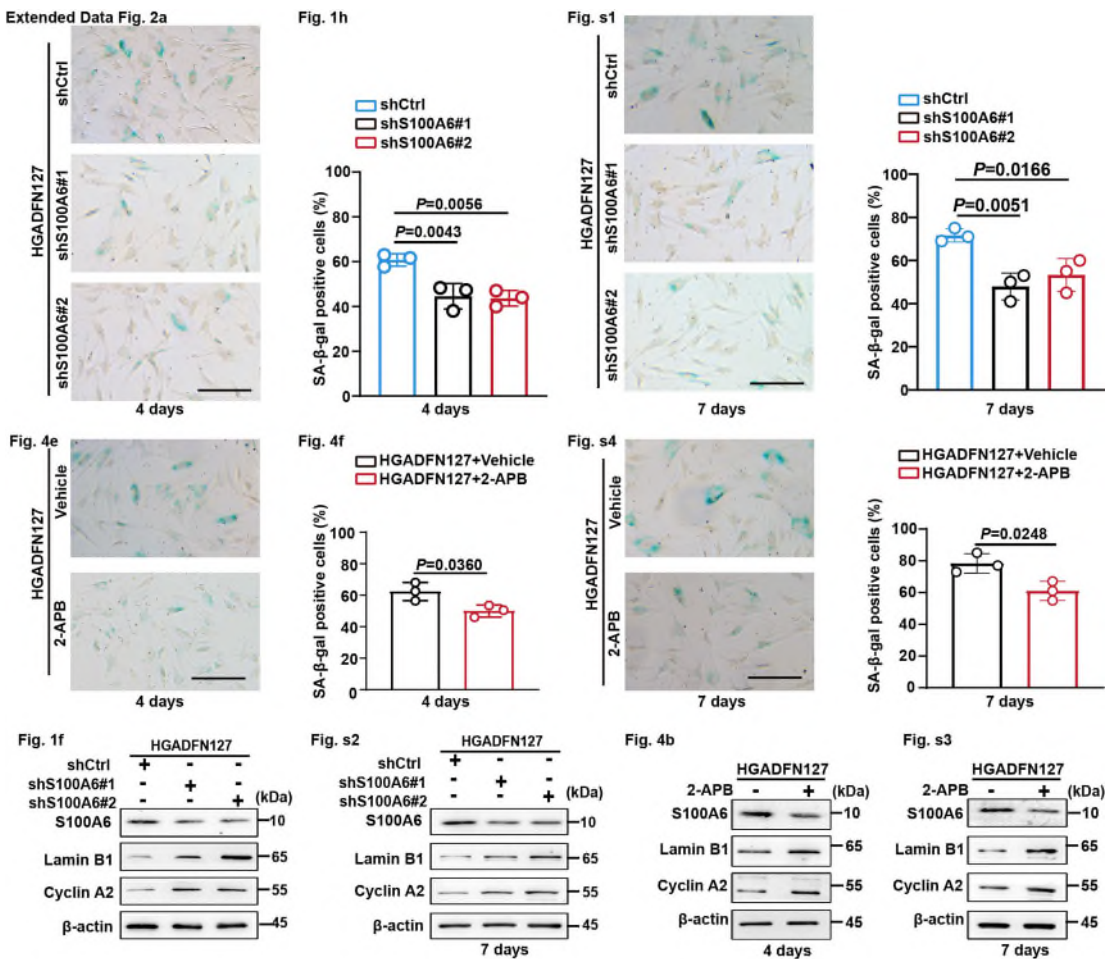
Q1: The authors argue that knockdown of S100A6 reduces SA- β -gal (Fig. 1k) and restores proliferation. However, a change from 60% to ~45% is of course not very likely to have a major impact. These cells should be followed (much) longer over time to see if the population fully gets into cell cycle progression, or whether they fall back into becoming largely senescent. Something similar holds true for 2ABP (Fig. 1y). How significant is the restoration of Lamin B1 (Fig. 1v) and a ~20% reduction in SA- β -gal?

Response: As suggested, after knocking down S100A6 in HGPS cells, we monitored the cells for 7 days to further clarify the role of S100A6 in senescence. We found that knocking down S100A6 for 4 or 7 days significantly reduced the proportion of SA- β -gal positive cells (Extended Data Fig. 2a, Fig. 1h and Fig. s1). Furthermore, to assess the sustained effects on cell cycle re-entry, we performed Western blot analysis, which

showed that the protein levels of Cyclin A2 and Lamin B1 were significantly restored at both 4 days and 7 days post S100A6 knockdown (Fig. 1f and Fig. s2).

Similarly, treatment with 2-APB for either 4 or 7 days significantly reduced the proportion of SA-β-gal positive HGPS patient cells (Fig. 4e, Fig. 4f and Fig. s4), accompanied by a marked recovery in the protein levels of Cyclin A2 and Lamin B1 (Fig. 4b and Fig. s3). Therefore, in subsequent experiments, we performed analyses at the 4-day time point to ensure timely and consistent evaluation while maintaining robust phenotypic changes.

We also provided detailed explanations in the revised manuscript (line 768-771, line 864-867 and line 1037-1038).



Extended Data Fig. 2 a, Fig. 1 h and Fig. s1. HGADFN127 cells were transfected with shS100A6 for 4 days (**Extended Data Fig. 2a**) and 7days (**Fig. s1**), followed by SA-β-gal staining. Scale bar: 100 μm. The percentage of SA-β-gal positive cells was calculated in HGADFN127 cells transfected with shS100A6 for 4 days (**Fig. 1h**) and 7 days (**Fig. s3**).

Fig. 4 e-f and Fig. s4. HGADFN127 cells were treated with 2-APB for 4 days (**Fig. 4e**) and 7 days (**Fig. s4**), followed by SA- β -gal staining. Scale bar: 100 μ m. The percentage of SA- β -gal positive cells was calculated in HGADFN127 cells treated with 2-APB for 4 days (**Fig. 4f**) and 7 days (**Fig. s4**).

Fig. 1 f and Fig. s2. Western blot analysis of the protein levels of cyclin A2 and lamin B1 was performed in HGADFN127 cells that had been transfected with shS100A6 for 4 days (**Fig. 1 f**) and 7 days (**Fig. s2**).

Fig. 4 b and Fig. s3. Western blot analysis of the protein levels of cyclin A2, lamin B1, and S100A6 was performed in HGADFN127 cells treated with 2-APB for 4 days (**Fig. 4 b**) and 7 days (**Fig. s3**).

Q2: It is not particularly common to see expression of IL6 and IL8 measured through western blot, whereas QPCR works well. To make sure the bands measured here are indeed IL6/8, at least for the most crucial figures a QPCR should be added.

Response: Thank you for your valuable suggestions. As suggested, we added qPCR analysis to the most critical datasets (These data have been incorporated into Fig. 3g and k). Consistent with the finding from Western blot, both inhibition of NF- κ B signal and S100A6 knockdown significantly reduced the mRNA levels of IL6 and IL8 in HGPS cellular senescence.

We also provided detailed explanations in the revised manuscript (line 840-841, and line 847-848).

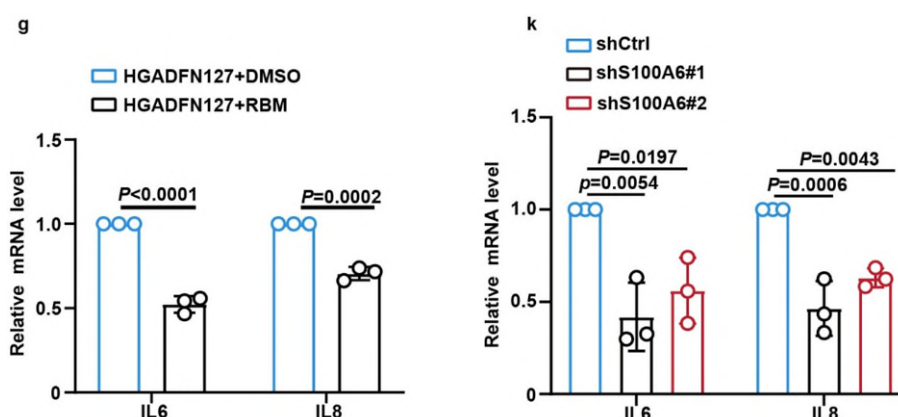


Fig. 3 (g-k). Quantitative RT-PCR analysis of IL6 and IL8 mRNA levels in HGADFN127 cells treated with p65 inhibitor RBM (10 μ M) for 24 h (**g**). Quantitative RT-PCR analysis of IL6 and IL8 mRNA levels in HGADFN127 cells with S100A6 deletion (**k**).

Q3: The in vivo experiment with 2ABP and MIA is strong, in principle. However, what is the expression in these tissues (Heart, Lung, Muscle) of SA- β -gal, Lamin B1, PARP1,

SASP etc? In other words; are the reduced drop in body weight, Overall Survival benefit etc linked to DDR, senescence/SASP, or might this be due to something else? PARP1 is measured in Fig. 8, adding credibility, but why not in Fig. 4 and 6? At least the obvious markers (SA- β -gal, PARP1, S100A6 and Lamin B1) should be added to the most crucial panels of these figures.

Response: Thank you for the valuable suggestions. As suggested, we supplemented immunohistochemical analysis of PARP1 in multiple tissue sections from *Lmna*^{G609G/G609G} mice following 2-APB treatment and found that 2-APB increased PARP1 levels in multiple tissues such as the heart and lung (Fig. 4t-v). In addition, we examined the levels of PARP1, IL-6, IL-8, and Lamin B1 in multiple tissues from *Lmna*^{G609G/G609G} mice after MIA treatment. MIA treatment increased PARP1 levels (Fig. 6u-w, Extended Data Fig. 6c-d), decreased IL-6 and IL-8 levels in multiple tissues of *Lmna*^{G609G/G609G} mice (Fig. 6u, x, and y, Extended Data Fig. 6e-f). MIA treatment also increased the proportion of Lamin B1 positive cells in tissues of *Lmna*^{G609G/G609G} mice, such as the lung and muscle (Extended Data Fig. 6g-h). These findings indicate that MIA upregulates PARP1 and Lamin B1 protein levels and suppresses SASP secretion in multiple tissues of *Lmna*^{G609G/G609G} mice. These results have been incorporated into Fig. 4, Fig. 6 and Extended Data Fig. 6.

We provided detailed explanations in the revision, as shown in line 886-887, line 965-967 and line 1100-1108.

Fig.4 t

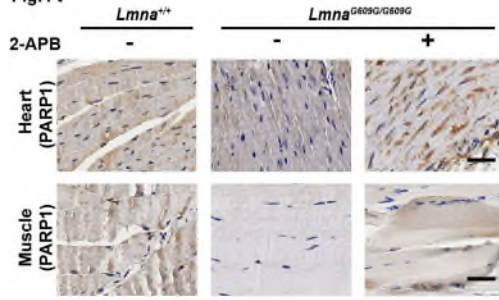


Fig.4 u

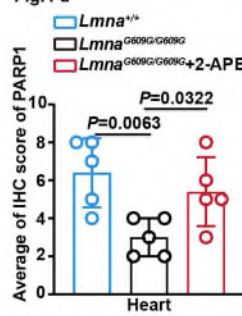


Fig.4 v

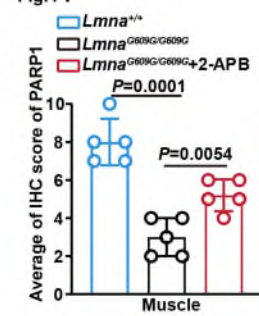


Fig. 6 u

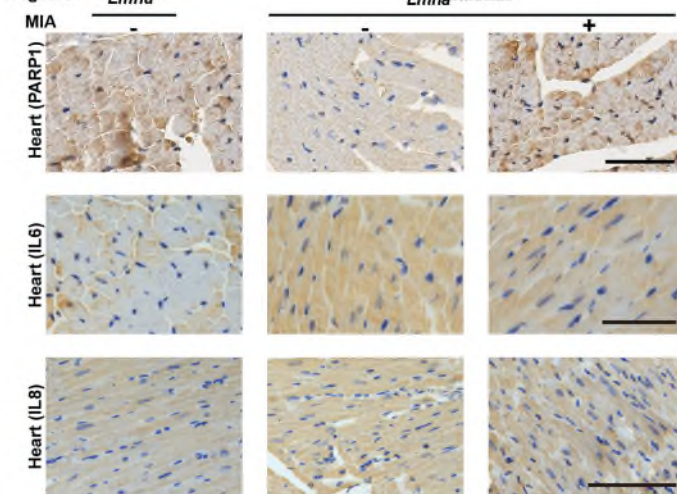


Fig. 6 w

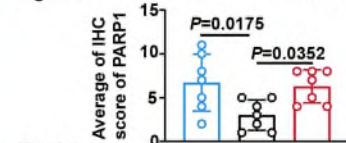


Fig. 6 x

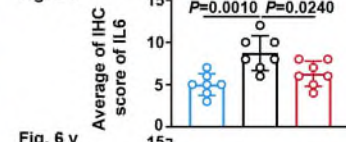
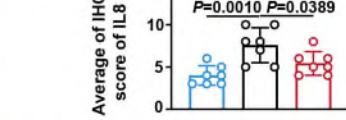
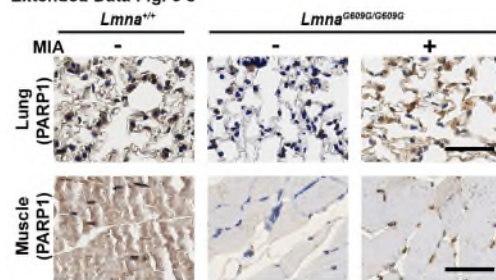


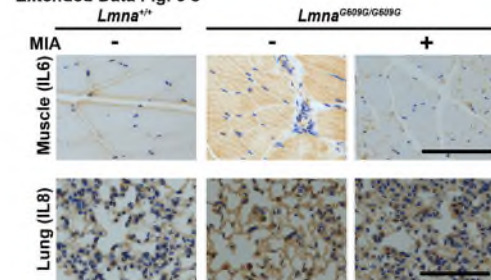
Fig. 6 y



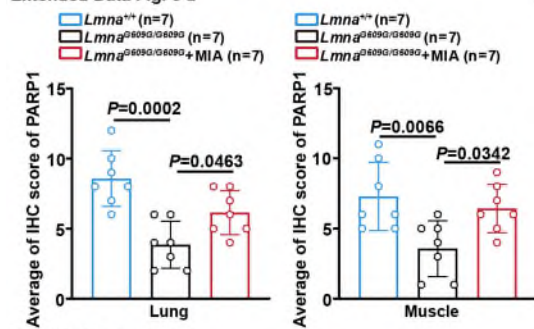
Extended Data Fig. 6 c



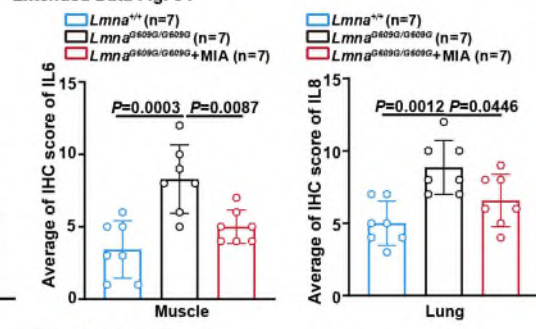
Extended Data Fig. 6 e



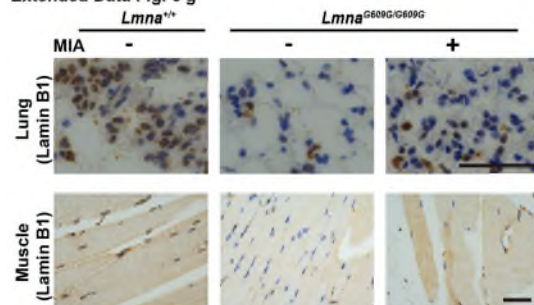
Extended Data Fig. 6 d



Extended Data Fig. 6 f



Extended Data Fig. 6 g



Extended Data Fig. 6 h

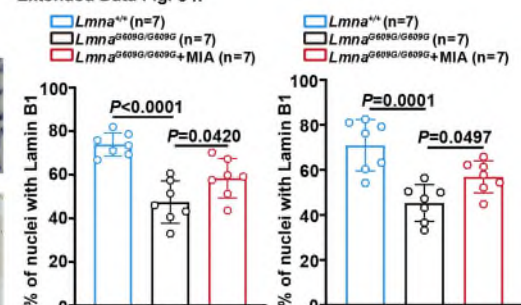


Fig. 4 (t-v). IHC of PARP1 in the heart and lung of *Lmna*^{G609G/G609G} mice (12 weeks), with or without 2-APB treatment (**t**, Scale bars, 50 μ m; n = 5). The quantification scores of PARP1 (**u-v**).

Fig. 6 (u-y). IHC was performed to detect S100A6, PARP1, IL6 and IL8 in the heart of *Lmna*^{G609G/G609G} mice, with or without MIA treatment (**u**) (Scale bars, 50 μ m; n = 7). The quantification scores of S100A6 (**v**), PARP1(**w**), IL6 (**x**) and IL8 (**y**) were also determined.

Extended Data Fig. 6 (c-h). IHC of PARP1 in lung and muscle of *Lmna*^{G609G/G609G} mice (12 weeks), treated with or without 10 mg/kg MIA (**c**) (Scale bars, 100 μ m; n = 7). The quantification scores of S100A6 in lung and muscle (**d**). IHC of IL6 in muscle and IL8 in lung of *Lmna*^{G609G/G609G} mice (12 weeks), treated with or without 10 mg/kg MIA (**e**) (Scale bars, 100 μ m; n = 7). The quantification scores of IL6 in muscle and IL8 in lung (**f**). IHC of Lamin B1 in lung and muscle of *Lmna*^{G609G/G609G} mice (12 weeks), treated with or without 10 mg/kg MIA (**g**) (Scale bars, 50 μ m; n = 7). The quantification scores of Lamin B1 in lung and muscle (**h**).

Q4: The connection to cGAS/STING is not unlikely, but also not exactly proven by this study. Either this pathway and expression of these proteins themselves should be measured, or the claims should be made weaker in that this is a hypothesis, but not proven.

Response: Thank you for the insightful suggestion. To more clearly validate the involvement of cGAS/STING pathway in the phenomena we observed, we detected the levels of cGAS, STING, p-STING and its downstream signaling molecules including IRF3, p-IRF3, p65 and p-p65. The results showed that cGAS protein level was unchanged, total STING level was reduced, and p-STING was elevated in HGPS patient cells. Consistently, downstream IRF3 and NF- κ B signaling pathways were activated (Fig. 3d). The HGPS patient HGADFN127 cells were further treated with IRF3 inhibitor Cyclo (phe-pro) (cFP) and p65 inhibitor Rubiadin-1-methyl-ether (RBM) respectively. The results showed that the mRNA levels of IL-6 and IL-8 in HGADFN127 cells decreased after treatment with p65 inhibitor RBM (Fig. 3e-g). These results indicate that during HGPS cellular senescence, the cGAS-STING-NF- κ B signaling pathway is activated, leading to the promotion of SASP factor transcription. These results have been incorporated into Fig. 3d-g in the revised manuscript.

We provided detailed explanations in the revision, as shown in line 834-841.

