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Sub-lethal apoptotic stress enables mtDNA release during senescence and drives the SASP

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Abstract:

Senescent cells drive age-related tissue dysfunction partially via the induction of a chronic senescence-associated secretory phenotype (SASP). Mitochondria are major regulators of the SASP; however, the underlying mechanisms have not been elucidated. Mitochondria also control apoptosis, a cell fate generally perceived as distinct from cellular senescence, which is apoptosis resistant.

Here, we show that mitochondrial outer membrane permeability (MOMP) occurring in a small subset of mitochondria is a feature of cellular senescence. This process, called minority MOMP (miMOMP), depends on the formation of BAX and BAK macropores and results in release of mitochondrial DNA (mtDNA) into the cytosol, which in turn activates the cGAS/STING pathway, a major regulator of the SASP. Importantly, we found that inhibition of MOMP *in vivo* decreases inflammatory markers and improves healthspan parameters in aged mice.

Our results propose the novel concept that apoptosis and senescence are regulated by similar mitochondrial-dependent mechanisms, and that sub-lethal mitochondrial apoptotic stress is a major driver of the SASP. We also provide proof-of-concept that inhibition of miMOMP may be a new therapeutic avenue to improve healthspan.

Introduction:

Cellular senescence refers to the irreversible growth arrest that occurs as a response to different stressors¹. Senescent cells secrete pro-inflammatory cytokines, chemokines, and extra-cellular matrix degrading proteins, which are collectively known as the senescence-associated secretory phenotype (SASP)². Senescent cells accumulate with age in different tissues and in multiple chronic diseases and clearance of senescent cells whether genetically or by using drugs which can specifically kill senescent cells alleviates the onset of a number of pathologies in aging mice³.

Therefore, these cells represent promising therapeutic targets to delay, or perhaps even reverse, functional deficits, and chronic disorders in aging.

Our group and others have demonstrated that mitochondrial dysfunction, characterized by alterations in morphology, dynamics, and function, is a hallmark of cellular senescence⁴⁻⁶. We have previously conducted a proof-of-principle experiment that revealed that clearance of mitochondria in senescent cells suppresses the SASP while preserving the cell-cycle arrest⁴. This work led us to propose that mitochondria may be promising targets for anti-senescence therapies and that by targeting them we may be able to suppress the detrimental aspects of the SASP known to contribute to aging, while maintaining the tumor suppressor capabilities of senescent cells⁷. Mitochondria are also majorly involved in apoptosis, a process which involves mitochondrial outer membrane permeability (MOMP) and subsequent release of cytochrome c through the actions of the pro-apoptotic proteins BAX and BAK⁸. Together with APAF-1, cytoplasmic cytochrome c forms part of the apoptosome complex, which activates caspase-9 and the apoptotic effector caspases, caspase-3, and caspase-7. Furthermore, apoptosis is accompanied by release of mtDNA into the cytosol. BAX and BAK pores gradually widen, allowing the extrusion of the inner mitochondrial membrane into the cytosol, where it becomes permeabilized, facilitating mtDNA release^{9,10}.

In this study, we sought to investigate the relationship between mitochondrial apoptosis and cellular senescence. We found that MOMP occurring in a small subset of mitochondria without inducing cell-death, a process known as minority MOMP (miMOMP)¹¹ is a feature of cellular senescence. During senescence, miMOMP, which depends on the formation of BAX and BAK macropores, results in release of mtDNA into the cytosol, which in turn activates the cGAS/STING pathway, a major regulator of the SASP¹². Finally, we found that inhibition of miMOMP *in vivo* decreases inflammatory markers and improves several parameters of healthspan in aged mice,

including frailty, motor coordination and physical endurance, grip strength and age-related bone loss.

Our studies support the novel concept that apoptosis and senescence are in fact regulated by similar mitochondrial-dependent mechanisms, and that miMOMP is a major contributor to senescence, specifically to the SASP. Our data supports that this can be explored as a new therapeutic avenue to improve healthspan.

Results:

miMOMP is a feature of cellular senescence

While originally considered as a binary, all-or-nothing event, MOMP can occur in a minority of mitochondria – termed minority MOMP (miMOMP) – in the absence of cell death ¹¹. To investigate whether miMOMP is a feature of cell senescence, we first conducted 3D super-resolution microscopy (SIM) on both proliferating and senescent human fibroblasts and analyzed co-localization between outer mitochondrial membrane protein TOM20 and cytochrome c (Cyt c). While in proliferating cells Cyt c and TOM20 show a reasonably good degree of co-localization, in senescent cells we observed the presence of a small number of peripheral mitochondria (dissociated from the network and exhibiting a globular structure) which are positive for TOM20 but not for Cyt c (**Figure 1a**). Unbiased quantification of the Pearson's correlation coefficient revealed decreased co-localization between Cyt c and TOM20 in both DNA damage-induced senescent cells (Sen IR) and replicatively senescent cells (**Figure 1b**). Confirming this observation, we found increased cytosolic Cyt c (**Figure 1c & d**) and increased expression of cleaved caspase-3 (**Figure 1e**) in senescent cells. We then investigated whether miMOMP was accompanied by BAX oligomerization, which is indicative of activation. Oligomerization of endogenous BAX was analyzed by size exclusion chromatography and revealed that BAX forms self-oligomers in senescent cells but not proliferating cells (**Figure 1f**). Additionally, using antibody

BAX6A7 which detects an active form of BAX, we found by 3D SIM microscopy that in senescent cells, peripheral globular mitochondria dissociated from the main network show increased expression of activated BAX (**Figure 1g**). Increased activation of BAX was observed in two independent human fibroblast strains (MRC5 and IMR90) and in the context of both Sen IR and ER-RAS oncogene-induced senescence (OIS) (**Figure 1h, i**).

Cytosolic mitochondrial DNA increases in senescent cells.