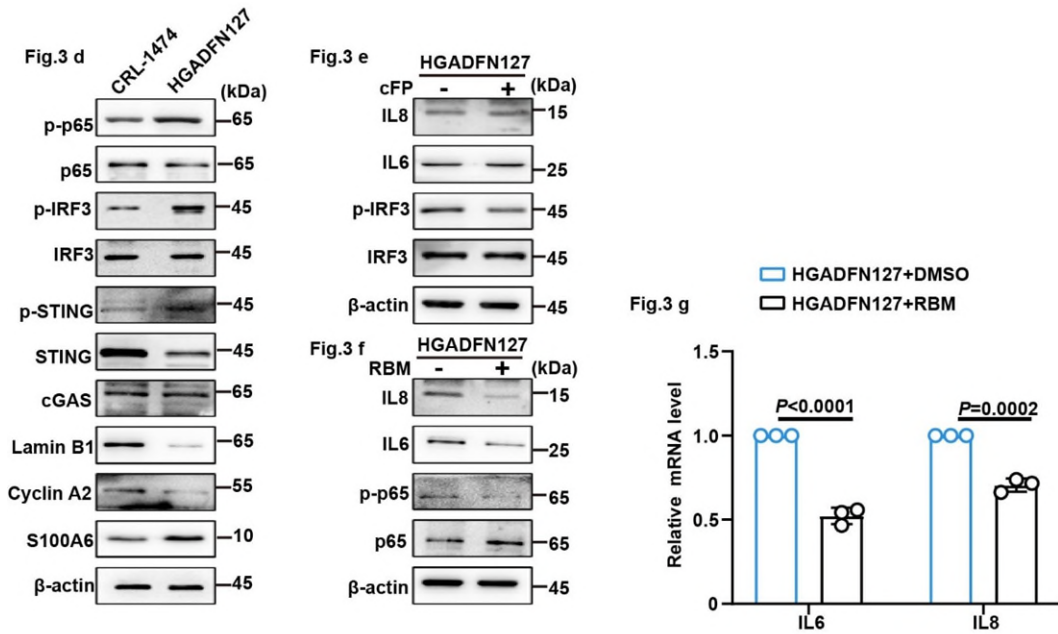


Fig. 3 (d-g). Western blot analysis of the protein levels of S100A6, cyclin A2, lamin B1, cGAS, STING, p-STING, IRF3, p-IRF3, p65, and p-p65 in CRL-1474 and HGADFN127 cells (**d**). Western blot analysis of the protein levels of IRF3, p-IRF3, IL-6 and IL-8 in HGADFN127 cells treated with IRF3 inhibitor cFP (2.5 mM) for 24 h (**e**). Western blot analysis of the protein levels of p65, p-p65, IL-6 and IL-8 in HGADFN127 cells treated with p65 inhibitor RBM (10 μ M) for 24 h (**f**). Quantitative RT-PCR analysis of IL6 and IL8 mRNA levels in HGADFN127 cells treated with p65 inhibitor RBM (10 μ M) for 24 h (**g**).

Minor:

Q1: Most figures addressing Ca^{2+} signaling show a measurement of its concentration in a sample. It would be helpful to examine this with a reporter system, or Calcein-AM as well.

Response: Thank you for the valuable suggestion. As suggested, we isolated cells from multiple tissues of *Lmna*^{G609G/G609G} progeroid mice and used Fluo-4 AM to measure cytoplasmic Ca^{2+} concentration. Ca^{2+} levels were significantly elevated in the cells from various tissues, including heart, lung, skin, (Fig. 1i-k), muscle and liver of *Lmna*^{G609G/G609G} progeroid mice (Extended Data Fig. 2c-d). Elevated Ca^{2+} concentrations were also found in the cytoplasm of HGPS patient cells (HGADFN127, HGADFN155 and HGADFN167) (Fig. 1l). Furthermore, we performed immunofluorescence detection of Ca^{2+} in CRL-1474 and HGADFN127 cells using Fluo-4 AM, which also revealed elevated cytoplasmic Ca^{2+} levels in HGADFN127 cells

(Fig. 1m). These results further demonstrate the dysregulation of Ca^{2+} homeostasis in HGPS senescence. The results have been incorporated into Fig. 1i-m and Extended Data Fig. 2c-d.

We also provided detailed explanations in the revision, as shown in line 772-778, and line 1041-1043.

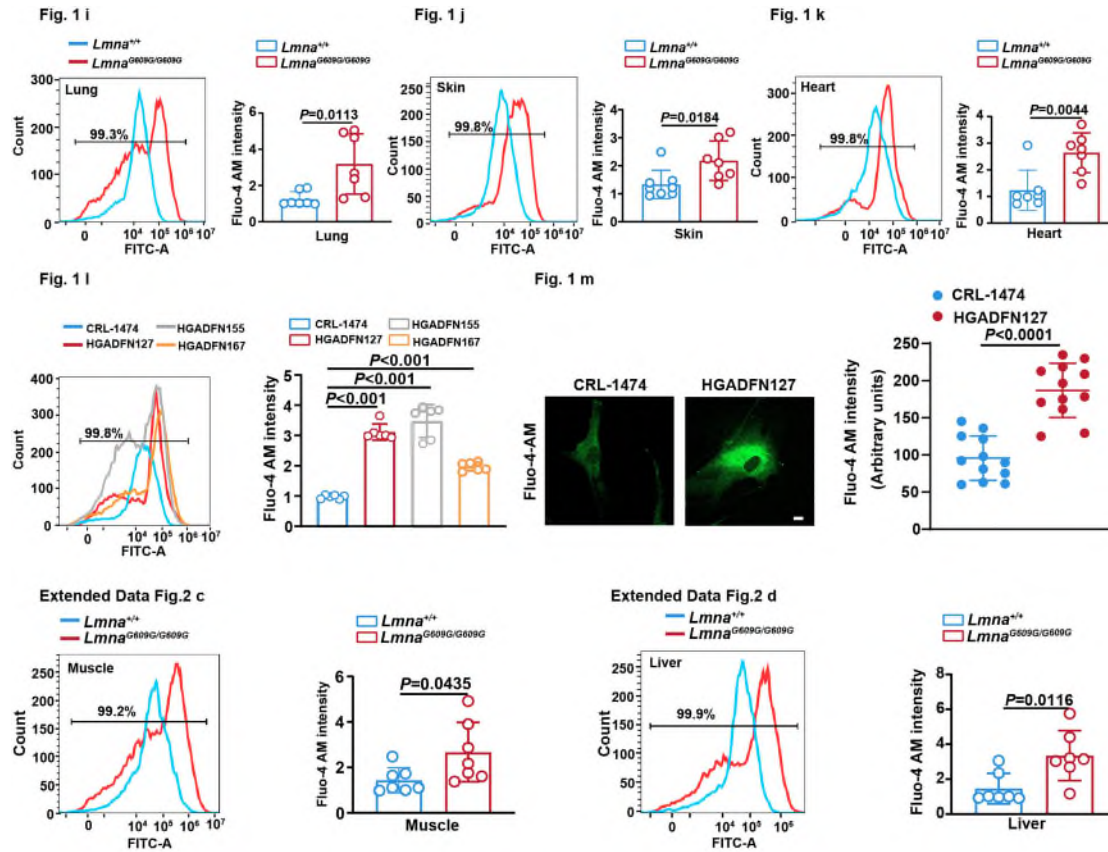


Fig. 1 (i-m). (i-k). Flow cytometry detection of Ca^{2+} concentration in cells isolated from multi-tissues was performed using Fluo-4 AM, including the lung (i), skin (j), and heart (k) of $\text{Lmna}^{+/+}$ and $\text{Lmna}^{\text{G609G/G609G}}$ mice (n=7). (l). Flow cytometry detection of Ca^{2+} concentration in HGPS patient cells was performed using Fluo-4 AM (CRL-1474, HGADFN127, HGADFN155 and HGADFN167 cells). (m). Immunofluorescence detection of Ca^{2+} in HGADFN127 and CRL-1474 cells was performed using Fluo4-AM. Scale bars: 10 μm .

Extended Data Fig. 2 (c-d). Flow cytometry detection of Ca^{2+} concentration in cells isolated from multi-tissues was performed using Fluo-4 AM, including the muscle (c) and liver (d) of $\text{Lmna}^{+/+}$ and $\text{Lmna}^{\text{G609G/G609G}}$ mice (n=7).

Q2: In Fig. 3a (and less so in 5o) it is unclear whether this was stable expression of Flag-progerin, or a transient expression. The latter is notoriously challenging in IMR90 and may well result in susceptibility to DNA damage per se, especially in case of

electroporation. An empty vector control would not to a similar response. A FLAG-GFP may transiently do something similar.

Response: We agree that distinguishing between stable and transient expression, as well as the potential confounding effects of transfection methods, is critical for proper interpretation of the results. The stable expression of progerin promotes cellular senescence and cell cycle arrest, making it extremely difficult to obtain stable cell lines through conventional selection. Instead, we used lentiviral transduction to offer high infection efficiency and enable stable expression of progerin after infection. In this study, Flag-progerin was expressed in IMR90 and CRL1474 cells via a lentiviral packaging system. We confirmed the stable progerin expression and the induction of a senescent phenotype in the cells after transduction before proceeding with subsequent experiments.

Q3: The figure order is off here and there (Fig. 1, l m k).

Response: Thank you for this kind reminder. Panels l, m, and k of Fig. 1 have been corrected.

Reviewer #2 (Remarks to the Author): In this study, Xiang et.al. sought to link cytoplasmic calcium homeostasis to aging and Hutchinson-Gilford progeria syndrome (HGPS), and claimed a mechanism involving S100A6-mediated PARP1 degradation, DNA damage and cGAS-STING pathway activation. The authors further demonstrated that mianserin (MIA), a tetracyclic antidepressant, ameliorated senescence phenotypes in HGPS and naturally aged models by restoring calcium homeostasis.

The major problem of this work is that the authors used wrong methods to measure cytoplasmic and ER Ca^{2+} . No reliable data related to cytoplasmic Ca^{2+} homeostasis was presented.

Response: We sincerely appreciate the reviewer's careful and critical evaluation of our manuscript. We believe the reviewer's comments are highly valuable in further confirming our conclusions especially regarding the detection of Ca^{2+} concentration. In the revised manuscript, we improved the detection methods of Ca^{2+} in cells and tissues. We also acknowledge that the reviewer has raised several important concerns regarding the mechanistic insights and methodological rigor. In response, we addressed each comment in a point-by-point manner.

Specific comments:

Q1: The authors measured the total Ca^{2+} in various tissues using a kit. However, as extracellular Ca^{2+} is 4 orders of magnitude higher than cytoplasmic Ca^{2+} , the variations in extracellular fluid (including blood) precludes a reliable estimation of intracellular Ca^{2+} . Total Ca^{2+} measurement of tissues should not be used to support any conclusion related to cytoplasmic Ca^{2+} .

Response: Thanks a lot for pointing out our mistake in detecting the cytoplasmic Ca^{2+} in mice tissues. We did ignore the source of Ca^{2+} in the tissues. To detect the Ca^{2+} level in the cells of tissues, we isolated cells from multiple tissues of *Lmna*^{G609G/G609G} mice and used Fluo-4 AM to measure the cytoplasmic Ca^{2+} concentration. Ca^{2+} levels were significantly elevated in the cells from various tissues, including heart, lung, skin,

muscle and liver of *Lmna*^{G609G/G609G} progeroid mice (Fig. 1i-k, Extended Data Fig. 2c-d). The results have been incorporated into Fig. 1i-m and Extended Data Fig. 2c-d.

Our results showed that both the cytoplasmic Ca²⁺ levels and the tissue Ca²⁺ levels were found elevated in HGPS mice, which was consistent with the reports indicating that increased intracellular calcium levels can also affect tissue calcium concentrations^[1-3].

We want to thank the reviewer again for pointing out our mistake to further improve our conclusion.

We also provided detailed explanations in the revision, as shown in line 772-774, and line 1041-1043.

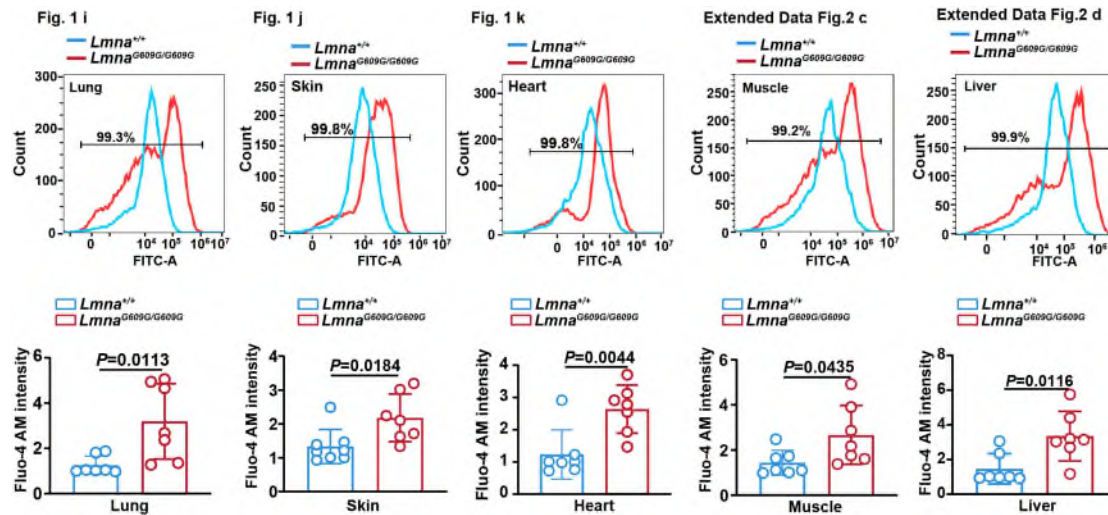


Fig. 1 (i-k). Flow cytometry detection of Ca²⁺ concentration in cells isolated from multi-tissues was performed using Fluo-4 AM, including the lung (i), skin (j), and heart (k) of *Lmna*^{+/+} and *Lmna*^{G609G/G609G} mice (n=7).

Extended Data Fig. 2 (c-d). Flow cytometry detection of Ca²⁺ concentration in cells isolated from multi-tissues was performed using Fluo-4 AM, including the muscle (c) and liver (d) of *Lmna*^{+/+} and *Lmna*^{G609G/G609G} mice (n=7).

- [1] Chao C T, Tsai Y T, Lee W T, et al. Deep Learning-Assisted Repurposing of Plant Compounds for Treating Vascular Calcification: An In Silico Study with Experimental Validation [J]. *Oxidative medicine and cellular longevity*, 2022, 2022: 4378413.
- [2] Princen K, Van Dooren T, van Gorsel M, et al. Pharmacological modulation of septins restores calcium homeostasis and is neuroprotective in models of Alzheimer's disease [J]. *Science (New York, NY)*, 2024, 384(6699).
- [3] Shroff R C, McNair R, Skepper J N, et al. Chronic mineral dysregulation promotes vascular smooth muscle cell adaptation and extracellular matrix calcification [J]. *Journal of the American Society of Nephrology : JASN*, 2010, 21(1): 103-112.
- [4] Bartok A, Weaver D, Golenár T, et al. IP(3) receptor isoforms differently regulate ER-

mitochondrial contacts and local calcium transfer [J]. Nature communications, 2019, 10(1): 3726.

- [5] Ye L, Zeng Q, Ling M, et al. Inhibition of IP3R/Ca²⁺ Dysregulation Protects Mice From Ventilator-Induced Lung Injury via Endoplasmic Reticulum and Mitochondrial Pathways [J]. Frontiers in immunology, 2021, 12: 729094.

Q2: The authors used Fluo-4 AM and ER-tracker to stain Ca²⁺ and the ER and stated that concentration of Ca²⁺ in the ER decreased while that in the cytoplasm increased. Fluo-4 is an indicator used to measure Ca²⁺ lower than micromolar, and is not able to detect ER Ca²⁺ which was much higher. Moreover, ER of many cell types lacks the enzyme to digest the AM ester, keeping Fluo-4 AM inactive in ER.

Response: We appreciate the reviewer's constructive comments. In the revised version, after finding elevated cytoplasmic Ca²⁺ in HGPS patient cells by using Fluo-4 AM (Fig. 1l and m), we further investigated the source of the elevated cytoplasmic Ca²⁺ using Mag-Fluo-4 AM and Rhod-2 AM to detect Ca²⁺ content in ER and mitochondria. Compared to CRL-1474 cells, the Ca²⁺ level in ER of HGADFN127 cells was decreased, while mitochondrial Ca²⁺ concentration remained unchanged (Fig. 1r-s), indicating that the increased cytoplasmic Ca²⁺ levels in HGPS cells are likely derived from ER. As a key factor regulating ER calcium efflux, we found that IP3R was highly expressed in HGPS patient HGADFN127 and HGADFN167 cells (Fig. 1t). Both IP3R inhibitors Xestospongine C (XeC) and 2-aminobenzyl diphenylborate (2-APB) reduced cytoplasmic Ca²⁺ concentration (Extended Data Fig. 2e-f). The above results suggest Ca²⁺ releases from ER into the cytoplasm during HGPS cellular senescence.

We also provided detailed explanations in the revision, as shown in line 775-778, line 786-791 and line 1044-1047.

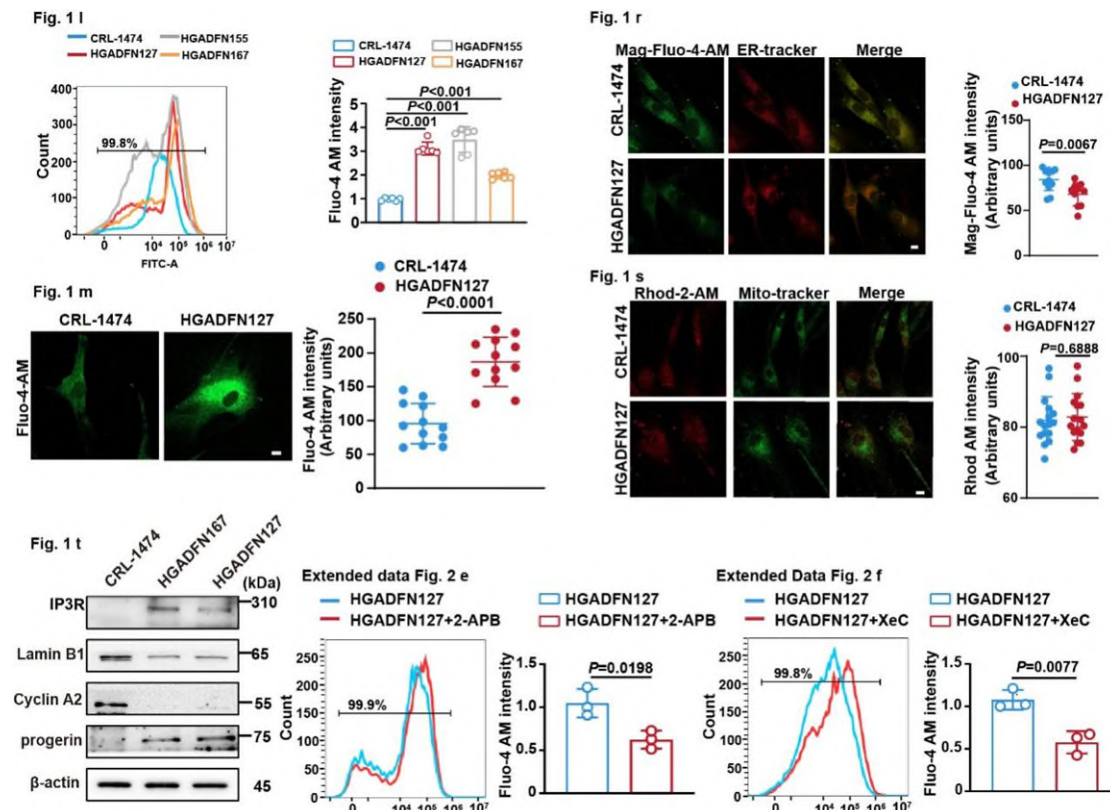


Fig. 1(l-m). Flow cytometry detection of Ca^{2+} concentration in HGPS patient cells was performed using Fluo-4 AM (CRL-1474, HGADFN127, HGADFN155 and HGADFN167 cells) (l). Immunofluorescence detection of Ca^{2+} in HGADFN127 and CRL-1474 cells was performed using Fluo4-AM (m). Scale bars: 10 μm. Immunofluorescence detection of Ca^{2+} in ER of HGADFN127 and CRL-1474 cells was performed using Mag-Fluo4 AM and ER-Tracker (r). Immunofluorescence detection of Ca^{2+} in the mitochondria of HGADFN127 and CRL-1474 cells was performed using Rhod-2 AM and Mito-tracker (s). Western blot analysis of the protein levels of progerin, cyclin A2, lamin B1, and IP3R was performed in CRL-1474, HGADFN167, and HGADFN127 cells (t).