During apoptosis it has been shown that MOMP leads to mtDNA being released into the cytosol. For that reason, we hypothesized that mitochondria undergoing miMOMP can release mtDNA into the cytosol of senescent cells.

By employing dual immunostaining of the mitochondria outer membrane protein TOM20 and DNA by 3D super-resolution Airyscan confocal microscopy (which allows the visualization of the mitochondrial outer membrane and mtDNA nucleoids as distinct entities under sub-diffraction-limiting conditions) we found that senescent fibroblasts contain an increased frequency of DNA nucleoids in the cytosol. This observation was consistent between different cell-types (human fibroblasts MRC5 and IMR90 and mouse adult fibroblasts) and occurred irrespectively of the senescence-inducing stimuli (Sen IR, replicative senescence or OIS) (**Figure 2a & b**). Similarly, we observed increased nucleoids in the cytosol of senescent cells by immunogold labelling transmission electron microscopy (**Figure 2c**).

To determine the origin of the DNA present in the cytosol, we performed Q-PCR to specifically detect mtDNA (D-loop region) and found a significant increase in the cytosolic fractions of Sen IR, OIS and replicative senescent cells (**Figure 2d**). Further supporting the mitochondrial origin of cytosolic DNA, we found that the majority of cytosolic DNA co-localized with TFAM (transcription factor A, mitochondrial), one of the core components of the mtDNA nucleoid, which is essential for the formation, abundance, and segregation of mtDNA (**Figure 2e & f**). Cytosolic

fractionation followed by western blotting confirmed an enrichment of TFAM in the cytosol of senescent cells (**Figure 2g**).

These results support that mtDNA is present in the cytosol of senescent cells, prompting us to investigate the functional consequences and underlying mechanisms leading to its release.

miMOMP is a driver of the SASP

Having observed that miMOMP occurs in senescent cells, we sought to investigate if miMOMP could solely drive senescence and the SASP. For that, we treated proliferating human fibroblasts with miMOMP inducer ABT-737, the prototypic BH3-mimetic compound that antagonizes anti-apoptotic BCL-2 family proteins, at a low concentration which does not induce any cell-death but leads to caspase activation (**Supplementary Figure 1a**). We found that chronic treatment with ABT-737 in proliferating human fibroblasts for 23 days resulted in a significant increase in the secretion of SASP components IL-6 and IL-8 (**Supplementary Figure 1b & c**). Additionally, we found a small but significant increase in senescent-associated markers γ H2A.X foci and SA- β -Gal (**Supplementary Figure 1d & e**). Consistent with the reported role of MOMP in the release of mtDNA to the cytosol, we found that treatment with ABT-737 resulted in increased number of extra-mitochondrial nucleoids (**Supplementary Figure 1f**).

We and others have previously reported that the escape of mtDNA from within the mitochondrial matrix occurs due to the formation of BAX and BAK macropores^{10,13}. Therefore, given our observation that senescent cells show BAX oligomerization and activation, we sought to address whether BAX and BAK (two proteins essential for MOMP) can facilitate the release of mtDNA to the cytosol in senescent cells. For that, we utilized the CRISPR-Cas9 gene editing tool to generate human fibroblasts which were deficient for both BAX and BAK (**Figure 3a**). We found that combined deletion of BAX and BAK suppressed the cytosolic leakage of mtDNA in senescent cells induced by DNA damage (**Figure 3b**). To investigate whether BAX and BAK impacted on

the SASP, we performed RNA-sequencing and found that commonly expressed SASP genes such as IL-1 α , IL-1 β , IL-6, IL-8 and others were significantly decreased following BAX and BAK deletion (**Figure 3c**). Furthermore, we performed a cytokine array using conditioned media from senescent cells expressing Empty^{CRISPR} and BAX/BAK^{CRISPR} and found that BAX and BAK deletion significantly inhibited the secretion of various SASP factors (**Figure 3d**). We confirmed the BAX/BAK-dependent decrease in different pro-inflammatory cytokines by independent ELISAs and qPCRs (**Supplementary Figure 2a-f**). We then investigated whether deletion of BAX and BAK impacted on pathways regulating the senescence cell-cycle arrest. We found that deletion of BAX and BAK did not alter the expression of cyclin-dependent kinase inhibitors p21 and p16^{INK4A} (**Figure 3e-g and 3m**), nor restored the senescence-associated loss of LaminB1 and HMGB1 (**Figure 3h**); SA- β -Gal activity (**Figure 3i and 3m**) or the number of γ H2A.X foci (**Figure 3j and 3m**). No change in expression of proliferation marker Ki-67 was observed (**Figure 3i and 3m**) as well as mRNA expression of genes involved in proliferation downregulated in senescent cells (**Figure 3l**), suggesting that BAX and BAK regulate the SASP but not the senescence-associated cell-cycle arrest.

Previous reports suggested that mitochondrial permeability transition pore (MPTP) could be involved in the release of mtDNA¹⁴. To investigate the role of the MPTP, we treated senescent cells with cyclosporin A, which blocks the MPTP by its binding to the mitochondrial cyclophilin D and then measured mtDNA release and secretion of the SASP factors IL-6 and IL-8. Treatment with Cyclosporin A did not alter the percentage of cells containing cytoplasmic DNA and increased secretion of SASP components IL-6 and IL-8 (**Supplementary Figure 2 g & h**).

Deletion of BAX and BAK can trigger other mechanisms which may contribute to the SASP, besides enabling release of mtDNA. For instance, dysfunctional mitochondria in senescent cells, have been shown to trigger a ROS-JNK retrograde signaling that results in the formation of cytosolic chromatin fragments (CCFs) and the SASP¹⁵. However, we did not find evidence that

BAX and BAK impacts on mitochondrial function, ROS generation or formation of CCF in senescent cells (**Supplementary Figure 2 i-k**). Additionally, we did not find significant differences in global mRNA expression of OXPHOS components due to BAX and BAK deletion in both proliferating and senescent cells (**Supplementary Figure 2l**).