Extended Data Fig. 2 (e-f). Flow cytometry detection of Ca^{2+} concentration in HGADFN127 cells transfected with 2-APB was performed using Fluo4-AM (e). Flow cytometry detection of Ca^{2+} concentration in HGADFN127 cells transfected with XeC was performed using Fluo4-AM (f).

Q3: The measurement of downstream signals of cGAS-STING, such as IL-6 and IL-8 by the whole-cell lysate is unreliable. Since these cytokines are secreted, the detected signal represents, to unknown extent, their inactive precursors, rather than functional secreted proteins.

Response: Thank you for your valuable suggestion on our study. IL-6 and IL-8 are downstream effectors of cGAS-STING and NF-κB signaling pathways, and their

expression is primarily regulated at the transcriptional level. In senescent cells, these two cytokines not only exhibit elevated level in their secreted forms, but also show significantly increased levels in their precursor forms ^[1-3]. To more precisely validate our results, we used qRT-PCR to detect mRNA levels of IL6 and IL8 (Fig.3 f-g, j-k). The results demonstrated that both inhibition of NF-κB activation and S100A6 knockdown significantly reduced the mRNA levels of IL6 and IL8 in HGPS cell senescence. The results have been incorporated into Fig. 3f-k.

We also provided detailed explanations in the revision, as shown in line 838-841, and line 845-858.

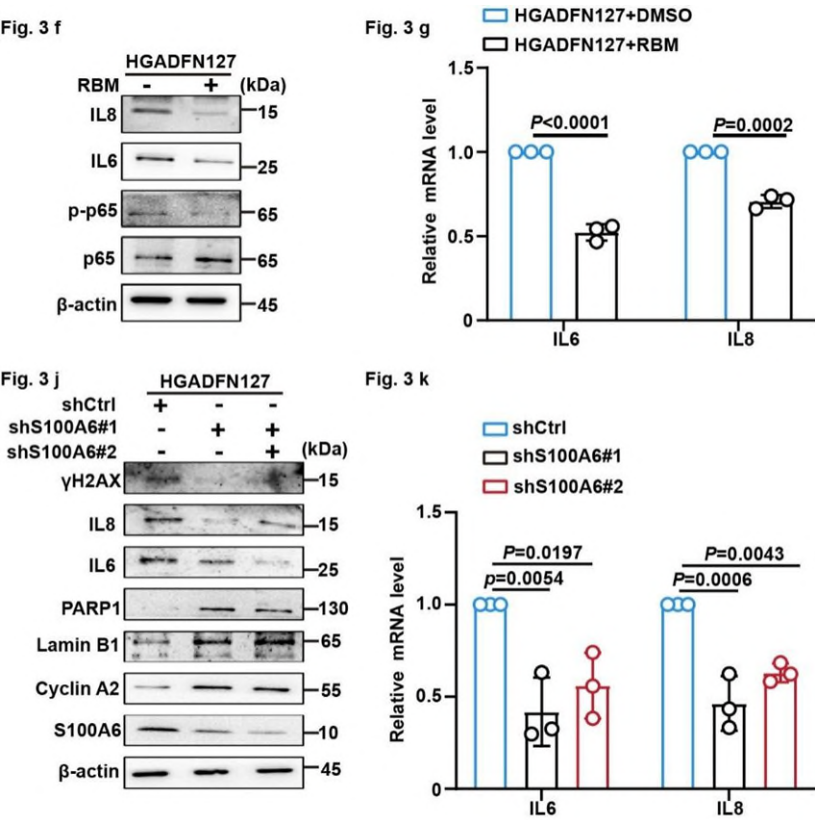


Fig. 3 (f-g). Western blot analysis of the protein levels of p65, p-p65, IL-6 and IL-8 in HGADFN127 cells treated with p65 inhibitor RBM (10 μM) for 24 h (f). Quantitative RT-PCR analysis of IL6 and IL8 mRNA levels in HGADFN127 cells treated with p65 inhibitor RBM (10 μM) for 24 h (g). Western blot analysis of the protein levels of S100A6, cyclin A2, lamin B1, IL6, IL8, and γH2AX in HGADFN127 cells infected with shRNA (shCtrl, shS100A6#1 and shS100A6#2) (j). Quantitative RT-PCR analysis of IL6 and IL8 mRNA levels in HGADFN127 cells infected with shRNA (shCtrl, shS100A6#1 and shS100A6#2) (k).

[1] Zhang R T, Zhang M, Wang G X, et al. Long-chain chlorinated paraffins (LCCPs) exposure causes senescence and inflammatory damage in cardiomyocytes [J]. Journal of

Environmental Management, 2025, 375.

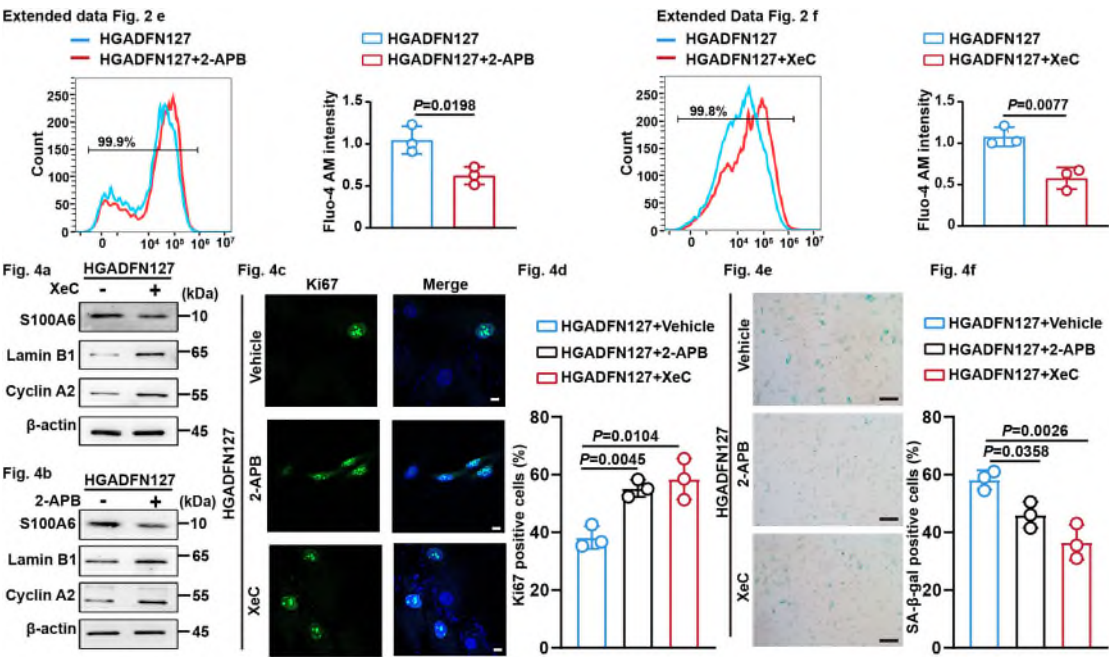
[2] Su T T, Li C, Zhang Y, et al. Upregulation of HMGB1 promotes vascular dysfunction in the soft palate of patients with obstructive sleep apnea via the TLR4/NF-κB/VEGF pathway [J]. *Febs Open Bio*, 2023, 13(2): 246-256.

[3] Chang J, Zhang W. Remifentanyl modulates the TLR4-mediated MMP-9/TIMP1 balance and NF-κB/STAT3 signaling in LPS-induced A549 cells [J]. *Experimental and therapeutic medicine*, 2023, 25(2): 79.

Q4: 2-APB is a “dirty” (without specificity) inhibitor of some Ca²⁺ permeable channels, not a “calcium inhibitor” as the authors said. Specific calcium signaling pathways (e.g., SOCE, IP3R or TRP channels), should be determined by reliable methods.

Response: We thank the reviewer for this important comment. As described in response to Q2, we found that the elevated cytoplasmic Ca²⁺ levels in HGPS cells originated from ER and found that IP3R, a key channel regulating ER calcium efflux, was upregulated. In the revised manuscript, in addition to 2-APB, we treated HGPS cells with another IP3R inhibitor Xestospongin C (XeC). Both IP3R inhibitors 2-APB and XeC significantly reduced intracellular Ca²⁺ levels (Extended Data Fig. 2e and f) and delayed cellular senescence (Fig. 4a-f). The results have been incorporated into Fig. 4a-f and Extended Data Fig. 2e-f.

We also provided detailed explanations in the revision, as shown in line 864-867 and line 1044-1047.



Extended Data Fig. 2 (e-f). Flow cytometry detection of Ca^{2+} concentration in HGADFN127 cells transfected with 2-APB was performed using Fluo4-AM (e). Flow cytometry detection of Ca^{2+} concentration in HGADFN127 cells transfected with XeC was performed using Fluo4-AM (f).

Fig. 4(a-f). Western blot analysis of the protein levels of cyclin A2, lamin B1, and S100A6 was performed in HGADFN127 cells treated with XeC and 2-APB for 4 days (a-b). Immunofluorescence staining of Ki67 in a-b (c). Scale bars: 10 μm . The percentage of Ki67 positive cells was calculated (d). The percentage of SA- β -gal positive cells was calculated (e-f). Scale bars: 100 μm .

Q5: Statistical issues. For example, many biochemical data lack statistical analysis. What statistical test was used for Kaplan-Meier survival curves? Why the control group has a high baseline SA- β -gal signal? SA- β -gal staining images should be provided.

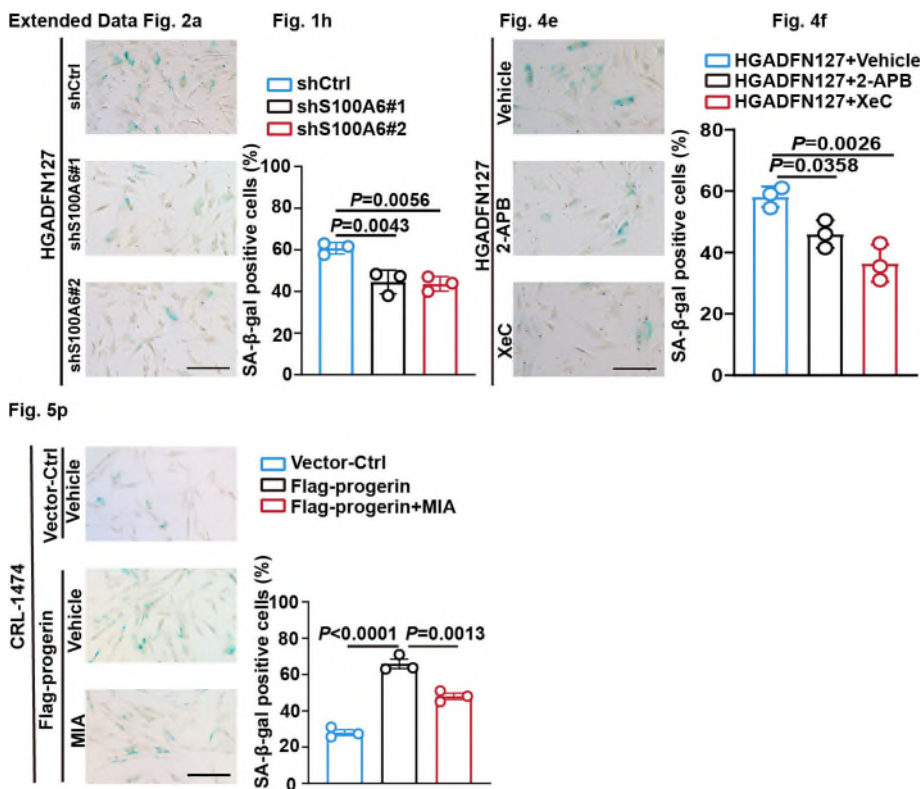
Response: We sincerely appreciate the reviewer's insightful comments regarding statistical analysis and data presentation in our study. In the revised manuscript, we carefully re-evaluated all biochemical experiments and performed appropriate statistical analyses for the datasets that were previously lacking such analysis. Specifically, data are presented as mean $\pm$ SEM. Statistical significance was assessed using an unpaired two-tailed Student's *t*-test for comparisons between two groups, or one-way ANOVA followed by appropriate post-hoc tests for multiple-group comparisons, as applicable. The details of replication and statistical analysis are described in the Methods section.

For the Kaplan-Meier survival curves, we used the Log-rank test to assess the significance of differences in survival rates between groups. We also provided detailed explanations in the revised manuscript (line 590-591).

In the case of SA- β -gal signal in control groups, because many of the control groups were performed using fibroblasts derived from HGPS patients (such as HGADFN127 cells), these cells inherently display elevated SA- β -gal activity (Extended Data Fig. 2a, Fig. 1h, Fig. 4e-f). Meanwhile, the fibroblasts derived from a healthy 7-year-boy (CRL-1474) displayed about 20% SA- β -gal positive cells, which was consistent with previous reports (Fig. 5p). As suggested, we have now included representative SA- β -gal staining images in the revised manuscript (Extended Data Fig. 2a, Fig. 1h, Fig. 4e-f and Fig. 5p),

which clearly showed the difference in staining intensity and percentage of positive cells between control and experimental groups.

We also provided detailed explanations in the revision, as shown in line 770-771, line 866-867, line 911-912 and line 1037-1038.



Extended Data Fig. 2a and Fig. 1h. HGADFN127 cells were transfected with shS100A6 for 4 days, followed by SA-β-gal staining (**Extended Data Fig. 2a**). Scale bar: 100 μm. Quantification of the percentage of SA-β-gal-positive cells (**Fig. 1h**).

Fig. 4e-f. HGADFN127 cells treated with XeC and 2-APB for 4 days, followed by SA-β-gal staining (**Fig. 4e**). Scale bar: 100 μm. Quantification of the percentage of SA-β-gal-positive cells (**Fig. 4f**).

Fig. 5p. CRL-1474 cells overexpressing progerin were treated with MIA (10 μM) for 4 days, and the percentage of SA-β-gal positive cells was calculated.

Reviewer #3 (Remarks to the Author): This is a very well-designed study identifies calcium (Ca^{2+}) homeostasis disruption as a key driver of aging and progeria. Specifically, the Authors revealed that elevated cytoplasmic calcium levels lead to accumulation of the Ca^{2+} -binding protein S100A6, which promotes aging by degrading PARP1, causing DNA damage, chromatin fragmentation, and secretion of SASP. With treatment of the antidepressant compound mianserin, the Authors measured restored calcium balance, decreased senescence, and extended lifespan in both progeric and naturally aging mice, highlighting the potential of MIA as an anti-aging therapeutic. Overall, the whole study appears to have been rigorously conducted and presented, and the manuscript is largely well-written. However, I still have some major concerns about this study that need to be addressed to better clarify the role of calcium homeostasis and its impact on cellular senescence.

Response: We gratefully acknowledge the reviewer for the thoughtful and constructive assessment of our manuscript. In response to the major concerns raised, we have carefully revised the manuscript accordingly and provide detailed point-by-point responses below, including new experimental data and updated analyses where appropriate.

Specific comments:

Q1: My primary concern lies in the use of the term “intracellular calcium” and the measurement of Ca^{2+} concentrations. Intracellular calcium encompasses both cytosolic Ca^{2+} and calcium stored in organelles such as the endoplasmic/sarcoplasmic reticulum (ER/SR). Based on the introduction and the reported localization of S100A6 in this study, it appears that the changes in “intracellular Ca^{2+} ” concentrations are actually referring specifically to cytosolic Ca^{2+} . However, cytosolic Ca^{2+} concentrations typically range in the nanomolar level (approximately 100–200 nM), whereas all the reported Ca^{2+} concentrations in this study are in the micromolar range (given muscle Ca^{2+} concentration in this study is from ~0.5-2 $\mu\text{g}/\text{ml}$, which is about 12-50 μM), which is inconsistent with established values for cytosolic Ca^{2+} in any cell type. I also noted that the authors used a calcium measurement kit from Abcam, which, according to the

manufacturer's instruction, is only capable of detecting calcium at micromolar levels. Therefore, the authors need to clarify both their method of Ca^{2+} measurement and the specific intracellular compartment to which the measured Ca^{2+} refers.

Response: We sincerely thank the reviewer for raising this important and insightful concern regarding the measurement of intracellular Ca^{2+} concentrations in our study. In our initial experiments, we used a calcium measurement kit from Abcam (colorimetric assay based on o-cresolphthalein complexone) to quantify total calcium content in both cells and mouse tissues. Importantly, these measurements reflect total Ca^{2+} per mg of tissue or per 2×10^6 cells, not the concentration of free cytosolic Ca^{2+} in a single cell. In the revised manuscript, we used Fluo-4 AM to monitor changes in intracellular Ca^{2+} levels. Fluorescence signals were normalized to reflect relative cytosolic Ca^{2+} dynamics. Specifically, to strengthen the reliability of our findings, we used Fluo-4 AM to assess cytoplasmic Ca^{2+} levels in cells derived from multiple tissues of *Lmna*^{G609G/G609G} mice and in HGPS patient cells. The results revealed significantly elevated Ca^{2+} concentrations in cells from various tissues—including the heart, lung, skin (Fig. 1i–k), muscle, and liver of *Lmna*^{G609G/G609G} progeroid mice (Extended Data Fig. 2c–d). Elevated Ca^{2+} concentrations were also found in the cytoplasm of HGPS patient cells (HGADFN127, HGADFN155 and HGADFN167) (Fig. 1l).

Furthermore, we performed immunofluorescence detection of Ca^{2+} in CRL-1474 and HGADFN127 cells using Fluo-4 AM, which revealed elevated cytoplasmic Ca^{2+} levels in HGADFN127 cells (Fig. 1m). These results further demonstrated the dysregulation of Ca^{2+} homeostasis in HGPS senescence (Fig. 1i–m and Extended Data Fig. 2c–d). In addition, we analyzed the source of the elevated cytoplasmic Ca^{2+} . We used Mag-Fluo-4 AM and Rhod-2 AM to detect Ca^{2+} content in the major intracellular Ca^{2+} stores ER and mitochondria. Compared to CRL-1474 cells, the Ca^{2+} level in ER of HGADFN127 cells was decreased, while mitochondrial Ca^{2+} concentrations remained unchanged (Fig. 1r–s), indicating that the increased cytoplasmic Ca^{2+} levels in HGPS cells are likely derived from ER. IP3R is the primary Ca^{2+} channel protein located on ER which controls the release of Ca^{2+} from ER into cytoplasm. We found that IP3R was highly

expressed in HGPS patient HGADFN127 and HGADFN167 cells (Fig. 1t). Both IP3R inhibitors Xestospongine C (XeC) and 2-aminobenzyl diphenylborate (2-APB) reduced Ca^{2+} concentration (Extended Data Fig. 2e-f). Above results suggest that the release of Ca^{2+} from ER into cytoplasm in HGPS cellular senescence.