It is possible that the SASP may be influenced by BAX and BAK-dependent activation of caspases, which in the context of apoptosis dampen the inflammatory response¹⁶⁻¹⁸. APAF1, which is a central hub in the regulation of apoptosis, binds to cytochrome c and forms the apoptosome, leading to activation of caspase 9, 3 and 7 (**Supplementary Figure 3a**). To investigate the role of APAF1 on senescence, we used CRISPR-Cas9 to delete APAF1 in human fibroblasts (**Supplementary Figure 3b**). We first observed that APAF1 deficient cells reach replicative senescence at the same population doubling as controls (**Supplementary Figure 3c**). Moreover, APAF1 deletion did not impact on expression of cyclin-dependent kinase inhibitors, proliferation marker Ki67, and SASP components when senescence was induced by irradiation (**Supplementary Figure 3d-k**). These results indicate that miMOMP-dependent caspase activation is not sufficient to dampen the SASP.

To further investigate the role of BAX and BAK in senescence *in vivo*, we used Bax^{fl/fl} Bak^{-/-} mice and performed tail vein injection with AAV-TBG-Cre virus, which allows loss of liver Bax expression. We subsequently exposed mice to 4Gy of ionizing irradiation, a dose previously shown to induce senescence and the SASP in liver^{12,15} (**Figure 4a**). We first confirmed by Immunohistochemistry (IHC) that BAX was absent in the liver (**Figure 4a**). We then characterized the mRNA expression of different pro-inflammatory components 6 days after ionizing radiation and found that while these were significantly increased in BAK^{-/-} mice, deletion of both BAX and BAK suppressed their induction (**Figure 4b**). Nonetheless, when we analyzed the senescent marker– telomere-associated foci (TAF), which denotes co-localization between the DNA damage response protein γ H2A.X and telomeres, we found that it was induced by irradiation irrespectively

of the genotype (**Figure 4 c-e**). Radiation had no significant effect on the % of caspase 3 high positive liver cells, which indicates that senescence rather than apoptosis was induced in these conditions (**Figure 4f**).

Cytosolic mtDNA is a contributor to the SASP via activation of cGAS/STING pathway

mtDNA is known to serve as a trigger for cGAS/STING pathway activation in the context of apoptosis^{9,10} and cGAS/STING have been shown to regulate the SASP¹². However, the link between mtDNA cytosolic release and the regulation of the SASP has not been investigated thus far. We hypothesized that during senescence, miMOMP mediated mtDNA cytosolic release contributes to the SASP by engaging the innate immunity cytosolic DNA-sensing cGAS-STING pathway.

To investigate the specific role of mtDNA in the regulation of the SASP, we first sought to generate cells devoid of mitochondria¹⁹. The rationale behind this experiment, was that this cellular system could be used as a *tabula rasa*, allowing the specific study of mtDNA in the regulation of the SASP uncoupled from other mitochondrial functions. Human fibroblasts were stably transduced with YFP-Parkin and senescence was induced by X-ray irradiation. Parkin-expressing senescent cells were treated with mitochondrial uncoupler CCCP which triggers widespread mitophagy and generates cells devoid of mitochondria¹⁹. Cells were then transfected with isolated mtDNA (**Figure 5a**). mtDNA levels were undetected by qPCR in mitochondria devoid senescent cells and significantly increased upon mtDNA transfection (**Figure 5b**). We confirmed by western blotting that mitochondrial proteins NDUFB8 and UQCRC2 were absent following Parkin-mediated mitophagy and not affected by transfection with mtDNA (**Figure 5c**). RNA-sequencing confirmed that Parkin-mediated clearance of mitochondria suppressed the expression of commonly known SASP components. Notably, we found that mRNA expression of SASP genes which were downregulated upon mitochondrial clearance were subsequently up regulated upon transfection

with mtDNA (**Figure 5d**). Secretion of IL-6 and IL-8 proteins was also partially restored in mitochondria devoid senescent cells upon reintroduction of mtDNA (**Figure 5e-f**). Aberrant mtDNA packaging due to TFAM deficiency was shown to enhance the expression of a subset of interferon-stimulated genes (ISG)²⁰. We used our RNA-seq dataset to investigate if this subset of mtDNA stress-induced ISG genes was up-regulated during senescence and if its expression was dependent on the presence of mitochondria or mtDNA. We found that majority of mtDNA stress-induced ISG genes were up-regulated in senescence, reduced upon mitochondrial clearance, and restored upon reintroduction of mtDNA (**Supplementary Figure 4a**). Using a more comprehensive database of both NF- κ B pathway regulated and interferon response genes we found similar patterns (**Supplementary Figure 4b-d**).

Given our data supporting a role for cytosolic mtDNA in cellular senescence, we cultured both wild-type and *Tfam*^{+/-} mouse embryonic fibroblasts (MEFs), which show enriched cytosolic mtDNA²⁰, until they reached senescence. We found that *Tfam*^{+/-} MEFs reached replicative senescence earlier than wild-type MEFs (**Supplementary Figure 5a**) and showed increased SA- β -Gal activity and mRNA expression of p16^{Ink4a} (**Supplementary Figure 5b & c**). *Tfam*^{+/-} MEFs also showed a passage-dependent increase in both NF- κ B-dependent activation of pro-inflammatory cytokines IL-6 and Cxcl15 (**Supplementary Figure 5d & e**) and genes associated with type I interferon responses (**Supplementary Figure 5f-h**). Altogether, these data are suggestive that mtDNA when present in the cytosol can accelerate senescence, possibly by enhancing pro-inflammatory responses.

Next, we sought to investigate if mtDNA activated the SASP via the cGAS-STING pathway. Consistent with this hypothesis, we found increased co-localization between cGAS-GFP reporter and cytosolic TFAM in senescent human fibroblasts (**Figure 5g**). Additionally, we confirmed that CRISPR/CAS9 mediated deletion of cGAS and STING significantly reduced the secretion of SASP proteins IL-6 and IL-8 (**Figure 5h-i**). We then transfected mtDNA into fibroblasts expressing

or not STING and found that transfection with mtDNA resulted in increased secretion of SASP components IL-6 and IL-8. However, this increase was significantly reduced in cells where STING was absent (**Figure 5m & n**). To further investigate if mtDNA could play a role in the regulation of the SASP, we induced senescence by X-ray irradiation in Rho (0) cells devoid of mtDNA by prolonged treatment with ethidium bromide. We found a significant reduction in the secretion of major SASP components IL-6 and IL-8 (**Figure 5o & p**).

Pharmacological inhibition of MOMP reduces mtDNA release, the SASP and improves healthspan during aging

Next, we sought to assess whether we could suppress mtDNA release and subsequently the SASP using pharmacological inhibitors of MOMP. We investigated the BAX channel blocker (BCB), which does not prevent BAX undergoing its conformational change during activation, but blocks pores that have formed. BCB has been demonstrated to prevent apoptosis and the release of inner membrane proteins such as Cyt c²¹. We observed that BCB was very effective at suppressing mRNA expression and secretion of IL-6 and IL8 in senescent human fibroblasts and the release of cytosolic DNA (**Figure 6a and Supplementary Figure 6a**).

The prevalence of frailty-related characteristics, such as decreased endurance and a decline in muscle strength, increases with aging. To investigate if MOMP inhibition can improve frailty phenotypes during aging, we treated aged mice (20 months-old) with Bax inhibitor (Bax i) BCB three times a week for three months (**Figure 6c**). We found that treatment with Bax i ameliorated age-related decline in neuromuscular coordination as demonstrated by a significant increase in rotarod latency (**Figure 6d**) and improved performance in the pole test, where Bax i-treated animals were able to maintain balance significantly longer on a raised rod (**Figure 6e & Supplementary Figure 6b**). Moreover, Bax i treatment improved forelimb grip strength in old mice (**Figure 6f**) and delayed the progression of age-associated frailty symptoms (**Figure 6g**;

Table 1)²². Notably, Bax i treatment improved healthspan of aged mice without affecting lifespan (**Supplementary Figure 6c**). We also performed a cytokine array in plasma from vehicle and Bax i-treated mice and although the differences were not statistically significant, we found tendencies for decreased levels of several circulating SASP factors, including IL-1 α , IL-6, MCP-1 and TNF- α (**Supplementary Figure 6d**).

Given the improvements in musculoskeletal phenotypes observed in Bax i-treated aged mice, and that senescent cells and the SASP have been shown to play a role in age-related bone loss²³, we performed microcomputed tomography (μ CT) to investigate the effects of Bax inhibition on bone microarchitecture. Treatment with Bax i effectively improved spine trabecular bone microarchitecture (**Supplementary Figure 6e**), such that animals treated with the Bax inhibitor have significantly increased spine trabecular number and decreased trabecular separation (**Figure 6h-j**), as well as a tendency towards higher bone volume fraction (**Supplementary Figure 6e**). Significant improvements in bone volume fraction, trabecular number and trabecular separation were also seen in trabecular bone at the femur (**Figure 6k-n & Supplementary figure 6e**). Analysis of femur cortical bone showed that BCB-treated animals have increased femur cortical thickness and polar moment of inertia (PMOI), which is a measure of the bone's resistance to torsion, although these differences were not statistically significant (**Supplementary Figure 6e**). Consistent with the hypothesis that miMOMP is a driver of the SASP, we found that BCB treatment was effective at significantly reducing mRNA expression of several pro-inflammatory SASP factors in the bone (**Figure 6o**) without affecting genes responsible for the senescence-associated cell-cycle arrest, such as p16^{Ink4a}, p21, and p53 (**Figure 6p**).

Discussion

Mitochondrial dysfunction is a hallmark of cellular senescence and has been shown to regulate the SASP^{15,24}. Mitochondrial DNA is present at thousands of copies per cell²⁵ and given its similarities to bacterial DNA, is recognized as foreign when found outside of mitochondria and

can trigger an inflammatory response^{26,27}. The presence of mtDNA in the cytosol leads to increased expression of NF- κ B-dependent and interferon-stimulated genes in a cGAS-STING - dependent manner^{9,10,20}. The presence of mtDNA in the cytosol has been associated with the process of apoptosis. Upon receiving apoptotic signals, BAX and BAK pores gradually widen, allowing the extrusion of the inner mitochondrial membrane into the cytosol, where it becomes permeabilized, facilitating mtDNA release^{9,10}.

Our work demonstrates that senescent cells display features of a limited mitochondrial apoptotic stress in the absence of cell-death, a phenomenon known as miMOMP¹¹, despite their widely reported resistance to apoptosis^{28,29}. miMOMP in senescent cells is characterized by the presence of several stages of the apoptotic cascade: BAX activation, formation of BAX and Bak macropores, leakage of intermembrane proteins, such as cytochrome c, leakage of the matrix components, such as mtDNA and TFAM, and activation of caspases. Our data indicates that BAX and BAK macropores in a small subset of mitochondria are responsible for leakage of mtDNA and contribute to the SASP. Cytoplasmic release of mtDNA fragments can occur through the opening of the mitochondrial permeability transition pore³⁰, however our results suggest that similarly to apoptosis¹⁰ this is not the case in senescent cells. Apoptosis, despite the release of mitochondrial content into cytoplasm and activation of cGAS-STING pathway, is a mode of cell death that is immunologically silent. This is due to the apoptotic caspases' ability to dampen the pro-inflammatory response resulting from mtDNA in the cytoplasm^{16,17}. Our data suggest that activation of caspases due to miMOMP, a process dependent on APAF1, is insufficient to dampen the SASP.