We provided detailed explanations in the revision, as shown in line 772-778, line 786-791 and line 1044-1047.

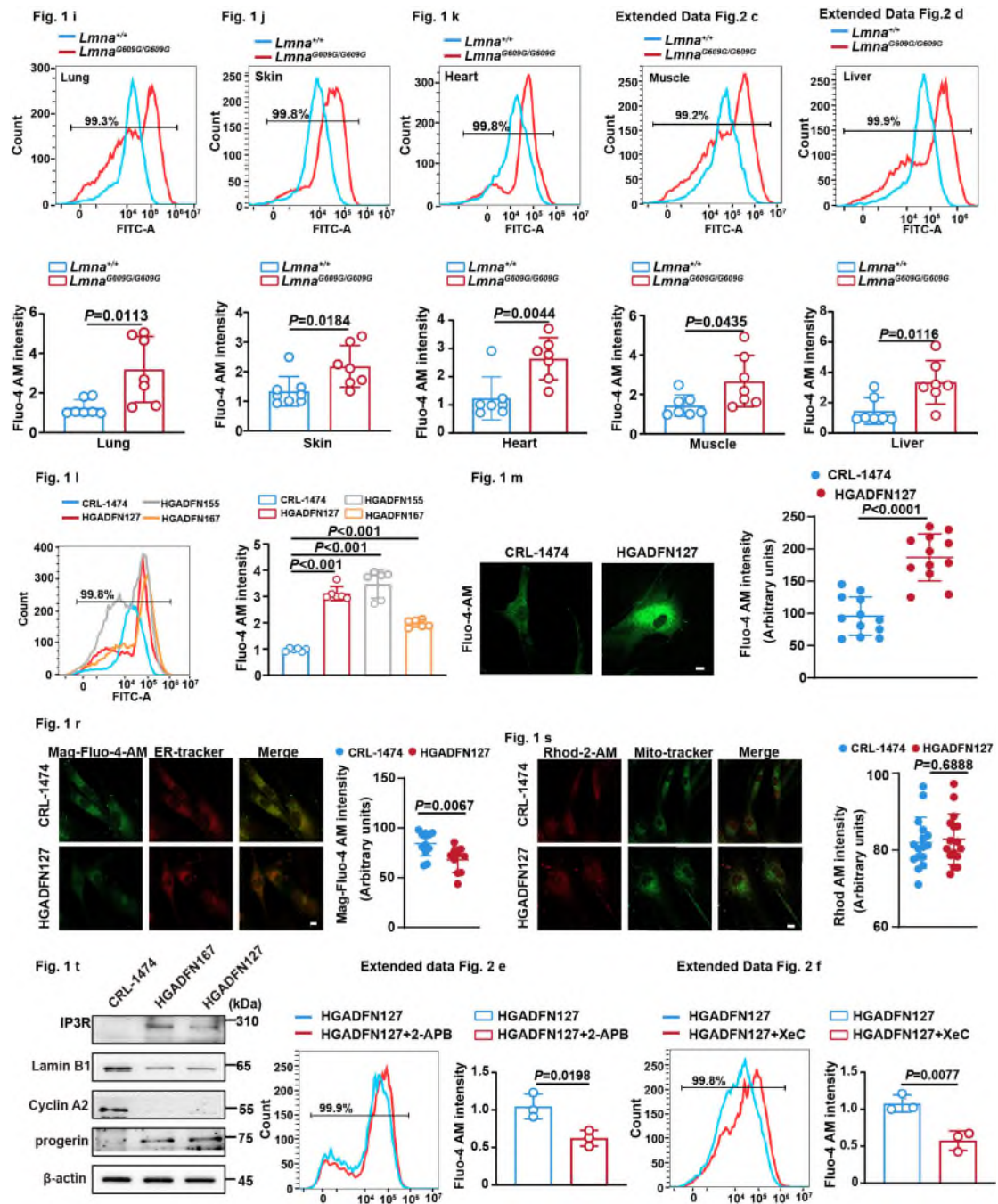


Fig. 1 (i-m), (r-u). Flow cytometry detection of Ca^{2+} concentration in cells isolated from multi-

tissues was performed using Fluo-4 AM, including the lung (i), skin (j), and heart (k) of *Lmna*^{+/+} and *Lmna*^{G609G/G609G} mice (n=7). Flow cytometry detection of Ca²⁺ concentration in HGPS patient cells was performed using Fluo-4 AM (CRL-1474, HGADFN127, HGADFN155 and HGADFN167 cells) (l). Immunofluorescence detection of Ca²⁺ in HGADFN127 and CRL-1474 cells was performed using Fluo4-AM (m). Scale bars: 10μm. Immunofluorescence detection of Ca²⁺ in ER of HGADFN127 and CRL-1474 cells was performed using Mag-Fluo4 AM and ER-Tracker (r). Immunofluorescence detection of Ca²⁺ in the mitochondria of HGADFN127 and CRL-1474 cells was performed using Rhod-2 AM and Mito-tracker (s). Western blot analysis of the protein levels of progerin, cyclin A2, lamin B1, and IP3R was performed in CRL-1474, HGADFN167, and HGADFN127 cells

Extended Data Fig. 2 (c-f). Flow cytometry detection of Ca²⁺ concentration in cells isolated from multi-tissues was performed using Fluo-4 AM, including the muscle (c) and liver (d) of *Lmna*^{+/+} and *Lmna*^{G609G/G609G} mice (n=7). Flow cytometry detection of Ca²⁺ concentration in HGADFN127 cells transfected with 2-APB was performed using Fluo4-AM (e). Flow cytometry detection of Ca²⁺ concentration in HGADFN127 cells transfected with XeC was performed using Fluo4-AM (f).

Q2: This study claims that S100A6 levels are closely associated with cytosolic Ca²⁺ concentrations, and the authors report that deletion of S100A6 leads to a reduction in cytosolic Ca²⁺ levels. However, the regulatory relationship between S100A6 and cytosolic Ca²⁺ is not clearly explained. Specifically, it remains unclear how the absence of S100A6 results in decreased cytosolic Ca²⁺—does S100A6 actively regulate calcium levels, or is its expression simply a response to elevated cytosolic Ca²⁺? In other words, is the increase in S100A6 a cause or a consequence of disrupted calcium homeostasis? A more detailed discussion of the mechanistic link between S100A6 and cytosolic Ca²⁺ regulation is needed to clarify this point.

Response: We sincerely appreciate the reviewer for raising this important mechanistic question regarding the relationship between S100A6 and cytosolic Ca²⁺. In our study, we observed that, consistent with elevated cytoplasmic Ca²⁺ levels in HGADFN127 cells, upregulated S100A6 was predominantly localized in the cytoplasm of these cells (Fig. 1n–o) and in multiple tissues of *Lmna*^{G609G/G609G} progeroid mice (Fig. 1p and q). As described in response to Q1, we have confirmed that the elevated cytoplasmic Ca²⁺ levels in HGPS cells originate from ER and found that IP3R, a key channel mediating ER calcium efflux, is upregulated. Then, we further treated HGPS cells with the IP3R inhibitor XeC or 2-APB. We found that both IP3R inhibitors significantly reduced

intracellular Ca^{2+} levels, led to a decrease in S100A6 levels in HGPS cells (Fig. 4a and b). As reported, S100A6 can also regulate cytoplasmic Ca^{2+} concentration [1-2]. In our study, we also found that knockdown of S100A6 led to a reduction in intracellular Ca^{2+} levels in HGPS cells (Fig. 1q). Our data demonstrate that S100A6 not only functions as a downstream effector but also actively participates in the regulation of Ca^{2+} homeostasis. We agree that the mechanism by which S100A6 regulates Ca^{2+} levels warrants further exploration, and this will be a focus of our future research. The results have been incorporated into Fig. 1n-q and Fig. 4a-b.

We also provided detailed explanations in the revision, as shown in line 779-785, and line 864-865.

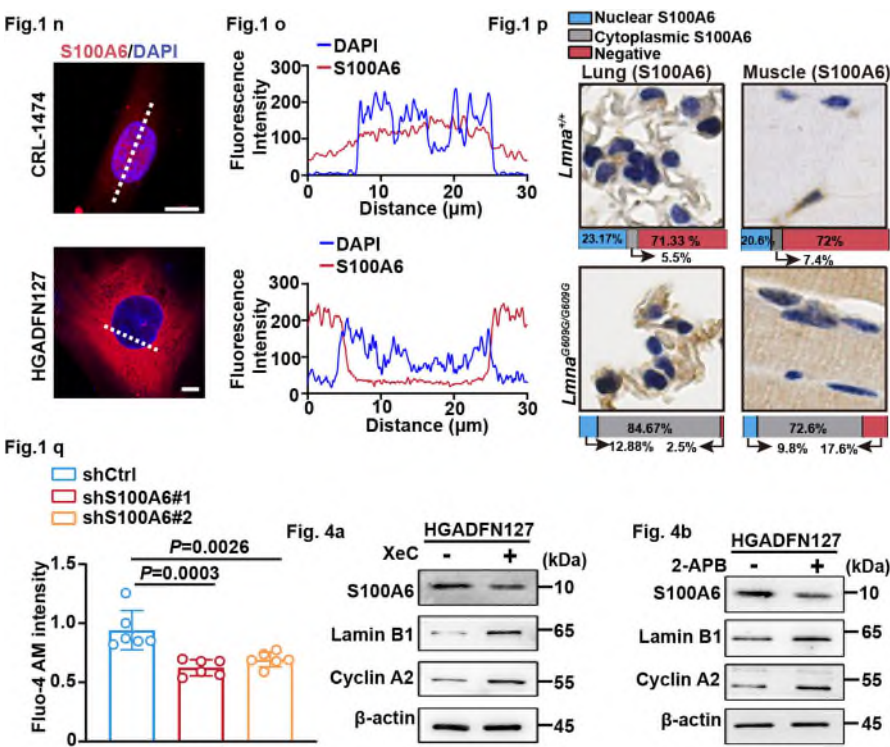


Fig. 1 (n-q). Immunofluorescence detection of S100A6 localization in HGADFN127 and CRL-1474 cells. Scale bars: 10 μm (n). Line graphs indicated the immunofluorescence signal intensity across the dotted lines in n (o). Immunohistochemistry detection of S100A6 localization in lung and muscle of *Lmna*^{+/+} and *Lmna*^{G609G/G609G} mice (p). Flow cytometry detection of Ca^{2+} concentration in HGADFN127 cells transfected with shS100A6 was performed using Fluo4-AM (q).

Fig. 4(a-b). Western blot analysis of the protein levels of cyclin A2, lamin B1, and S100A6 was performed in HGADFN127 cells treated with XeC and 2-APB for 4 days (a-b).

[1] Donato R. S100: a multigenic family of calcium-modulated proteins of the EF-hand type

with intracellular and extracellular functional roles [J]. The international journal of biochemistry & cell biology, 2001, 33(7): 637-668.

[2] Schäfer B W, Heizmann C W. The S100 family of EF-hand calcium-binding proteins: functions and pathology [J]. Trends in biochemical sciences, 1996, 21(4): 134-140.

Q3: Regarding the muscle force measurements, the authors state that force was measured as specific force, which refers to force production normalized to muscle size. The standard unit for specific force is typically mN/mm² or N/cm². However, in this study, the reported units are in mN, indicating that only absolute force was measured. This needs to be clarified. Furthermore, the force values should be normalized to muscle size, especially given that muscle size varies across the experimental groups. Normalizing force to muscle cross-sectional area would provide a more accurate representation of muscle quality and function in this study.

Response: We sincerely thank the reviewer for this important and insightful comment regarding the muscle force measurements in our study. We acknowledge that the original description of the force measurements was not sufficiently clear. In our initial submission, however, we reported absolute force values in mN without normalization to muscle cross-sectional area, which could potentially confound comparisons between experimental groups with varying muscle sizes. To report specific force, we have revised the manuscript to normalize muscle force measurements to the physiological cross-sectional area in Fig. 6n-o.

We also provided detailed explanations in the revision, as shown in line 953-954.

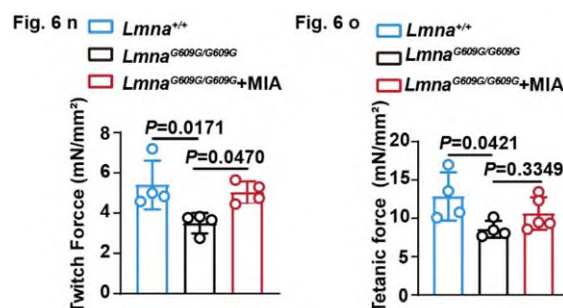


Fig. 6 (n-o). The twitch force (n) and tetanic force (o) of muscles derived from *Lmna*^{G609G/G609G} mice (12 weeks), treated with or without MIA, (n=4).

Q4: Following up on the previous point, the tetanic force in this study was measured

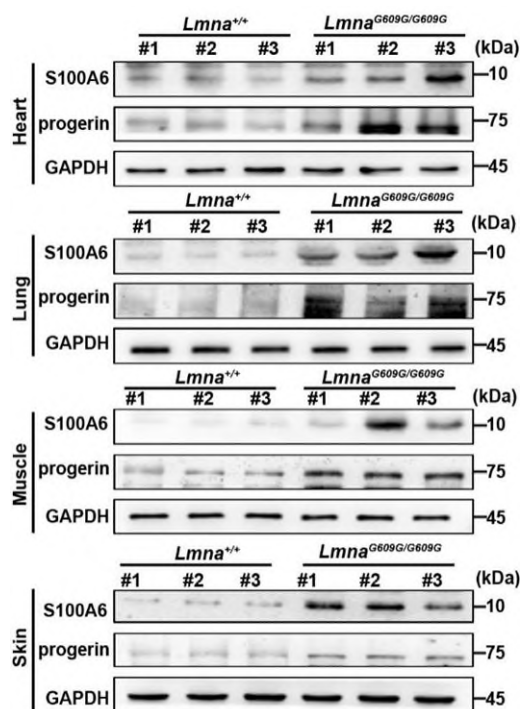
using a 100 Hz tetanic stimulus. However, according to prior studies, tetanic force is typically elicited using stimulation frequencies of around 150–200 Hz to ensure maximal force production. The authors should provide a justification for using 100 Hz in this context and clarify whether this frequency is sufficient to induce maximal tetanic force in the muscle type studied. Without this clarification, it remains uncertain whether the reported force measurements truly reflect the muscle's maximum contractile capacity.

Response: In our study, we chose a stimulation frequency of 100 Hz to elicit tetanic contraction. This decision was based on experimental considerations related to the mouse model used, the 12-week-old *Lmna*^{G609G/G609G} mice, which exhibit significant frailty and muscle weakness at this age. Prior to the main experiments, we conducted pilot studies to determine the optimal stimulation frequency for inducing maximal force in these animals. Our preliminary data showed that 100 Hz was sufficient to evoke maximal force production in their muscle fibers. This likely reflects altered muscle physiology due to the disease state and may differ from what is observed in healthy, wild-type animals.

Q5: β -actin is commonly considered a housekeeping protein; however, its suitability as a loading control in aging tissues is questionable for several reasons. Studies have shown that β -actin expression can vary with age, and aging-related changes in cytoskeletal dynamics may further affect actin levels. In fact, beta-actin levels appear to vary in several panels in this study, such as Figures 1d–1g. Therefore, in aging tissues, a more appropriate approach for quantifying target protein levels would be to use total protein staining as a loading control. If the authors choose to use beta-actin, they should first validate its expression stability across different experimental groups and tissue types.

Response: We thank the reviewer for raising this important concern regarding the suitability of β -actin as a loading control in aging tissues. In response to this concern, we repeated the tissue experiments using GAPDH as an alternative internal control across multiple tissue samples from HGPS progeric mice. Our updated results

consistently demonstrate that S100A6 is significantly upregulated in multiple organs such as the heart, liver, and lung of HGPS mice compared to wild-type controls.



Western blot analysis of the protein levels of S100A6 in tissues including the heart, lung, muscle, and skin of *Lmna*^{+/+} and *Lmna*^{G609G/G609G} mice (n=3).

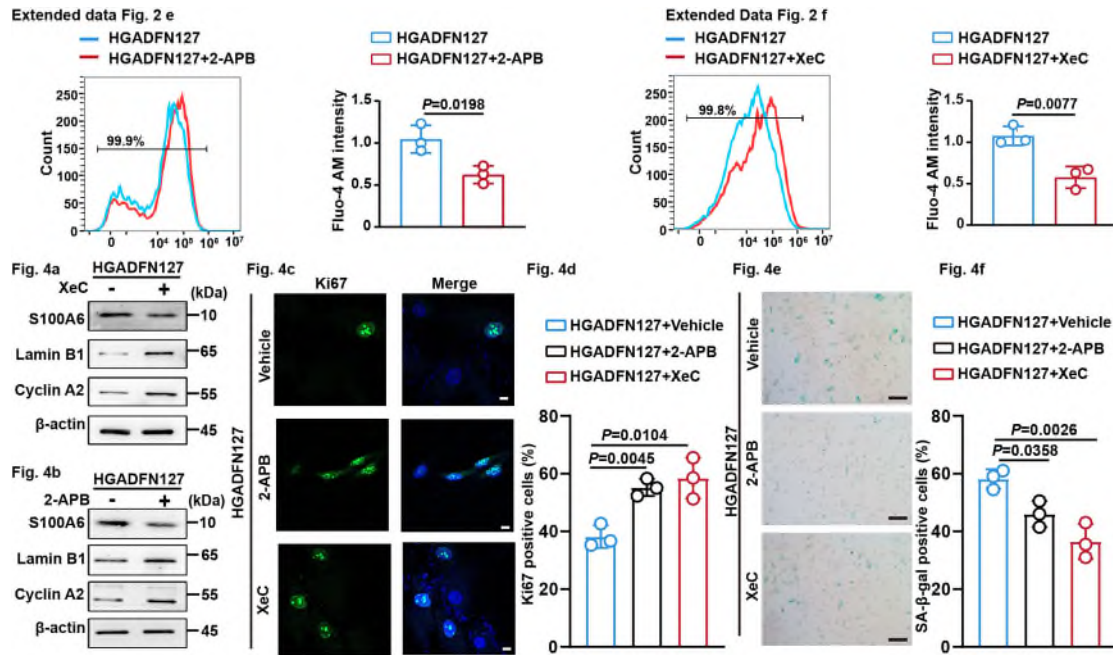
Q6: Two Ca²⁺ inhibitors are used in this study. For 2-APB, its mechanism as a Ca²⁺ inhibitor is briefly described in the manuscript as an IP₃ receptor blocker. However, for the other inhibitor, AML—although it is a commonly used antihypertensive drug—the authors should still provide a more detailed explanation of its mechanism of action and relevant signaling pathways.

Response: We thank the reviewer for this insightful comment regarding the mechanisms of action of the Ca²⁺ inhibitors used in our study. Following the observation of elevated cytoplasmic Ca²⁺ in HGPS cellular senescence primarily originated from IP3R mediated Ca²⁺ release from ER, as described in Q1, we further treated HGPS cells with IP3R inhibitor XeC or 2-APB and found that both IP3R inhibitors significantly reduced intracellular Ca²⁺ levels and attenuated HGPS cellular senescence (Extended Data Fig. 2e-f and Fig. 4a-f).

AML is a dihydropyridine-class L-type voltage-gated calcium channel (VGCC) blocker

that primarily inhibits Ca^{2+} influx through VGCCs in the plasma membrane. Based on our finding that elevated cytosolic Ca^{2+} in HGPS cells results from Ca^{2+} release from ER, we have removed the experimental data related to AML from the manuscript.

We also provided detailed explanations in the revision, as shown in line 864-867 and line 1044-1047.