Several reports indicate that nuclear derived cytosolic chromatin fragments (CCFs) and activation of retrotransposons play a role in senescence and the SASP, by activating the DNA sensing cGAS-STING pathway^{12,31}. Our work demonstrates that similarly, mtDNA is present in the cytosol of senescent cells and can also engage this pathway. In fact, we found that senescent cells show

a gene expression signature consistent with mtDNA stress²⁰, which can be suppressed by Parkin-mediated clearance of mitochondria and is restored by reintroduction of mtDNA. Consistent with our hypothesis, previous work had shown that elimination of senescent cells from old donor animals was shown to reduce circulating mtDNA, improved age-associated inflammation and prolonged the survival of mice receiving organ transplant from old animals³² suggesting that senescent cells are major sources of cell-free mtDNA.

Mechanistically, it is still unclear why senescent cells acquire miMOMP. Mitochondria in senescent cells show several features which intersect with mitochondrial-driven apoptosis such as decreased mitochondrial membrane potential, increased production of mitochondrial ROS, inefficient oxidative phosphorylation and decreased mitochondrial dynamics^{5,6,33}. Also, senescent cells show decreased mitophagy³³, which may impede the elimination of mitochondria undergoing MOMP.

In summary, MOMP is often essential for apoptotic cell death, a cell fate generally considered independent from cellular senescence. Our findings indicate that miMOMP occurs during cellular senescence and can drive the SASP, dependent, in part, on the release of mitochondrial DNA (mtDNA) into the cytosol. Importantly, we show that inhibition of miMOMP *in vivo* may be a novel therapeutic target to counteract inflammation and improve healthspan.

Material and Methods:

Cell culture and treatments

Human embryonic lung MRC5 fibroblasts and IMR90 fibroblasts were grown in Dulbecco's Modified Eagle's Medium (Sigma, D5796) supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 units/ml penicillin, 100 µg/ml streptomycin and 2 mM L-glutamine and maintained at 37°C with 5% CO₂. MRC5 fibroblasts were cultured in atmospheric oxygen conditions and IMR90 fibroblasts were cultured under low oxygen (3%) conditions.

HEK293T cells were used for lentiviral transduction and were cultured in DMEM as described above and further supplemented with 1% non-essential amino acids (Sigma, M7145), 500µl/µl G418 antibiotic (Sigma, A1720) and 1 mM sodium pyruvate (Sigma, S8636).

Parental and rho (0) osteosarcoma 143B cells were grown in DMEM as described above with further supplementation using 5% non-essential amino acids and 25 µg/ml of Uridine.

Mouse adult fibroblasts (MAF) were cultured in DMEM/F12 (Thermo Fisher Scientific, 12634010) supplemented with 10% heat-inactivated FBS, 100 µg/ml streptomycin, 100 units/ml penicillin (Sigma, P4333) and 2mM L-glutamine (Sigma, G3126), and maintained at 37°C in low oxygen conditions (3% oxygen) with 5% CO₂.

WT and *Tfam*^{+/-} mouse embryonic fibroblasts (MEFs) were generated from E12.5-14.5 mouse embryos and cultured in DMEM (Corning #10-013-CV) supplemented with 10% FBS (Atlanta Biological). The *Tfam*^{+/-} mice were originally derived from *Tfam*fl_{ox} mice obtained from Dr. Navdeep Chandel (Northwestern University) and generated as described previously²⁰.

Stress-induced senescence was achieved by exposing cells to X-ray irradiation at 10 Gy (MAFs) or 20 Gy (human fibroblasts). Replicative senescence was achieved by serially passaging cells until they reached their replicative potential and performed less than 0.5 population doublings for at least 4 weeks. Senescence was confirmed by presence of p16 and p21, SA-β-Gal positivity, and absence of proliferation markers Ki67 or EdU incorporation. Oncogene-induced senescence was achieved in ER-RAS-IMR90 fibroblasts by treating cells with 100nM of 4-hydroxy-tamoxifen (4-OHT). 4-OHT was maintained in culture medium until cells were harvested.

For induction of miMOMP, proliferating cells were treated with 2.5µM ABT-737 (Abcam, ab141336) for 9 and 23 days. Treatment was refreshed every 48-72 hours. For inhibition of the mitochondrial permeability transition pore (MPTP), cells were irradiated with 20 Gy X-irradiation and treated with 1 µM cyclosporin A (Sigma, SML1575) for 10 days. For Bax inhibition, MRC5 fibroblasts were irradiated with 20 Gy X-irradiation and treated with Bax Channel Blocker (BCB) (Adooq Biosciences, A15335) at the concentrations indicated. Cyclosporin A and BCB were added straight after irradiation and maintained in cell culture medium for 10 days (refreshed every 48-72 hours).

Parkin-mediated mitochondria clearance

Parkin-mediated widespread mitochondrial clearance was performed as in^{19,24}. Briefly, proliferating, or irradiated Parkin-expressing IMR90 were treated with 12.5 μ M CCCP (Sigma, C2759) (3 days after irradiation) for 48 hours (CCCP was refreshed every 24 hours). Mitochondria-depleted cells were then transfected with isolated mitochondrial DNA (as described below) at 7 days post-irradiation and harvested at 10 days after irradiation.

Parkin-expressing IMR90 were transfected with 15 μ g of isolated mitochondrial DNA, 7 days after irradiation using DharmaFECT kb DNA transfection reagent (Horizon, #T-2006-01), according to the manufacturer's instructions.