Extended Data Fig. 2(e-f). Flow cytometry detection of Ca^{2+} concentration in HGADFN127 cells transfected with 2-APB was performed using Fluo4-AM (e). Flow cytometry detection of Ca^{2+} concentration in HGADFN127 cells transfected with XeC was performed using Fluo4-AM (f).

Fig. 4(a-f). Western blot analysis of the protein levels of cyclin A2, lamin B1, and S100A6 was performed in HGADFN127 cells treated with XeC and 2-APB for 4 days (a-b). Immunofluorescence staining of Ki67 in a-b (c). Scale bars: 10 μm . The percentage of Ki67 positive cells was calculated (d). The percentage of SA-β-gal positive cells was calculated (e-f). Scale bars: 100 μm .

Reviewer #4 (Remarks to the Author): In this study, Weifang Xiang et al. explore the role of S100A6-mediated calcium dysregulation in the pathogenesis of Hutchinson-Gilford progeria syndrome (HGPS) and propose MIA as a therapeutic intervention. While the topic is of substantial interest in aging biology, the manuscript suffers from significant mechanistic, methodological, and interpretational limitations that undermine the strength of its central claims and are not suitable for publication in its current form.

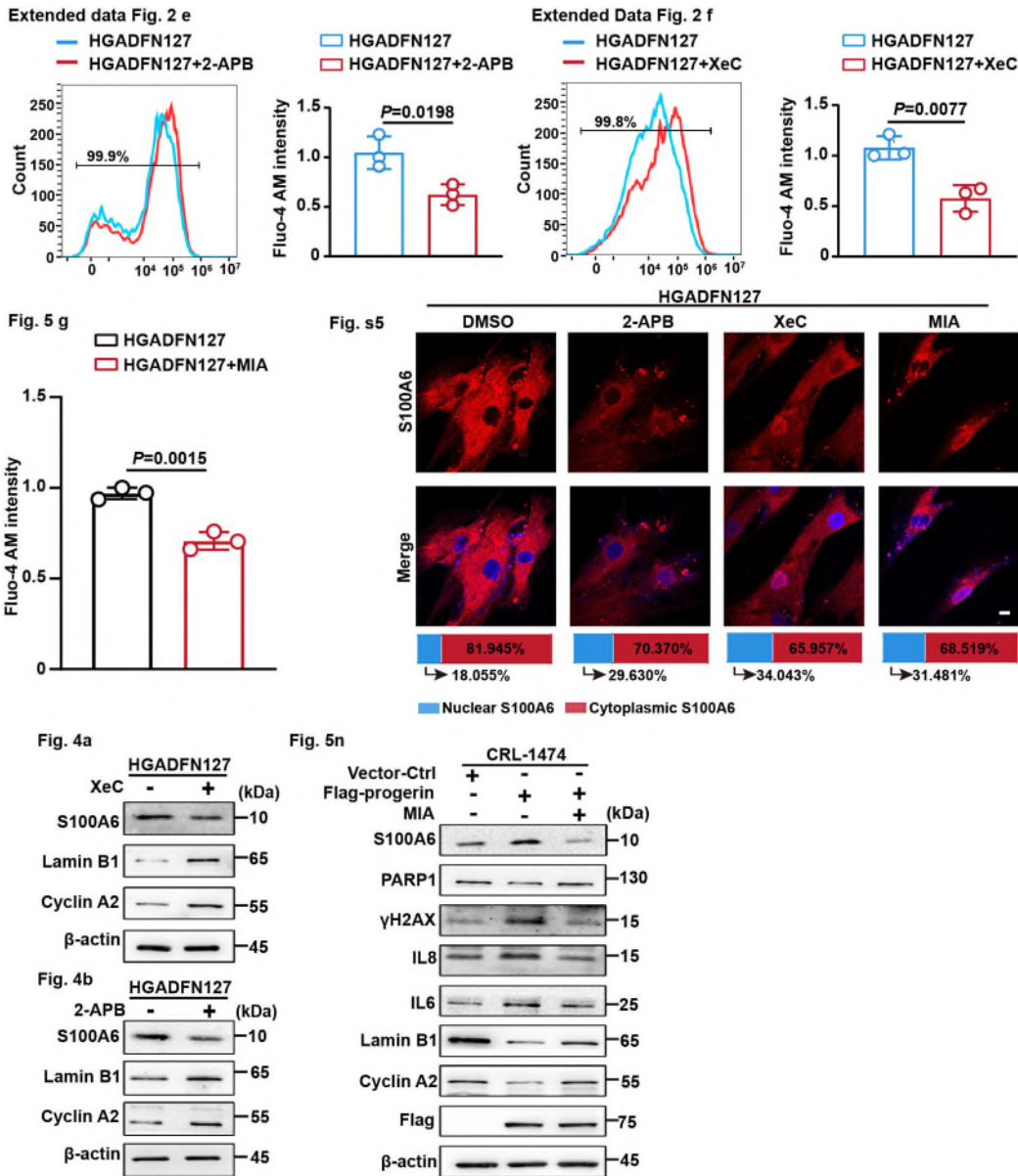
Response: We sincerely appreciate the reviewer's careful and critical evaluation of our manuscript. We acknowledge that the reviewer has raised several important concerns regarding the mechanistic insights, methodological rigor, and interpretation of our findings. In response, we addressed each comment in a point-by-point manner.

Specific comments:

Q1: The authors propose that disrupted calcium homeostasis leads to cytoplasmic S100A6 accumulation and its aberrant interaction with PARP1, contributing to PARP1 degradation. However, the causality of this pathway is not convincingly established. The manuscript lacks direct experimental evidence demonstrating that calcium fluxes actively drive S100A6 subcellular redistribution or binding to PARP1. No perturbation experiments (e.g., calcium chelation, ionophore treatment) were performed to dynamically modulate calcium levels and observe resulting changes in S100A6 localization or interaction strength with PARP1. Additionally, the involvement of the proposed chaperone CacyBP/SIP remains speculative, as no biochemical or genetic interrogation of this factor was conducted. The mechanistic link between calcium signaling and S100A6-PARP1 complex formation requires substantial reinforcement.

Response: We sincerely thank the reviewer for the constructive suggestions. To further investigate the evidence for Ca²⁺-dependent activation of S100A6 translocation and dynamic S100A6-PARP1 interaction, we treated HGADFN127 cells with MIA and two IP3R inhibitors 2-APB and XeC to reduce intracellular calcium levels (Extended Data Fig. 2e-f and Fig. 5g) and then examined S100A6 localization and levels. The results

showed that, following Ca^{2+} inhibition, parts of S100A6 relocated from the cytoplasm to the nucleus. Meanwhile, S100A6 protein levels were reduced in a subset of HGPS cells (Fig. s5, Fig. 4a-b and Fig. 5n). These results indicate that Ca^{2+} levels influence both the level and distribution of S100A6 in HGPS cells.



Extended Data Fig. 2e-f. Flow cytometry detection of Ca^{2+} concentration in HGADFN127 cells transfected with 2-APB was performed using Fluo4-AM (e). Flow cytometry detection of Ca^{2+} concentration in HGADFN127 cells transfected with XeC was performed using Fluo4-AM (f).

Fig. 5g and n. Flow cytometry detection of Ca^{2+} concentration in HGADFN127 cells treated with or without MIA (10 μM) for 4 days was performed using Fluo-4 AM (g). Western blot analysis of the protein levels of cyclin A2, lamin B1, IL-6, IL-8, γ H2AX, PARP1, and S100A6

in CRL-1474 cells overexpressing progerin and treated with MIA (10 μ M) for 4 days (n).

Fig. 4 a-b. Western blot analysis of the protein levels of cyclin A2, lamin B1, and S100A6 was performed in HGADFN127 cells treated with XeC and 2-APB for 4 days (a-b).

Fig. s5. Immunofluorescence detection of S100A6 localization in HGADFN127 with 2-APB, XeC or MIA for 4 days.

Additionally, we examined the subcellular localization of S100A6 and PARP1 in both CRL-1474 and HGPS cells, and found that S100A6 and PARP1 co-localized and formed a complex in the cytoplasm of HGPS patient HGADFN127 cells (Fig. 2c-e).

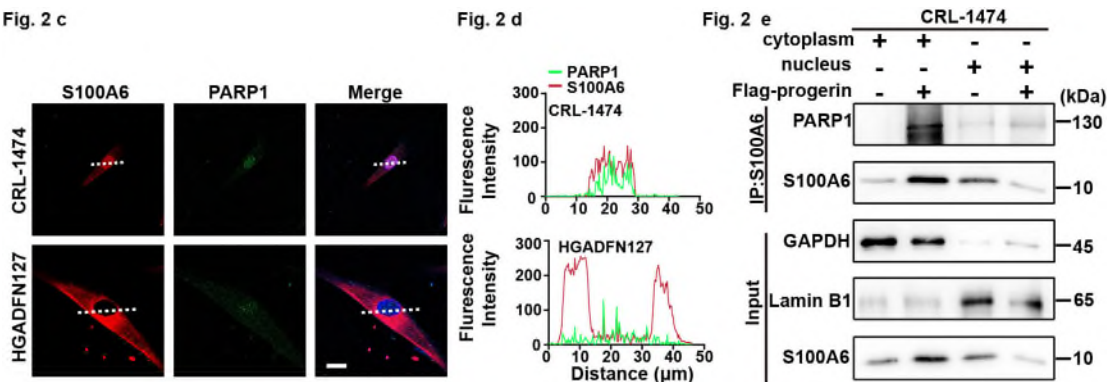


Fig. 2(c-e). Immunofluorescence detection of S100A6 and PARP1 localization in HGADFN127 and CRL-1474 cells (c). Scale bars: 20 μ m. Line graphs indicated the immunofluorescence signal intensity across the dotted lines in c (d). Co-IP assay was performed to analyze the interaction between S100A6 and PARP1 in the nucleus and cytoplasm of CRL-1474 cells overexpressing Flag-progerin (e).

To further investigate the role of CacyBP in S100A6 mediated PARP1 degradation, we examined the interactions among S100A6, PARP1, and CacyBP in HGPS cells. The results showed that S100A6, PARP1, and CacyBP form a complex in HGADFN127 cells (Fig. 2l). Knowdown of CacyBP led to a significant upregulation of PARP1 protein levels and a marked decrease in its ubiquitination levels (Fig. 2m-n). Further investigation showed that knocking down S100A6 weakened the binding between CacyBP and PARP1 (Fig. 2o). However, interfering with CacyBP didn't affect the interaction between S100A6 and PARP1 (Fig. 2p). These results indicate that upregulated S100A6 recruits CacyBP to ubiquitinate and degrade PARP1 in HGPS cells (Fig. 2q). The results have been incorporated into Fig. 2l-q.

We also provided detailed explanations in the revision, as shown in line 801-805, line

818-828, line 864-865, line 897-898, line 907-910 and line 1044-1047.

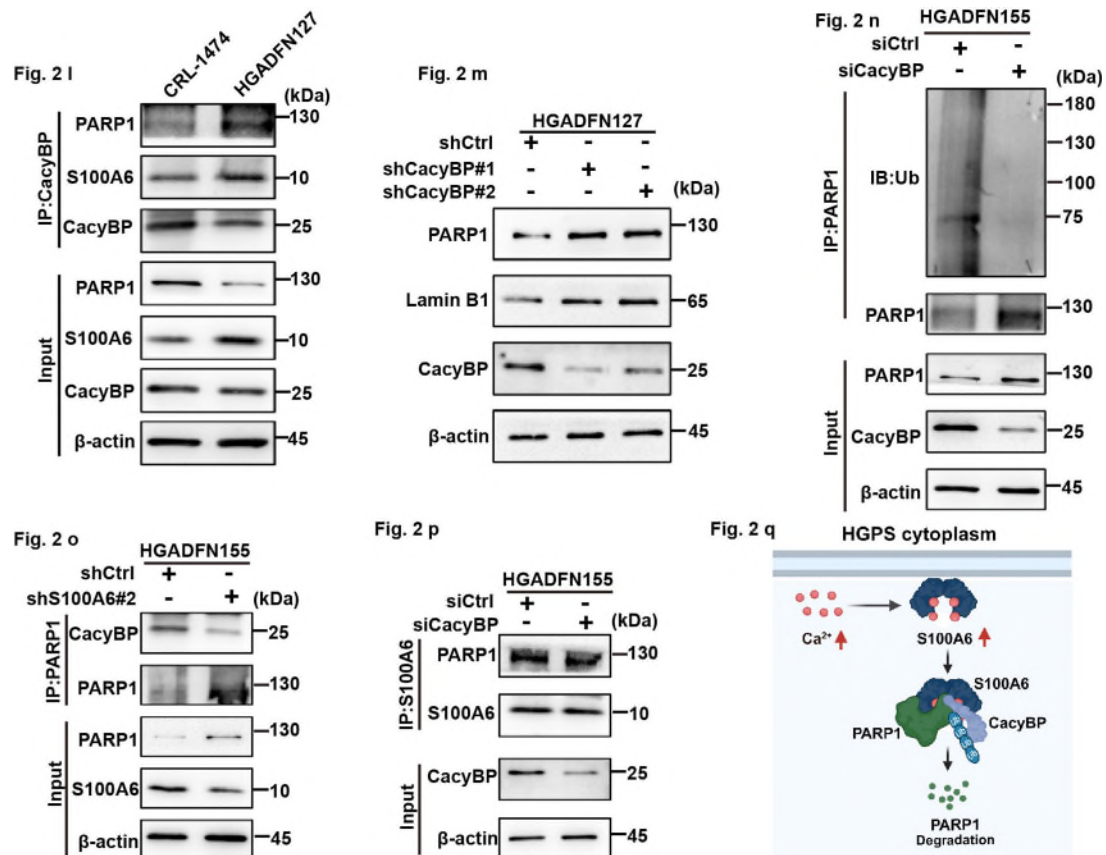


Fig. 2(l-q). Co-IP assay was performed to analyze the interaction between S100A6, CacyBP and PARP1 in CRL-1474 and HGADFN127 cells (**l**). Western blot analysis of the protein levels of CacyBP, Lamin B1, and PARP1 in CacyBP-deleted HGADFN127 cells was performed (**m**). Co-IP assay was performed to analyze the PARP1 ubiquitination level in HGADFN155 cells that were transfected with siCacyBP for 48 h (**n**). Co-IP assay was performed to analyze the interaction between PARP1 and CacyBP in HGADFN155 cells that were transfected with shS100A6 for 48 h (**o**). Co-IP assay was performed to analyze the interaction between S100A6 and PARP1 in HGADFN155 cells that were transfected with siCacyBP for 48 h (**p**). Schematic diagram for S100A6 recruiting CacyBP to ubiquitinate and degrade PARP1 (**q**).

Q2: The study relies heavily on bulk colorimetric calcium assays, which are inherently limited in both spatial and temporal resolution. These measurements do not distinguish between cytosolic, ER, or mitochondrial calcium pools, nor do they provide dynamic insights into calcium signaling alterations in HGPS cells. More precise tools—such as fluorescent calcium indicators (e.g., Fluo-4, Fura-2) or genetically encoded calcium

sensors (e.g., GCaMP)—should have been employed to monitor intracellular calcium fluxes in live cells. This represents a critical gap in validating the calcium dysregulation hypothesis.

Response: We sincerely appreciate the reviewer's insightful suggestions. As suggested, we strengthened the Ca^{2+} measurements. First, we used Fluo-4 AM to assess cytoplasmic Ca^{2+} levels in cells derived from multiple tissues of *Lmna*^{G609G/G609G} mice and in HGPS patient cells. The results revealed significantly elevated Ca^{2+} concentrations in cells from various tissues—including the heart, lung, skin, muscle, and liver of *Lmna*^{G609G/G609G} progeroid mice (Fig. 1i–k, Extended Data Fig. 2b–c). Elevated Ca^{2+} concentrations were also found in the cytoplasm of HGPS patient cells (HGADFN127, HGADFN155 and HGADFN167) (Fig. 1l). Furthermore, we performed immunofluorescence detection of Ca^{2+} in CRL-1474 and HGADFN127 cells using Fluo-4 AM, which also revealed elevated cytoplasmic Ca^{2+} levels in HGADFN127 cells (Fig. 1m). These results further demonstrated the dysregulation of Ca^{2+} homeostasis in HGPS senescence. The results have been incorporated into Fig. 1i–m and Extended Data Fig. 2b–c.

Furthermore, we analyzed the source of elevated cytoplasmic Ca^{2+} . We used Mag-Fluo-4 AM and Rhod-2 AM to detect Ca^{2+} content in the major intracellular Ca^{2+} stores ER and mitochondria. Compared to CRL-1474 cells, the Ca^{2+} level in ER of HGADFN127 cells was decreased, while mitochondrial Ca^{2+} concentrations remained unchanged (Fig. 1r–s), indicating that the increased cytoplasmic Ca^{2+} levels in HGPS cells are likely derived from ER. IP3R is the primary Ca^{2+} channel protein located on ER which controls the release of Ca^{2+} from ER into cytoplasm. We found that IP3R was highly expressed in HGPS patient HGADFN127 and HGADFN167 cells (Fig. 1t). Both IP3R inhibitors XeC and 2-APB reduced Ca^{2+} levels of HGPS cells (Extended Data Fig. 2d–e). Above results suggest the release of Ca^{2+} from ER into cytoplasm in HGPS cellular senescence.

We also provided detailed explanations in the revision, as shown in line 772–774, line 797–804 and line 1057–1059.