Subcellular fractionation and mitochondrial DNA extraction

For cytosolic fraction analysis: a total of 7×10^6 cells were collected and centrifuged at 900xg for 5 minutes. Supernatant was discarded, cells were resuspended in PBS and then divided into 2 1.5ml Eppendorf tubes. Following another centrifugation at 600xg for 5 minutes, the pellet from one tube was frozen and considered as the "whole cell fraction". The pellet from the other tube was incubated in 500 μ l of Buffer 1 (150 mM NaCl, 50mM HEPES pH 7.4, 25 μ g/ml digitonin (Sigma, D141)) for 10 minutes at room temperature. Cells were centrifuged at 150xg at 4°C. The supernatant was next centrifuged twice at 150xg at 4°C and once at 17000xg for 10 minutes, obtaining a cytosolic fraction. Extraction of DNA from the "whole cell fraction" was performed using the DNeasy Blood & Tissue Kit (Qiagen, 69504) following manufacturer's instructions. The "cytosolic fraction" was cleaned up using Qiaquick Nucleotide Removal Kit (Qiagen, 28115) according to manufacturer's instructions and DNA concentration was measured using a Nanodrop ND-1000 Spectrophotometer.

For mitochondrial-enriched fraction: Followed by a rinse in ice-cold PBS, cells were collected by scraping the flask with 5ml of ice-cold PBS. Cells were centrifuged at 800xg for 5 minutes at 4°C and resuspended in Mitochondrial Isolation Solution (MIS) (20mM HEPES-KOH pH 7m 220mM mannitol, 70mM sucrose, 1mM EDTA, 0.5 mM PMSF, 2mM DTT). Samples were transferred to a glass homogenizer and cells were broken open using 60 strokes. The homogenate was centrifuged at 800xg for 5 minutes at 4°C. The supernatant was further centrifuged at 800xg for 5 minutes at 4°C. An aliquot of the supernatant was collected and stored as the "whole cell extract". The remaining was centrifuged at 16100xg for 10 minutes at 4°C. The supernatant was collected as the "cytosolic fraction". The pellet containing mitochondria was resuspended in 1ml of MIS and centrifuged again at 16100xg for 10 minutes at 4°C. This step was repeated, and the resulting pellet was resuspended in 100 μ l of MIS. For mitochondrial DNA extraction, the mitochondrial pellet was centrifuged at 16100xg for 10 minutes at 4°C and the pellet was resuspended in 200 μ l PBS. DNA extraction was performed using the DNeasy Blood & Tissue Kit (Qiagen, 69504) following manufacturer's instructions.

Seahorse

Cellular oxygen consumption rate (OCR) was measured using Agilent Seahorse XFe96 Analyzer, according to manufacturer's instructions. Cell culture medium was replaced by unbuffered basic medium, 45 mg/L dextrose, 110 mg/L sodium pyruvate (Sigma, S8636), 4 mM L-glutamine

(Sigma, G3126). The following compounds were added to test mitochondrial activity: 0.5 μ M oligomycin, 2.5 μ M FCCP, 0.5 μ M rotenone with 2.5 μ M antimycin A. The resulting OCR values were normalized by cell numbers quantified following the assay using Automated Cell Counter.

CRISPR/CAS9-based genome editing

The following plasmids were used: LentiCRISPR v2 hBak (Addgene, 129579), LentiCRISPR v2 hBAX (Addgene, 129580), LentiCRISPR v2-puro (Addgene, 52961), APAF1 CRISPR, cGAS CRISPR, LentiCRISPR v2-Blasti.

The following sequences were used to create CRISPR/Cas9-mediated deletion of STING, APAF1 and cGAS:

hTMEM173_1 5'-GCAAGCATCCAAGTGAAGGG-3'

hTMEM173_2 5'-CGGGCCGACCGCATTTGGGA-3'

APAF1 5'-ACAGCCTGCCATTCCATGTA-3'

CGAS 5'-AAAGTAATATGCACGAGTGT-3'

For lentiviral transduction, HEK293FT cells were transfected with the plasmids above together with the packaging and envelope plasmids VSVG and Gag-Pol (Sigma) using Lipofectamine 3000 (Invitrogen, L3000015) according to the manufacturer's instructions. Two days later, the supernatant from the transfected HEK293FT cells containing viral particles was filtered using a 0.45 μ m pore PVDF filter, mixed with 10 μ g/ml polybrene and used to infect the cells of interest. Following infection, cells were selected for successful CRISPR/Cas9 deletion by using the following antibiotics: 1 μ g/ml Puromycin (for Bax, Bak and cGAS) or Blasticidin 10 μ g/ml (for APAF1 and STING).

Cytokine array

Detection of cytokines and chemokines in cell culture supernatant and mouse plasma was performed by Eve Technologies Corporation (Calgary, AB, Canada). The following assays were used: Human Cytokine/Chemokine 41-Plex Discovery Assay (HD41) and Mouse Cytokine/Chemokine 31-Plex Discovery Assay Array (MD31), respectively.

Confirmatory ELISAs were performed using the following kits: Human IL-6 DuoSet ELISA (R&D Systems, DY206), Human IL-8 DuoSet ELISA (R&D Systems, DY208) and Human CXCL10/IP-10 DuoSet ELISA, according to manufacturer's instructions. Optical density at 450nm was determined using a Multiskan FC microplate reader (ThermoFisher Scientific) and corrected by subtracting the readings at 540nm.

Senescence-associated- β -Galactosidase activity

Cells grown on coverslips were fixed in 0.2% glutaraldehyde in 2% PFA in PBS for 5 minutes. Sen- β -Gal staining solution (150 mM sodium chloride, 2 mM magnesium chloride, 40 mM citric acid, 12 mM sodium phosphate pH 6.0, 1 mg/ml 5-bromo-4-chloro-3-inolyl- β -d-galactosidase (X-

gal), 5 mM potassium hexacyanoferrate (II) trihydrate, 5 mM potassium hexacyanoferrate (III) trihydrate) (pH 6.0) was applied and incubated overnight at 37°C in the dark overnight. Cells were washed in PBS three times and were then mounted onto glass microscope slides using ProLong Gold Antifade Mountant with DAPI (Invitrogen).