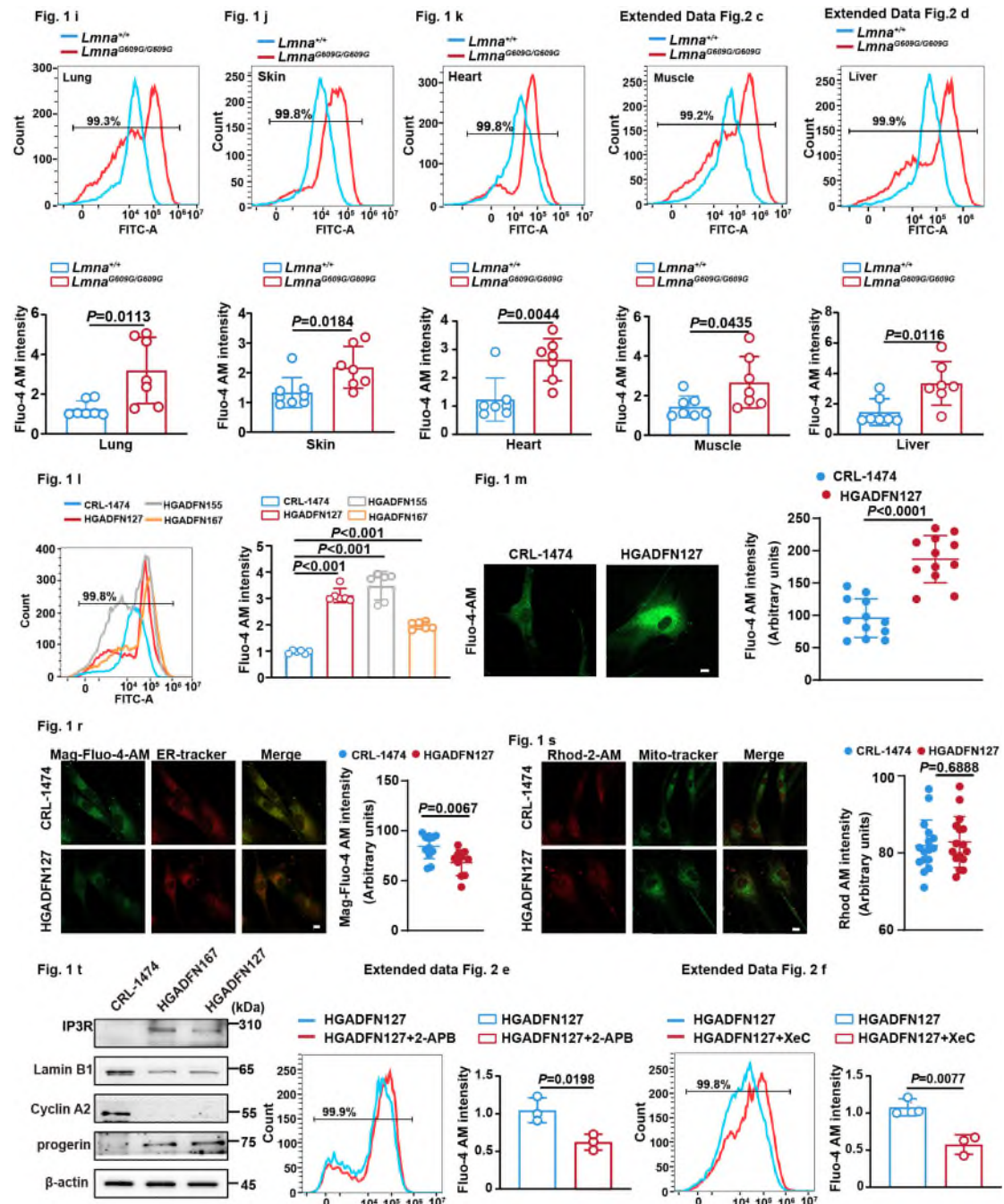


Fig. 1 (i-m). (r-t). Flow cytometry detection of Ca^{2+} concentration in cells isolated from multi-tissues was performed using Fluo-4 AM, including the lung (i), skin (j), and heart (k) of $\text{Lmna}^{+/+}$ and $\text{Lmna}^{\text{G609G/G609G}}$ mice (n=7). Flow cytometry detection of Ca^{2+} concentration in HGPS patient cells was performed using Fluo-4 AM (CRL-1474, HGADFN127, HGADFN155 and HGADFN167 cells) (l). Immunofluorescence detection of Ca^{2+} in HGADFN127 and CRL-1474 cells was performed using Fluo4-AM (m). Scale bars: 10 μ m. Immunofluorescence detection of Ca^{2+} in ER of HGADFN127 and CRL-1474 cells was performed using Mag-Fluo4 AM and ER-Tracker (r). Immunofluorescence detection of Ca^{2+} in the mitochondria of HGADFN127 and CRL-1474 cells was performed using Rhod-2 AM and Mito-tracker (s). Western blot analysis of the protein levels of progerin, cyclin A2, lamin B1, and IP3R was performed in CRL-1474, HGADFN167, and HGADFN127 cells (t).

Extended Data Fig. 2 (c-f). Flow cytometry detection of Ca^{2+} concentration in cells isolated from multi-tissues was performed using Fluo-4 AM, including the muscle (c) and liver (d) of *Lmna*^{+/+} and *Lmna*^{G609G/G609G} mice (n=7). Flow cytometry detection of Ca^{2+} concentration in HGADFN127 cells transfected with 2-APB was performed using Fluo4-AM (e). Flow cytometry detection of Ca^{2+} concentration in HGADFN127 cells transfected with XeC was performed using Fluo4-AM (f).

Q3: The therapeutic effects of MIA are attributed to its modulation of serotonin receptors (HTR2B/HTR2C), leading to lowered intracellular calcium. However, this conclusion is weakly supported. The study lacks receptor-specific validation using genetic knockout models or receptor-selective antagonists. It is also unclear whether the observed calcium-lowering effect of MIA is specific to HTR2B/HTR2C inhibition or a consequence of broader, off-target actions. Additionally, no dose-response data or comparisons with known calcium modulators (e.g., BAPTA-AM, thapsigargin) are included, making it difficult to attribute MIA's anti-aging effects solely to calcium modulation. The therapeutic relevance of MIA in the context of HGPS, therefore, remains speculative.

Response: Thank you for the insightful suggestion. We found that MIA reduced intracellular Ca^{2+} level in HGPS cells (Fig. 5g). Since it has been reported that MIA can inhibit IP3R activation by antagonizing 5-HT receptors HTR2B and HTR2C, thereby reducing intracellular Ca^{2+} concentration. To further elucidate the mechanism underlying this effect, we first knocked down HTR2B or HTR2C in HGADFN127 cells, which resulted in a reduction of intracellular Ca^{2+} levels (Fig. 5l). To further explore the relationship between MIA and HTR2B/2C in HGPS senescence, we treated HGADFN127 cells with MIA and simultaneously added agonists for HTR2B or HTR2C. The results showed that both HTR2B and HTR2C agonists restored Ca^{2+} levels reduced by MIA (Fig. 5m). We further examined the effect of MIA on the interactions between HTR2B/HTR2C and their downstream G proteins. The results showed that the binding of HTR2B or HTR2C to G- α was reduced by MIA treatment (Fig. 5o). Indeed, as the reviewer pointed out, MIA is a tetracyclic antidepressant known to increase synaptic serotonin (5-HT) levels by blocking reuptake, while antagonizing 5-HT and adrenergic receptors. Although our current work highlights its role in regulating Ca^{2+}

homeostasis via HTR2B/2C in HGPS cells, we acknowledge that MIA may exert anti-aging effects through additional mechanisms. This mechanism will be investigated in future research. Above data showed that MIA reduced the intracellular Ca^{2+} concentration of HGPS cells, at least in part, by targeting HTR2B and HTR2C. The results have been incorporated into Fig. 5g and l-m.

We also provided detailed explanations in the revision, as shown in line 897-898, line 903-906 and line 909-910.

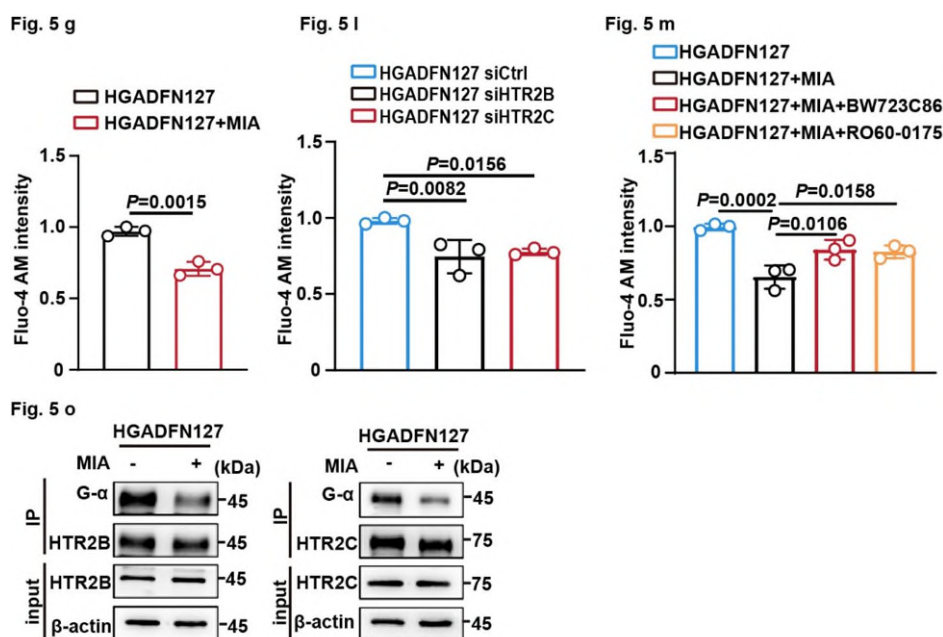


Fig. 5 (g-m). Flow cytometry detection of Ca^{2+} concentration in HGADFN127 cells treated with or without MIA (10 μM) for 4 days was performed using Fluo-4 AM (g). Flow cytometry detection of Ca^{2+} concentration in HGADFN127 cells transfected with siHTR2B was performed using Fluo4-AM (l). Flow cytometry detection of Ca^{2+} concentration in HGADFN127 cells transfected with siHTR2C was performed using Fluo4-AM (m). Co-IP assay was performed to analyze the interaction between HTR2B or HTR2C and G- α in HGADFN127 cells treated with or without MIA (10 μM) for 4 days (o).

Q4: Mouse studies are underpowered, especially for lifespan and endpoint analyses. Survival curves derived from small cohorts ($n < 30$) are not statistically reliable. Similarly, endpoint assays (e.g., histology, protein expression) conducted with fewer than 10 animals per group lack robustness. The authors must increase sample sizes in accordance with standard practices for aging and intervention studies.

Response: Thank you for this valuable suggestion. In response to the concern about

the reliability of survival analysis, we expanded the cohort size for the survival study to 30 mice per group (Fig. 6t).

Regarding the endpoint analyses, we increased the number of animals per group to 7 for histological assays. The updated cohort size ($n = 7$ per group) provided improved statistical power and reproducibility (Fig. 6i, m, r, u-y and Extended Data Fig. 6 a-h). Due to the limited availability of *Lmna*^{G609G/G609G} homozygous mice and the prolonged time required for the animals to reach the appropriate age for displaying senescent phenotypes, it's difficult to establish larger cohort sizes within a typical research timeline. In future studies, we plan to further increase the sample size when time and resources permit.

We also provided detailed explanations in the revision, as shown in line 945-947, line 950-953, line 958-960, line 963-964, line 965-967 and line 1097-1108.

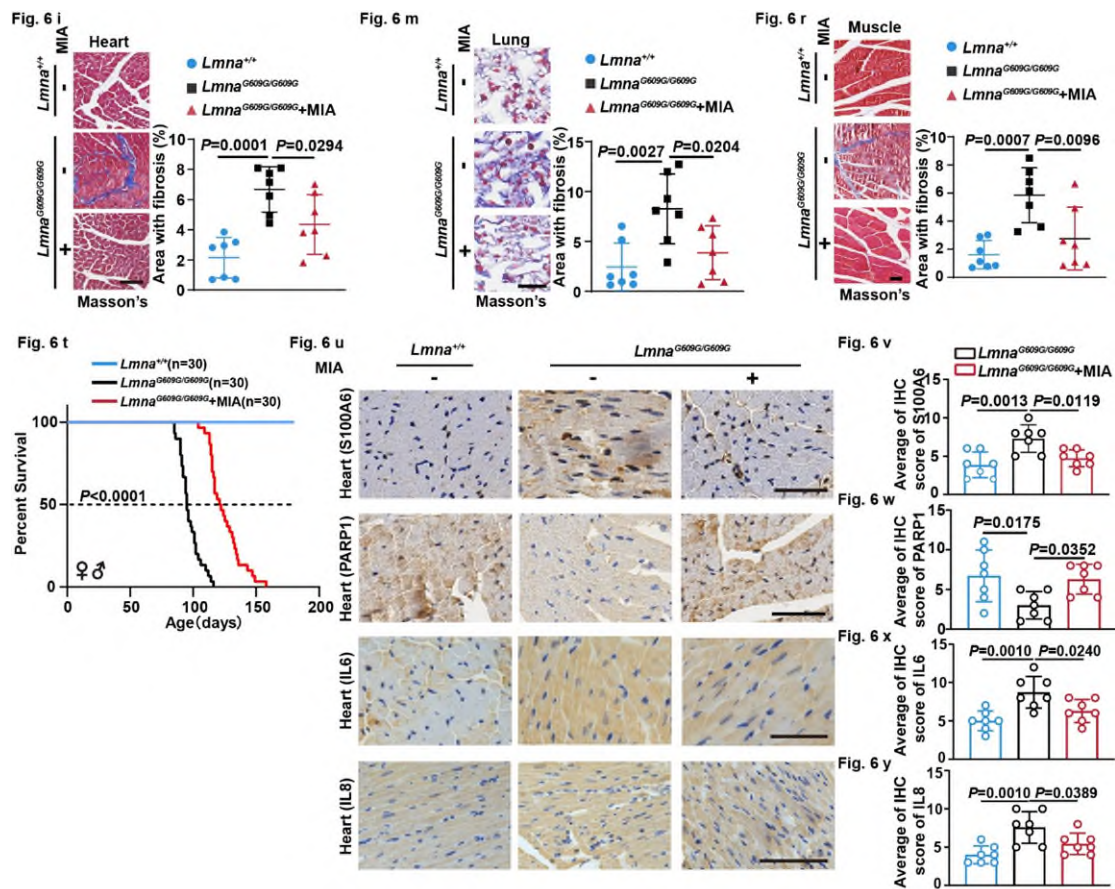
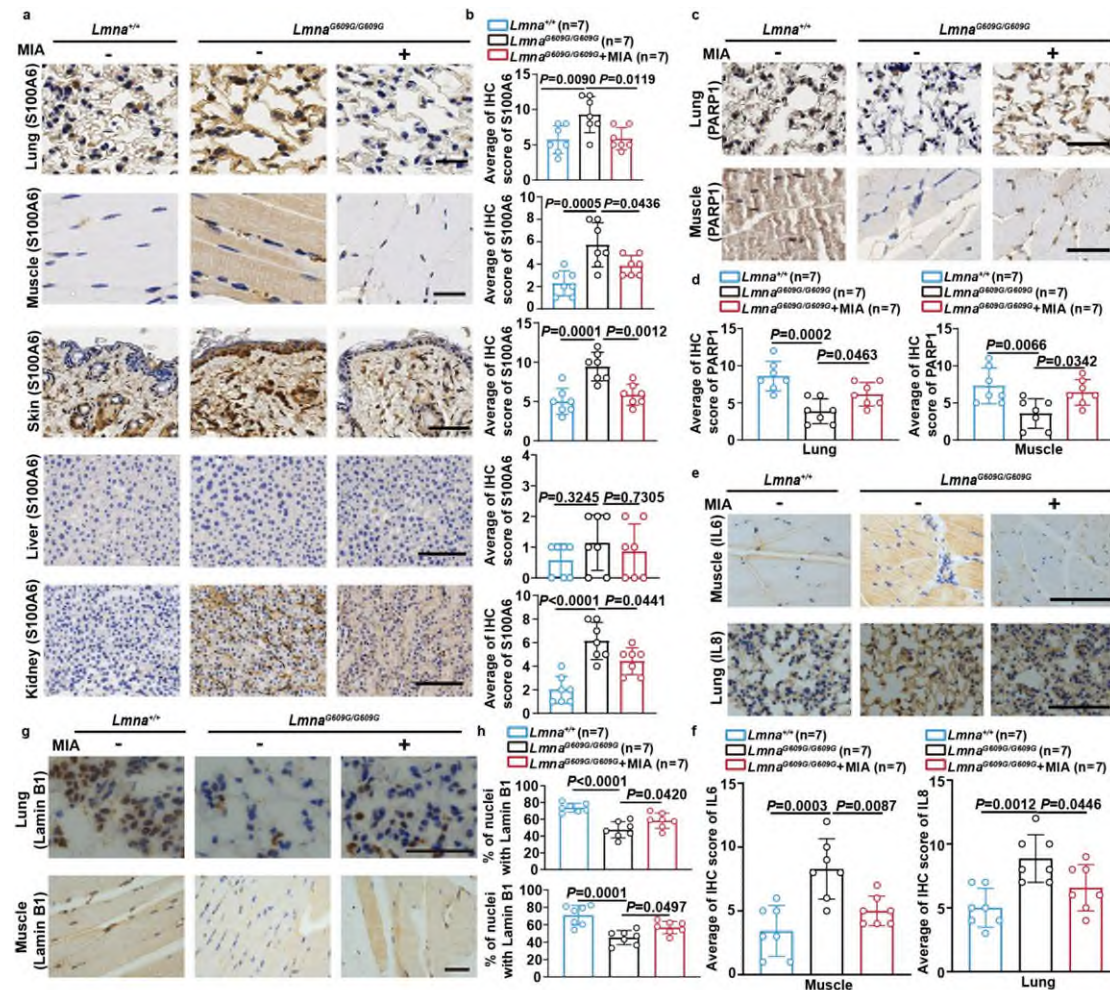


Fig. 6 (i-y). Masson's trichrome staining was performed on muscle tissue derived from *Lmna*^{G609G/G609G} mice (12 weeks), treated with or without MIA. Representative photographs of fibrosis in the muscle are shown (i). (Scale bars, 50μm; $n = 7$). Masson's trichrome staining was

performed on lung tissue derived from *Lmna*^{G609G/G609G} mice (12 weeks), treated with or without MIA. Representative photographs of fibrosis in the lung are shown (m). (Scale bars, 50µm; n = 7). Masson's trichrome staining was performed on muscles derived from *Lmna*^{G609G/G609G} mice (12 weeks) treated with or without MIA. Representative photographs of muscle fibrosis are presented (r). (Scale bars, 50µm; n = 7). Kaplan-Meier survival curves for *Lmna*^{+/+} and *Lmna*^{G609G/G609G} mice, treated with or without MIA, were analyzed (t). (n=30). IHC was performed to detect S100A6, PARP1, IL6 and IL8 in the heart of *Lmna*^{G609G/G609G} mice, with or without MIA treatment (u, Scale bars, 50 µm; n = 7). The quantification scores of S100A6 (v), PARP1(w), IL6 (x) and IL8 (y) were also determined.



Extended Data Fig. 6 (a-h). IHC of S100A6 in lung, muscle, skin, liver and kidney of *Lmna*^{G609G/G609G} mice (12 weeks), treated with or without 10 mg/kg MIA (a, Scale bars, 100 µm; n = 7). The quantification scores of S100A6 (b). IHC of PARP1 in lung and muscle of *Lmna*^{G609G/G609G} mice (12 weeks), treated with or without 10 mg/kg MIA (c, Scale bars, 100 µm; n = 7). The quantification scores of S100A6 in lung and muscle (d). IHC of IL6 in muscle and IL8 in lung of *Lmna*^{G609G/G609G} mice (12 weeks), treated with or without 10 mg/kg MIA (e, Scale bars, 100 µm; n = 7). The quantification scores of IL6 in muscle and IL8 in lung (f). IHC of Lamin B1 in lung and muscle of *Lmna*^{G609G/G609G} mice (12 weeks), treated with or without 10 mg/kg MIA (g, Scale bars, 50 µm; n = 7). The quantification scores of Lamin B1 in lung and muscle (h).

Q5: Western blot data are presented without quantification or normalization controls, preventing proper evaluation of protein expression levels. Full-length blots, along with molecular weight markers and loading controls, should be included. The manuscript also lacks essential statistical annotations and confidence intervals for many datasets.

Response: Thank you for this valuable suggestion. As suggested, we have quantified the band intensities of the key blots from three independent experiments using ImageJ. The blots shown in the manuscript are representative images, and the corresponding statistical results have been submitted together with the Source Data file. As suggested, we also included full-length, unprocessed Western blot images in the Source Data file, which is provided alongside the revised manuscript. We also fully agree that clear statistical reporting is crucial for data interpretation.

To this end, we conducted a comprehensive review of all datasets in the manuscript and completed the following revisions: data are now presented as mean $\pm$ SEM; statistical significance was determined by unpaired two-tailed Student's t-test for two-group comparisons or one-way ANOVA with appropriate post-hoc tests for multiple comparisons. Exact p-values (or statistical significance annotations) have been added to all relevant figures and graphs; all figure legends have been updated to clearly specify the sample size (n) and the statistical methods used; furthermore, the Methods section has been revised to describe the detailed statistical analysis procedures, rationale for the choice of tests, and data presentation approaches, thereby enhancing the transparency and reproducibility of our results.

Point-by-Point Response to NCOMMS-25-10890A-Z

Title: Ameliorating calcium homeostasis improves longevity and healthspan in progeroid and naturally aged mice

Reviewer #1 (Remarks to the Author):

Specific comments:

Q1: The authors argue that knockdown of S100A6 reduces SA- β -gal (Fig. 1k) and restores proliferation. However, a change from 60% to ~45% is of course not very likely to have a major impact. These cells should be followed (much) longer over time to see if the population fully gets into cell cycle progression, or whether they fall back into becoming largely senescent. Something similar holds true for 2ABP (Fig. 1y). How significant is the restoration of Lamin B1 (Fig. 1v) and a ~20% reduction in SA- β -gal?

These results are the same data s my comment was based on. In the new Fig. 1h, the reduction in SA- β -gal by shS100A6 is only some 15-20% (from ~60% to ~45%); this is not likely to be biologically meaningful. Hence I asked to do this over a longer period of time, which does not seem to have been done. This needs to be added.

Response: We sincerely apologize that our description of the experimental setup was insufficiently clear in the first revised manuscript. Following your previous suggestion, we extended the S100A6 knockdown duration in HGPS cells from 4 to 7 days and assessed SA- β -gal positive proportion. The results showed that both 4-day and 7-day S100A6 knockdown reduced the proportion of SA- β -gal-positive cells and the reduction is appropriate 18% (4 days) and 20% (7 days) (Fig.1h and Supplementary Fig. 2b-c). We think the modest change of SA- β -gal might be attributed to the fact that HGPS patient cells are already in a stable state of profound senescence, making it difficult to achieve a more substantial SA- β -gal reversal even after S100A6 knockdown. Actually, we observed that knockdown of S100A6 reduced the staining intensity of SA- β -gal-positive cells (Fig.1h and Supplementary Fig. 2b-c); however, in our quantification, these cells were still counted as positive, which may partly explain why SA- β -gal-positive proportion did not show a very significant decrease.

To further confirm the role of S100A6 in HGPS senescence, we added more senescent markers in this version. Consistently, protein levels of Cyclin A2 and Lamin B1 were markedly restored, while IL-6, γ H2AX, p16, and p21, was downregulated after S100A6 knockdown for both 4 and 7 days (Fig.1f and Supplementary Fig. 2d). All these results showed that shS100A6 alleviated HGPS cellular senescence.

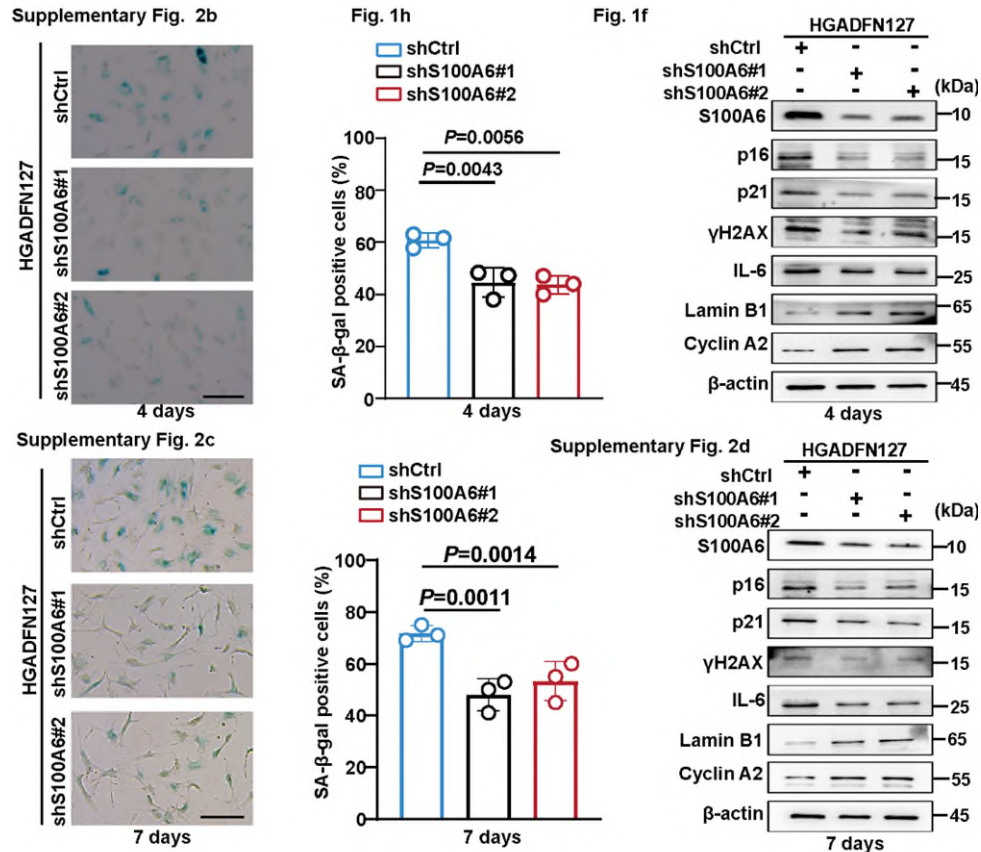


Fig. 1 f. Western blot analysis of the protein levels of Cyclin A2, Lamin B1, IL-6, γH2AX, p21, p16 and S100A6 was performed in HGADFN127 cells that had been transfected with shS100A6 for 4 days.

Fig. 1 h. The percentage of SA-β-gal positive cells was calculated in HGADFN127 cells transfected with shS100A6 for 4 days.

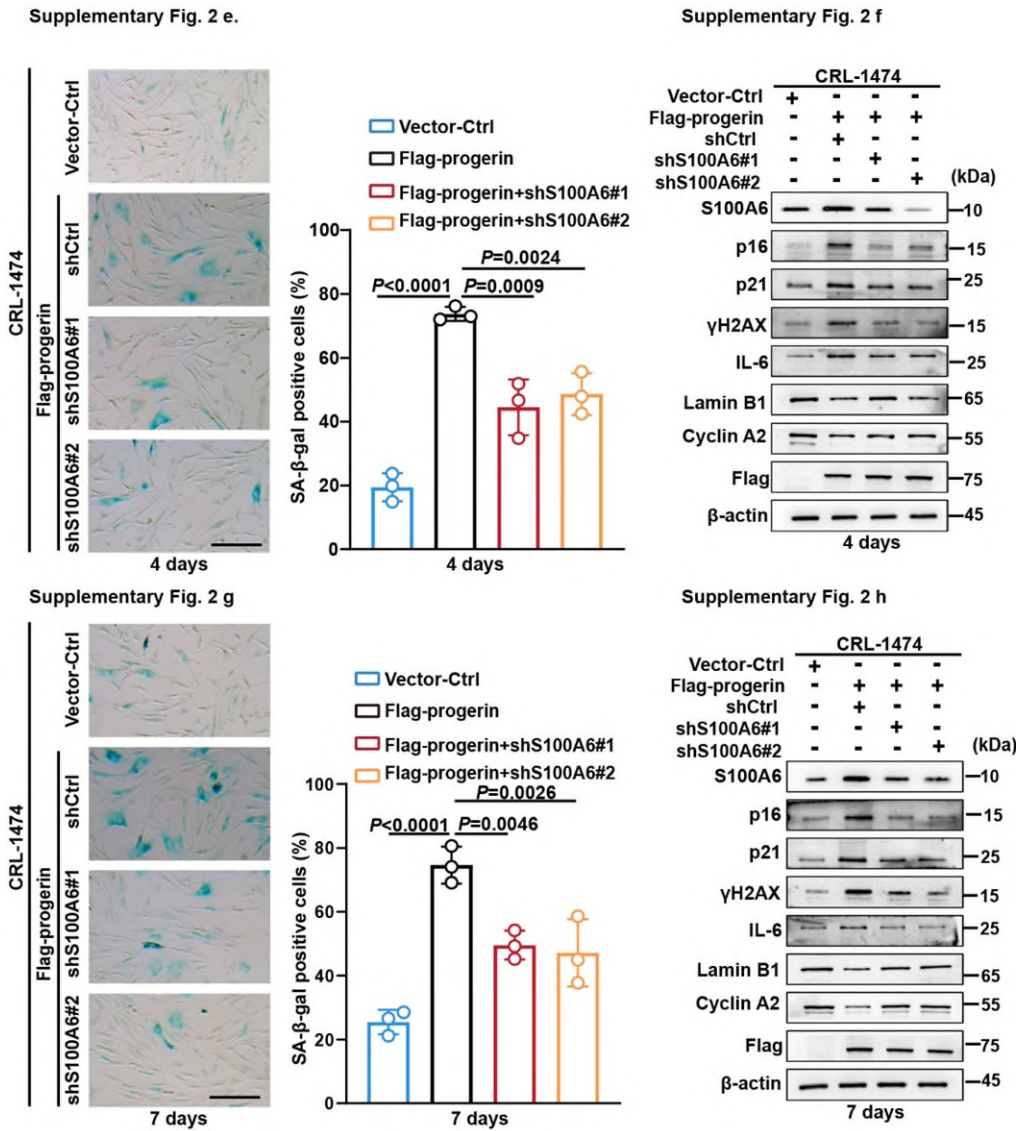
Supplementary Fig. 2 b-c. HGADFN127 cells were transfected with shS100A6 for 4 days and 7days, followed by SA-β-gal staining. Scale bar: 100 μm. The percentage of SA-β-gal positive cells was calculated in HGADFN127 cells transfected with shS100A6 for 4 days (**b**) and 7 days (**c**).

Supplementary Fig. 2 d. Western blot analysis of the protein levels of Cyclin A2 and Lamin B1 was performed in HGADFN127 cells that had been transfected with shS100A6 for 7 days.

Further, we also knocked down S100A6 in CRL-1474 cells overexpressing progerin and evaluated its effects on cellular senescence at 4 and 7 days. The results showed that S100A6 knockdown for either 4 or 7 days reduced the proportion of SA-β-gal–positive cells by approximately 30% to 35% (Supplementary Fig. 2e and g). Consistent with the findings in HGPS patient cells, Cyclin A2 and Lamin B1 protein levels were

substantially restored by shS100A6 in progerin-expressing CRL-1474 cells, accompanied by significant downregulation of IL-6, γ H2AX, p16, and p21 (Supplementary Fig. 2f and h).

Collectively, these results indicate that S100A6 knockdown also alleviates cellular senescent phenotype caused by progerin overexpression.



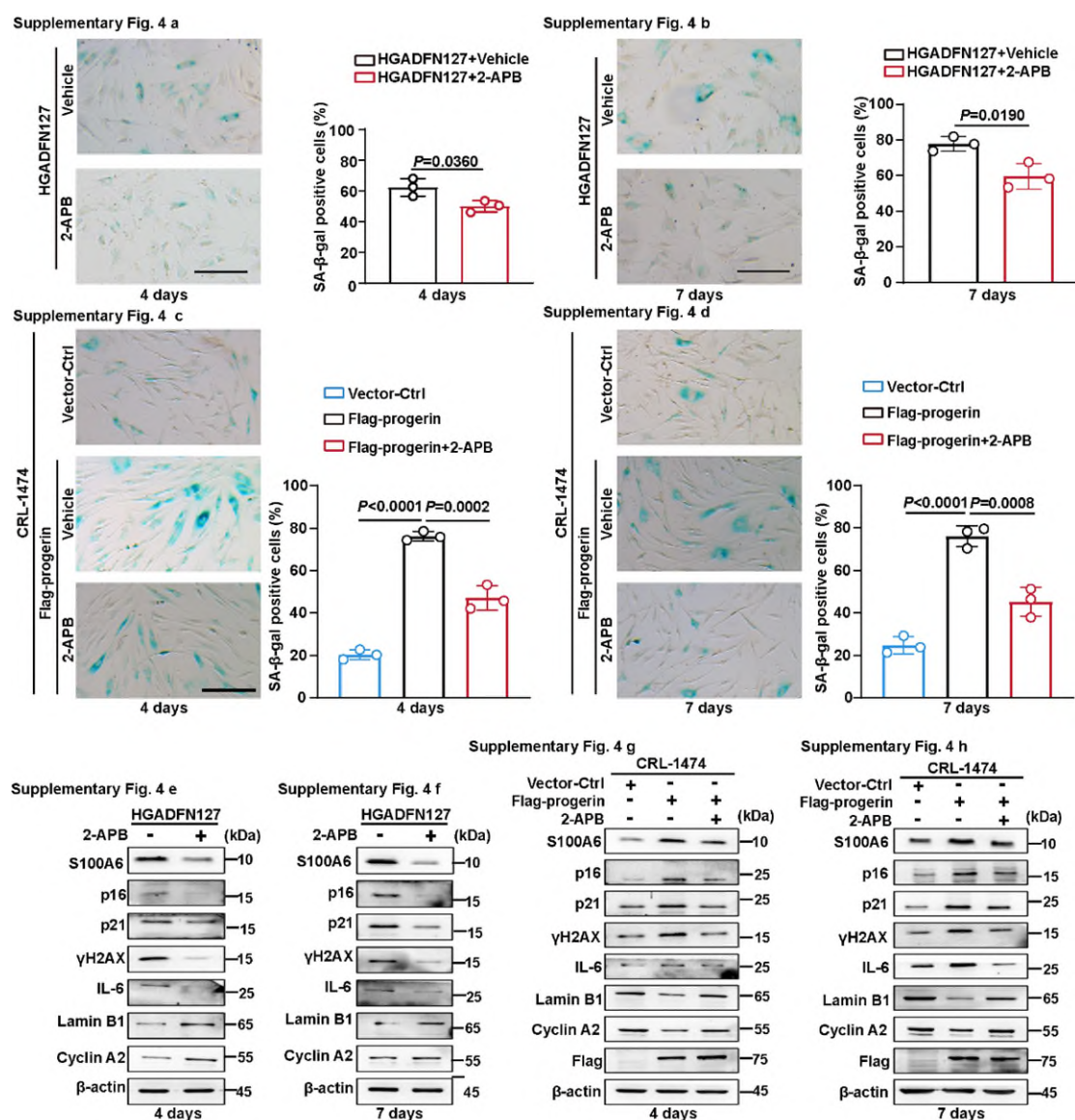
Supplementary Fig. 2 e-h. CRL-1474 cells overexpressing progerin were transfected with shS100A6 for 4 days and 7days, followed by SA- β -gal staining. Scale bar: 100 μ m. The percentage of SA- β -gal positive cells was calculated in 1474 cells overexpressing progerin were transfected with shS100A6 for 4 days (e) and 7 days (g). Western blot analysis of Cyclin A2, Lamin B1, IL-6, IL-8, γ H2AX, PARP1, and S100A6 in CRL-1474 cells overexpressing progerin were transfected with shS100A6 for 4 days (f) and 7 days (h).

Similarly, treatment with 2-APB for both 4 and 7 days in cells derived from HGPS patients reduced the proportion of SA- β -gal positive cells (Fig. 4e-f, Supplementary Fig.

4a-b). Meanwhile, the levels of Cyclin A2 and Lamin B1 proteins were markedly restored, and the levels of IL-6, γ H2AX, p16, and p21 were significantly decreased (Supplementary Fig. 4e-f). Consistent results were observed in CRL-1474 cells overexpressing Flag-progerin (Supplementary Fig. 4c, d, g and h). Based on these observations, we chose a 4-day treatment duration for subsequent experiments to ensure robust phenotypic changes while facilitating timely and consistent evaluation.

We have incorporated the additional results into Supplementary Fig. 4.

We also provided detailed explanations in the revised manuscript (line 1064-1074).



Supplementary Fig. 4 a-b. HGADFN127 cells were treated with 2-APB for 4 days and 7 days, followed by SA- β -gal staining. Scale bar: 100 μ m. The percentage of SA- β -gal positive cells was calculated in HGADFN127 cells treated with 2-APB for 4 days (**a**) and 7 days (**b**).

Supplementary Fig. 4 c-d. CRL-1474 cells overexpressing progerin treated with 2-APB for 4 days

and 7 days, followed by SA- β -gal staining. Scale bar: 100 μ m. The percentage of SA- β -gal positive cells was calculated in 1474 cells overexpressing progerin treated with 2-APB for 4 days (c) and 7 days (d).

Supplementary Fig. 4 e-f. Western blot analysis of Cyclin A2, Lamin B1, IL-6, IL-8, γ H2AX, PARP1, and S100A6 in HGADFN127 cells treated with 2-APB for 4 days (e) and 7 days (f).

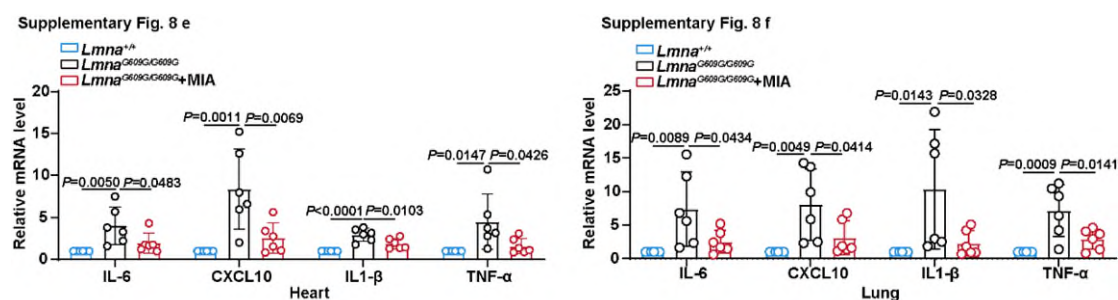
Supplementary Fig. 4 g-h. Western blot analysis of Cyclin A2, Lamin B1, IL-6, IL-8, γ H2AX, PARP1, and S100A6 in CRL-1474 cells overexpressing progerin treated with 2-APB for 4 days (g) and 7 days (h).

Q3: IL6 (and IL8) staining is notoriously hard on (mouse) FFPE tissue sections. If this staining is real, it would be a major asset to the field. However, this also raises the bar as to whether it is real or not. Controls should be added. In all fairness, I think these should be moved to the supplementary figures as they will lead to discussion of an otherwise strong figure.

Response: Thank you very much for your careful review and valuable comments. The concern regarding the reliability of IL-6 (and IL-8) immunohistochemical staining in mouse tissue is entirely reasonable. We have taken this issue very seriously and re-evaluated the relevant experimental data. Upon thorough re-examination, we found that our previous IL-6 and IL-8 immunohistochemistry results contained false-positive signals. To ensure the rigor and reliability of our conclusions, we removed this data from the manuscript.

Instead, we performed qPCR to measure the mRNA levels of inflammatory cytokines, including IL-6, CXCL10, IL-1 β and TNF- α in multiple tissues of HGPS mice. MIA treatment reduced the mRNA level of IL-6, CXCL10, IL-1 β and TNF- α across several tissues (Supplementary Fig.8e and f). This provides transcriptional evidence that MIA suppresses mRNA levels of these inflammatory mediators in the heart and lung of HGPS mice, which was consistent with our findings in HGPS patient cells.

These data have been included in the revised manuscript (Supplementary Fig.8e and f).



Supplementary Fig.8e-f. Quantitative RT-PCR analysis of IL6, CXCL10, IL-1 β and TNF- α mRNA levels in heart and lung of $Lmna^{G609G/G609G}$ mice, with or without MIA treatment.

Q4: The HGPS patient HGADFN127 cells were further treated with IRF3 inhibitor Cyclo (phe-pro) (cFP) and p65 inhibitor Rubiadin-1-methyl-ether (RBM) respectively. The results showed that the mRNA levels of IL-6 and IL-8 in HGADFN127 cells decreased after treatment with p65 inhibitor RBM (Fig. 3e-g). These results indicate that during HGPS cellular senescence, the cGAS-STING-NF- κ B signaling pathway is activated, leading to the promotion of SASP factor transcription. These results have been incorporated into Fig. 3d-g in the revised manuscript.

[3f+g are convincing, 3e is not; this should be removed or improved. Why no QPCR with cFP in that regard?]