Western blotting

Cells were lysed in lysis buffer (150 mM NaCl, 1% NP40, 0.5% NaDoC, 0.1% SDS, 50 mM Tris pH 7.4, 1x phosphatase and protease inhibitors cocktail in H₂O) and protein concentration was determined using BioRad Protein Assay (BioRad, Reagent A, 500-0113; Reagent B, 500-0114; Reagent C, 500-0115). Equal amounts of protein (at least 15 µg) from each sample were resolved on Tris-Glycine gels and samples were then blotted onto a 0.45 µm polyvinylidene difluoride (PVDF) membrane (Millipore) using Trans-Blot SD Semi-Dry Transfer Cells (Bio-Rad). Membranes were blocked with PBS-Tween blocking buffer (5% milk powder, 0.05% Tween-20 in PBS) and then incubated with primary antibodies at 4°C overnight. Following washes in distilled water, membranes were incubated with a peroxidase-conjugated secondary antibody for 1 hour at room temperature. Membranes were then incubated with either Clarity ECL Western Blot Substrate (Bio-Rad, 170-5060) or KwikQuant Western blot detection kit (Kindle Bioscience, R1100) according to manufacturer's instructions, and visualized using the LAS4000 (Fujifilm) or KwikQuant Imager (Kindle Bioscience, D1001). Signal intensity of protein bands was analyzed using ImageJ analysis software.

FPLC

Cells grown in 150 cm² flasks were trypsinized and pooled to obtain sufficient material for the assay. Following PBS washes, cells were centrifuged at 900xg for 5 minutes. Cell pellets were then lysed using CHAPS lysis buffer (1% [w/v] CHAPS, 20 mM HEPES at pH 7.4, 150 mM NaCl, 1% [v/v] glycerol, 1 mM PMSF, 10 µg/mL leupeptin, 10 µg/mL pepstatin, 100 mM NaF, 10 mM sodium pyrophosphate, 1 mM sodium vanadate, 20 nM microcystin) for 30 minutes at 4°C. Samples were diluted to contain 10 mg/ml of protein and 200 µl was injected onto a Superdex S200 size exclusion column. 20 500 µl fractions were collected. Protein precipitation using trichloroacetic acid (TCA) was then performed. Briefly, samples were incubated with 1/10th sample volume of 10% Triton X-100 and 1/5th sample volume of 100% ice-cold TCA for 20 minutes on ice. Samples were then centrifuged for 5 minutes at 800xg at 4°C, the supernatant was discarded, and the pellet was washed once with 1ml ice-cold 10% TCA and twice with 1ml acetone at -20°C. Pellets were left to air dry at room temperature and were then solubilized in sample buffer (4X Laemmli Sample Buffer, Bio-Rad, 1610747) with 1% 2-mercaptoethanol (Bio-Rad, 1610710). Protein was separated using 4-20% gradient acrylamide Tris-Glycine gel generated using Gradient Former (Bio-Rad, Model 230, 165-2700). Protein was transferred to BioTrace NT nitrocellulose membrane (Pall Corporation, 66485) and immunoblotted according to the method described above.

RNA-sequencing

Sequencing libraries were made from poly-A RNA, as recommended by Illumina, and sequenced using either an Illumina GAII-X or a NextSeq 500 sequencer. RNA-seq paired-end reads were assessed for quality using the 'FastQC' algorithm, then aligned to the human genome using the splice-aware aligner STAR with a two-pass alignment pipeline. Reference splice junctions were provided by a reference transcriptome from the GenCode GRCh38 (hg38) build. BigWig files were generated using DeepTools. Raw read counts per gene were calculated using htseq-count. The read count matrix was then used for differential expression analysis with the linear modeling tool DESeq2. Significantly changing expression was defined as an FDR-corrected p-value ≤ 0.05 . FPKM (Fragments Per Kilobase of transcript per Million mapped reads) values were generated using Cufflinks. Gene ontology analysis was performed using Gene Set enrichment Analysis (GSEA) and Ingenuity® Pathway Analysis (IPA) software.

Mouse models and treatments

All animal experiments were performed according to protocols approved by the Institutional Animal Care and Use Committee (IACUC) at Mayo Clinic, unless specified otherwise. Aged wild-type C57BL/6 mice were acquired from the National Institute on Aging (NIA) and were maintained in a pathogen-free facility under a 12-hour light, 12-hour dark cycle at 23-24°C with free access to regular chow and water. Mice were housed in same-sex cages in groups of 3-5. Animals were randomly assigned into vehicle or treatment group. Mice were injected intraperitoneally with 10 mg/kg of Bax Channel Blocker (BCB; Tocris Bioscience, 2160) three times a week for three months, at which point animals were euthanized and tissues were collected for analysis. For lifespan study, BCB injections were administered three times weekly until death. Frailty assessment was conducted every two months because these measurements are noninvasive. Mice were euthanized and considered as being dead if they met humane end points. Survival was assessed by right-censored Kaplan-Meier curve analysis using the Log-Rank test.

Bak1^{-/-};Bax^{fl/fl} mice (mixed background) were kindly donated by Owen Sansom. Mice were monitored daily and kept in conventional animal facilities. Experiments conducted with *Bak1^{-/-};Bax^{fl/fl}* mice were performed under UK Home Office license and approved by the University of Glasgow Animal Welfare and Ethical Review Board. Mice were genotyped by Transnetyx INC. (Cordova, Tennessee). AAV-Cre virus in 100ul PBS (AAV8.TBG.PI.Cre.rBG, UPenn Vector Core, Catalogue number: AV-8-PV1091) was delivered by tail vein injection (2×10^{11} PFU per mouse) in mice aged between 6 and 8 weeks. A week post-injection, mice were irradiated with 4Gy. Mice were sacrificed 6 days later, and liver harvested in 10% neutral buffered formalin.