Response: We thank the reviewer for this insightful observation. We have now included qPCR data with cFP treatment in Fig. 3f to comprehensively assess the impact of IRF3 inhibition on the mRNA level of IL6 and IL8. The results showed that IL-6 and IL-8 mRNA levels in HGADFN127 cells remained unchanged after treatment with the IRF3 inhibitor cFP (Fig. 3f), consistent with the protein level (Fig. 3e). On the other hand, treatment with p65 inhibitor RBM significantly reduced both the mRNA and protein levels of these cytokines. These findings indicate that NF- κ B (p65) pathway, not IRF3, is essential for the upregulation of IL-6 and IL-8 in HGPS cells.

We also provided detailed explanations in the revision, as shown in line 168-170.

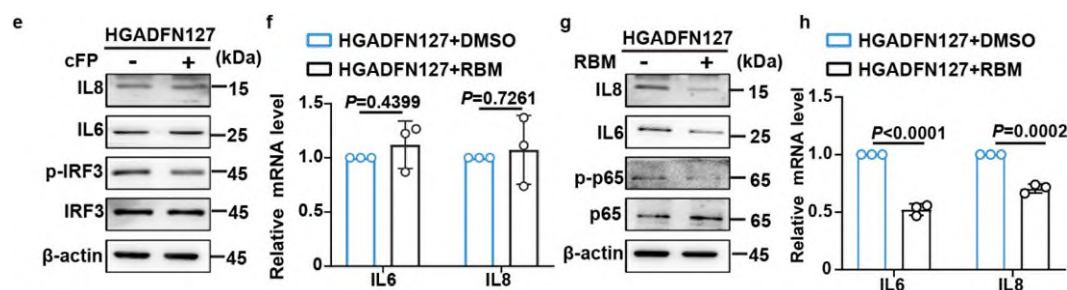


Fig. 3e and g. Western blot analysis of IRF3, p-IRF3, p65, p-p65, IL-6, and IL-8 in HGADFN127 cells treated for 24 h with the IRF3 inhibitor cFP (2.5 mM; **e**) or the p65 inhibitor RBM (10 μ M; **g**).

Fig. 3f-h. Quantitative RT-PCR analysis of IL6 and IL8 mRNA levels in HGADFN127 cells treated with cFP (**f**) and RBM (**h**) for 24 h.

Minor:

Q2: In Fig. 3a (and less so in 5o) it is unclear whether this was stable expression of Flag-progerin, or a transient expression. The latter is notoriously challenging in IMR90

and may well result in susceptibility to DNA damage per se, especially in case of electroporation. An empty vector control would not to a similar response. A FLAG-GFP may transiently do something similar.

Response: We agree that distinguishing between stable and transient expression, as well as the potential confounding effects of transfection methods, is critical for proper interpretation of the results. The stable expression of progerin promotes cellular senescence and cell cycle arrest, making it extremely difficult to obtain stable cell lines through conventional selection. Instead, we used lentiviral transduction to offer high infection efficiency and enable stable expression of progerin after infection. In this study, Flag-progerin was expressed in IMR90 and CRL1474 cells via a lentiviral packaging system. We confirmed the stable progerin expression and the induction of a senescent phenotype in the cells after transduction before proceeding with subsequent experiments.

[Please indicate this in the text. Lentiviral transduction of proteins (i.e. not merely a vector control) can also cause DNA damage on its own, so the fact this data was generated as such is relevant to note].

Response: We thank the reviewer for this important point. We fully acknowledge that lentiviral transduction per se may induce cellular stress or DNA damage, and therefore requires appropriate experimental controls to distinguish vector-related effects from those specifically caused by the transgene. In our experiments, all comparisons were performed between cells transduced in parallel under identical conditions: one group with lentivirus expressing Flag-progerin and the other with the empty lentiviral vector (pCDH-CMV-3×Flag). The results showed that DNA damage markers (e.g., γ H2AX foci), senescence phenotypes, and cell cycle arrest were consistently and significantly higher in Flag-progerin-expressing cells than in empty vector-transduced controls.

As suggested, we have now explicitly stated in the figure legends for Fig. 3a and Fig. 5p-q as follow: Flag-progerin was expressed via lentiviral transduction; control cells were transduced with empty lentiviral vector (pCDH-CMV-3×Flag) under identical conditions.

We also provided detailed explanations in the revision, as shown in line 160-162, line 850-851, and line 923-924.

Reviewer #3 (Remarks to the Author):

I thank the authors for making thorough revisions in response to my initial comments. Most of my concerns have been addressed to my satisfaction, and I appreciate the authors' use of fluorescence intensity to demonstrate changes in calcium concentrations. However, there are still several areas where further clarification or revision is needed.

1. Specific Force Calculation (related to Q3)

In my third comment, I suggested reporting specific force instead of absolute force, and I'm glad to see that the authors have made this change. However, in the Methods section (lines 572–573), the manuscript incorrectly states that specific force was calculated by normalizing to the TA muscle mass. Specific force should instead be normalized to the cross-sectional area (mm²) of the muscle, which is indeed the unit used in the figures, but introduced incorrectly in the method. Moreover, there is currently no description in the Methods of how the muscle cross-sectional area was determined. The authors should add this methodological detail. Additionally, in the figure legend (lines 953–954), it should be clearly stated that the forces presented are specific forces normalized to muscle cross-sectional area.

Response: We sincerely thank the reviewer for this careful and insightful comment. We apologize for the inappropriate description in the Methods section (lines 572–584). As the reviewer pointed out, specific force must be normalized to physiological cross-sectional area (CSA), not muscle mass. We wish to clarify that this is indeed the approach we used in our analysis, and the values shown in the figure are correctly presented as specific force (in mN/mm²). We have revised the Methods section in the manuscript to accurately reflect this normalization procedure.

We have now corrected the Methods section to accurately describe how CSA was calculated. Specifically, we added the following:

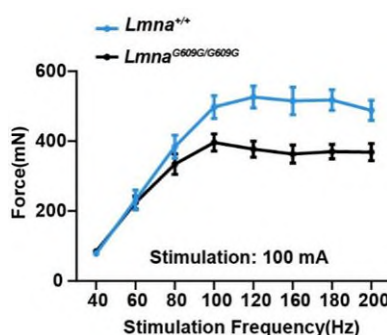
“Specific force was normalized to the physiological cross-sectional area (CSA) of the tibialis anterior (TA) muscle, calculated using the formula: $CSA = \text{muscle mass} / (L_f \times 1.06)$, where L_f is the fiber length estimated as $L_o \times 0.6$ (with L_o being the optimal muscle length), based on the established fiber length-to-muscle length ratio for mouse TA, and 1.06 g/cm³ is the assumed density of skeletal muscle.”

These revisions ensure methodological clarity and consistency between the text, methods, and data presentation. Thank you again for your valuable feedback.

2. Force-Frequency Relationship (related to Q4)

In response to my fourth comment, the authors mentioned that their pilot study data showed that 100 Hz stimulation is sufficient to evoke maximal force production. However, to determine the optimal stimulation frequency for maximum force generation, a force-frequency curve should be established. I do not see such data presented in the current manuscript. The authors should either include this data within the main figures or at least provide it in the supplementary materials as supporting evidence.

Response: We thank the reviewer for this important suggestion. As requested, we have now included the force-frequency curve data in Supplementary Fig. 7k. This curve was generated using the same experimental setup and muscle preparations described in the manuscript (line 576-580), which demonstrated that stimulation at 100 Hz elicited near-maximal force production, consistent with our pilot observations. We believe these data provides essential supporting evidence for our choice of stimulation.



Supplementary Fig. 7k. Force–frequency relationship in tibialis anterior muscle from *Lmna*^{+/+} and *Lmna*^{C609G/C609G} mice (n=6). TA muscle force was measured at stimulation frequencies ranging from 40 to 200 Hz under a constant current of 100 mA (n=6).

3. Loading Control for Western Blot (related to Q5)

I appreciate the authors' revision in changing the loading control from β -actin to GAPDH. However, GAPDH is also not an ideal housekeeping protein under certain physiological conditions, particularly those involving altered redox balance. For example, during aging, oxidative stress can significantly affect GAPDH content. Ideally, total protein normalization remains the gold standard for controlling sample loading. That said, since the authors used two different loading controls and obtained consistent results, I consider the current data acceptable.

Response: Thank you for your thoughtful comment regarding loading controls. We appreciate your recognition of our revision from β -actin to GAPDH, and we fully agree that no single housekeeping protein is universally stable, particularly under conditions involving oxidative stress or aging, where GAPDH expression can indeed be affected. To address this concern rigorously, we would like to clarify that all protein samples

were quantified using the BCA assay prior to electrophoresis, and equal amounts of total protein were loaded. This approach ensures consistent sample loading independent of individual protein expression changes. We acknowledge that total protein normalization represents the current gold standard, and we will adopt such methods in future studies.

4. Minor Concerns

I still have several minor concerns from my initial review for which I did not see a formal point-by-point response. Although I noticed that some of these issues have been revised, others appear to remain unaddressed. I encourage the authors to list all the minor concerns, similar to how they addressed the major comments, and indicate how each has been revised or resolved.

Response: We sincerely apologize for not having provided a point-by-point response to all of the detailed comments you raised in your initial review. We addressed these issues during the editing process. However, we fully recognize that without a clear point-by-point response list, it would be difficult to verify whether each concern has been adequately addressed. To ensure clarity, we now provide a point-by-point response to all minor comments from the original review (listed below).

Thank you again for your diligence and for the opportunity to improve our work.

Q1: Line 59-61, Reference needed for the introduction of mutations in LMNA gene.

Response: We thank the reviewer for this suggestion. We have now added references to support the statement regarding disease-causing mutations in the *LMNA* gene.^[1-3]

- [1] Eriksson M, Brown W T, Gordon L B, et al. Recurrent de novo point mutations in lamin A cause Hutchinson-Gilford progeria syndrome [J]. *Nature*, 2003, 423(6937): 293-298.
- [2] De Sandre-Giovannoli A, Bernard R, Cau P, et al. Lamin a truncation in Hutchinson-Gilford progeria [J]. *Science (New York, NY)*, 2003, 300(5628): 2055.
- [3] Merideth M A, Gordon L B, Clauss S, et al. Phenotype and course of Hutchinson-Gilford progeria syndrome [J]. *The New England journal of medicine*, 2008, 358(6): 592-604.

Q2: In Lines 63–65, the manuscript contains the only statement regarding Ca²⁺ concentration in HGPS patients. Since this study emphasizes calcium homeostasis and its role in HGPS, it would be beneficial to include additional background or evidence of calcium dysregulation in HGPS in this paragraph.

Response: Thank you for this insightful comment. As suggested, we expanded the background in the Introduction to include additional evidence regarding calcium dysregulation in HGPS as follow: Specifically, we have added references to several

studies. For example, transcriptomic analyses comparing cell lines derived from HGPS patients and healthy controls revealed significant dysregulation of calcium signaling-related genes, such as ITPR1, ITPR3, CACNA2D1, and CAMK2N1^[1]. Moreover, progerin-expressing myoblasts exhibited enhanced store-operated calcium entry (SOCE), along with increased co-localization of the key regulatory proteins STIM1 and ORAI1 at the plasma membrane, indicating aberrant activation of calcium signaling pathways ^[2]. (line 68-71).

- [1] Fafián-Labora JA, Morente-López M, de Toro FJ, Arufe MC. High-Throughput Screen Detects Calcium Signaling Dysfunction in Hutchinson-Gilford Progeria Syndrome. *Int J Mol Sci*. 2021 Jul 7;22(14):7327.
- [2] Wang WP, Wang JY, Lin WH, Kao CH, Hung MC, Teng YC, Tsai TF, Chi YH. Progerin in muscle leads to thermogenic and metabolic defects via impaired calcium homeostasis. *Aging Cell*. 2020 Feb;19(2): e13090.

Q3: Line 119, definition needed for HEK-293T and CRL-1474 cells.

Response: We thank the reviewer for this suggestion. We have now added brief definitions for both HEK-293T and CRL-1474 cells in the revised manuscript: HEK-293T cells (human embryonic kidney cells expressing SV40 large T antigen); CRL-1474 cells (human skin fibroblasts derived from a 7-year-old boy) (line 137-139).

Q4: Line 148, the first appearance of shS100A6, need to define shS100A6 as short hairpin RNA (shRNA) designed to knock down the expression of the S100A6 gene.

Response: We thank the reviewer for this suggestion. We have defined "shS100A6" at its first appearance (line 174) as a short hairpin RNA (shRNA) designed to knock down S100A6 level.

Q5: The animal experimental data presented in the main results appear to be based solely on male mice. However, I noticed that both male and female mice were included in the extended data, and the outcomes seem comparable between both sexes. Please clarify why only male mice data are shown in the main results and provide a justification for this choice in the Methods section.

Response: We sincerely thank the reviewer for their careful attention to the issue of sex selection in our animal experiments. To ensure the comprehensiveness and representativeness of our conclusions, the majority of experiments in HGPS mice were conducted in both male and female animals. Due to space constraints, the main figures primarily present pooled data from both male and female mice, except for representative images of external morphology and Micro-CT, which show male mice. The complete sex-stratified results are provided in the Supplementary Fig. 5,

Supplementary Fig. 6 and Supplementary Fig. 7. The results for body weight, locomotor activity, and survival from both male and female mice show consistent trends, collectively supporting the sex-independent nature of our key conclusions.

Q6: For all the Western blots, “kDa” is a standalone unit, so there should be a space between the numerical value and “kDa” (e.g., “25 kDa” instead of “25kDa”).

Response: We thank the reviewer for their careful correction. We have now uniformly revised all Western blot images and corresponding figure legends throughout the manuscript to ensure a space between the numerical value and “kDa.”

Point-by-Point Response to NCOMMS-25-10890B

Title: Ameliorating calcium homeostasis improves longevity and healthspan in progeroid and naturally aged mice

Reviewer #1 (Remarks to the Author):

Specific comments:

Q1: The results in Fig. 1h, sup Fig 2b and Sup. Fig 2c are not convincing to argue that shS100A6 reduces senescence. This claim should therefore be removed from the manuscript.

Regarding this response “The results showed that both 4-day and 7-day S100A6 knockdown reduced the proportion of SA- β -gal-positive cells and the reduction is appropriate 18% (4 days) and 20% (7 days) (Fig.1h and Supplementary Fig. 2b-c).”: The results in Fig. 1h, sup Fig 2b and Sup. Fig 2c are not convincing to argue that shS100A6 reduces SA- β -gal/Senescence. This claim should therefore be removed from the manuscript.

Regarding this response “We think the modest change of SA- β -gal might be attributed to the fact that HGPS patient cells are already in a stable state of profound senescence, making it difficult to achieve a more substantial SA- β -gal reversal even after S100A6 knockdown. Actually, we observed that knockdown of S100A6 reduced the staining intensity of SA- β -gal-positive cells (Fig.1h and Supplementary Fig. 2b-c); however, in our quantification, these cells were still counted as positive, which may partly explain why SA- β -gal-positive proportion did not show a very significant decrease.” The last sentence here confirms there is not a very significant effect. Therefore the claim is an overstatement. If cells were counted as positive that are not, the quantification should be performed differently. With these figures, the statement that shS100A6 reduces SA- β -gal /Senescence is simply not correct.

Response: We sincerely thank the reviewer for this critical and constructive assessment. We agree with your evaluation that the SA- β -gal staining data (Fig. 1h, Supplementary Fig. 2b–c), showing a modest reduction in positive cells, are not sufficiently robust on their own to definitively conclude that shS100A6 reduces HGPS patient cells senescence. As suggested, we removed the Fig. 1h, Supplementary Fig. 2b, and Supplementary Fig. 2c. Accordingly, we removed the description of the SA- β -gal rescue by shS100A6 in HGPS patient cells, while retaining the improvement in SA- β -gal staining caused by shS100A6 in the presence of progerin overexpressed CRL-1474 cells.

Specifically, the Results section now reads: “Knockdown of S100A6 in HGPS patient cells increased the expression of Cyclin A2 and Lamin B1, downregulated γ H2AX, p16, and p21 (Fig. 1f), and restored the cell proliferative capacity (Fig. 1g). Additionally, knockdown of S100A6 in CRL-1474 cells overexpressing progerin increased the expression of Cyclin A2 and Lamin B1, downregulated γ H2AX, p16 (Supplementary Fig. 2c and e), and reduced proportion of SA- β -gal-positive cells (Supplementary Fig. 2b and d).”

We believe this revised framing accurately represents the data without overstating the effects of the SA- β -gal assay in shS100A6 in HGPS patient cells.

Q2: Regarding this response “To further confirm the role of S100A6 in HGPS senescence, we added more senescent markers in this version. Consistently, protein levels of Cyclin A2 and Lamin B1 were markedly restored, while IL-6, γ H2AX, p16, and p21, was downregulated after S100A6 knockdown for both 4 and 7 days (Fig. 1f and Supplementary Fig. 2d). All these results showed that shS100A6 alleviated HGPS cellular senescence.” These western blot results also show marginal effects (one would like to see the full blots to exclude exposure artefacts and a positive control lane next to them). Also, IL-6 is not something the field shows through western blot. All in all, the data to support the argument that shS100A6 reduces senescence is not convincing.

Response: We sincerely thank the reviewer for these rigorous and constructive comments. We agree that our interpretation of the Western blot data requires refinement, particularly regarding the modest magnitude of the observed effects and the methodological limitations of using Western blotting for IL-6 quantification. To address these concerns comprehensively, we have taken the following actions:

Taking into account the concerns regarding the marginal effects of the experimental results and potential exposure artifacts, we carefully re-examined two additional independent replicates for Fig. 1f and Supplementary Fig. 2d, and replaced the bands affected by these marginal effects. Furthermore, we have provided complete, uncropped Western blot membranes for all target proteins (Cyclin A2, Lamin B1, γ H2AX, p16, and p21) within the Source Data file.

We fully agree with the reviewer's assessment. While we acknowledge that some previous studies have utilized Western blotting to detect IL-6, we recognize that ELISA and qPCR are the more established and standard methods in this field. To adhere to best practices and ensure the robustness of our data, we have removed the IL-6 Western blot results from the revised manuscript.

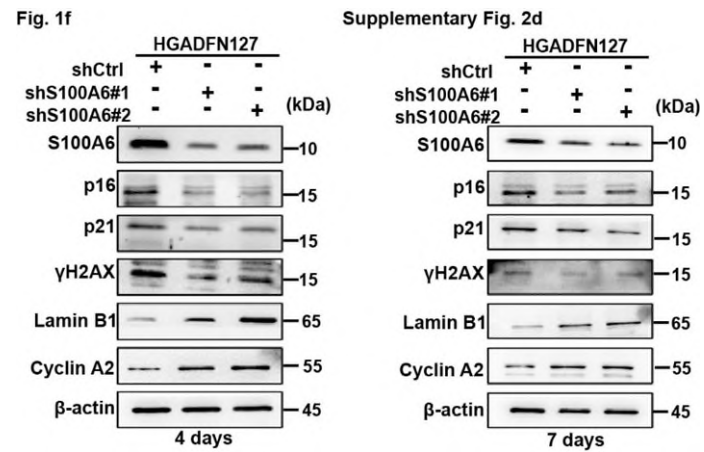


Fig. 1 f. Western blot analysis of the protein levels of Cyclin A2, Lamin B1, IL-6, γ H2AX, p21, p16 and S100A6 was performed in HGADFN127 cells that had been transfected with shS100A6 for 4 days.

Supplementary Fig. 2 d. Western blot analysis of the protein levels of Cyclin A2 and Lamin B1 was performed in HGADFN127 cells that had been transfected with shS100A6 for 7 days.