Rotarod

Assessment of maximal walking speed and latency was performed using an accelerating RotaRod system (Ugo Basile, Rota Rod 47650). Mice were trained on the RotaRod for 3 consecutive days prior to test day. Training consisted of mice remaining on RotaRod at speeds of 4, 6 and 8 r.p.m. for 200 seconds on days 1, 2 and 3, respectively. On the test day, mice were placed on the rotating cylinder, which increased in speed from 4 to 40 r.p.m over a 200 second interval. The speed and latency at which a mouse fell off the cylinder were recorded. Results were the average of 3 trials.

Neuromuscular coordination analysis

Assessment of neuromuscular coordination was done using the tightrope test³⁴. Animals were placed on a horizontal bar, which was 1.5 cm in diameter and situated 60 cm off the ground. The time animals were able to spend on top of the bar was recorded. A trial was deemed successful if an animal could remain on top of the bar for 60 seconds without falling. Each mouse was given five trials with a 30 second rest between trials.

Forelimb grip strength analysis (Hanging test)

Assessment of forelimb grip strength was carried out by allowing animals to grip a suspended wire coat hanger, which was 2 mm in diameter and 30 cm in length, by using their forelimbs. The time a mouse was able to hang from the wire following grip was recorded, and each mouse was given 10 attempts (with a 20 second rest in between) up to a total of 90 seconds. Success was defined as being able to hang for a total sum of 90 seconds. Trial was defined as failure if the animal fell from the wire.

Frailty measurements

Frailty was assessed using a 30-parameter index based on²². For each parameter, mice were given a score of 0, 0.5 or 1 corresponding to absence, mild or severe phenotype, respectively. Body weight was recorded, and surface body temperature was measured using an infrared temperature probe. For dystonia assessment, a score of 1-4 was given, where a score of 1 was equivalent to clasping with one limb whereas a score of 4 was given if the animal showed clasping with all four limbs.

Skeletal imaging

All bone imaging and analysis was done in a blinded fashion. Quantitative *ex vivo* analyses of bone microarchitecture of the lumbar vertebrae (L₅) and femur (proximal metaphysis/mid-shaft diaphysis) were performed using a μ CT system (Skyscan 1276 Scanner, Bruker, Kontich, Belgium). Scan settings were as follows: 55 kV, 200 μ A, 10 μ m voxel size, 0.4-degree rotation steps for 360°, and 4 frames average imaging with a 0.25-mm A1 filter. Skyscan NRecon software was used to reconstruct scans and for post-alignment and beam hardening corrections. μ CT parameters were derived using the manufacturer's protocols for Bruker CtAN software, which permits assessments of trabecular and cortical bone parameters. Trabecular bone volume fraction (BV/TV; %) was assessed at the lumbar spine (200 slices) and proximal metaphysis (100 slices) of the femur. Further, at the proximal metaphysis and mid-diaphysis (50 slices) of the femur, cortical thickness (Ct.Th; mm), endocortical circumference (E.C; mm), periosteal circumference (P.C; mm), and cortical porosity (Ct.Po, %) were assessed. In addition, an estimate of bone torsional strength (*i.e.*, polar moment of inertia, PMOI; mm⁴) was derived.

q-PCR

For bone, osteocyte-enriched cell samples were generated as described³⁵. Samples were homogenized in QIAzol Lysis Reagent (QIAGEN, Valencia, CA) and immediately stored at -80°C. Total RNA was extracted using QIAzol Lysis Reagent followed by purification with RNeasy Mini

Columns (QIAGEN, Valencia, CA). For liver, a section measuring approximately 3mm³ stabilized in RNAlater (Qiagen) was used. RNA from liver and cells was extracted using the RNAeasy Mini Kit (Qiagen, 74106) according to manufacturer's instructions (an additional DNase I treatment was done in liver to remove genomic DNA contamination). Complementary DNAs were synthesized using the High Capacity cDNA Reverse Transcription Kit (ThermoFisher, 4368814) following manufacturer's instructions. Quantitative real-time PCR was carried out using either Power SYBR® Green PCR Master Mix (Invitrogen, 4367659) in a C100™ Thermal Cycler (BioRad), ToughMix Perfecta (PerfeCTa qPCR ToughMix, QuantaBio, 95112-250) using CFX96™ Real-Time System (Bio-Rad), Brilliant III Ultra-Fast SYBR Green qPCR Master Mix (Agilent Technologies) (for liver samples) or with the ABI Prism 7900HT Real Time System (Applied Biosystems, Carlsbad, CA) using SYBR green (QIAGEN, Valencia, CA) as the detection method (for bone). mRNA levels were calculated using the 2- $\Delta\Delta$ CT method and normalized to a housekeeping gene.

Immunocytochemistry

Cells grown on coverslips were fixed using 2% paraformaldehyde in PBS for 10 minutes. Cells were washed in PBS and then permeabilized in PBG-Triton (PBS, 0.4% Fish skin gelatin, 0.5% BSA, 0.5% Triton X-100) for 45 minutes. Subsequently, cells were incubated with primary antibody overnight at 4°C. Following PBS washes, secondary antibody was applied and incubated for 45 minutes at room temperature. Coverslips were mounted onto glass microscope slides with ProLong Gold Antifade Mountant with DAPI (Invitrogen).

For mitochondrial proteins, cells were fixed in 0.02% glutaraldehyde in 4% paraformaldehyde for 10 minutes, washed in PBS and permeabilized in 0.5% Triton-X in PBS for 10 minutes. Cells were blocked in 5% normal goat serum (NGS) for one hour. Incubation with primary and secondary antibodies was done as above.

Immunohistochemistry

Formalin-fixed paraffin-embedded (FFPE) tissue sections (5 μ m) were deparaffinized in xylene (3 times for 3 minutes each) and hydrated using 100% ethanol (twice for 5 minutes), 70% ethanol (5 minutes) and distilled water (twice for 3 minutes each). Antigen retrieval was done by heating sections to 98°C in citrate buffer pH 6.0 (Agilent-Dako, S236984) for 30 minutes. Slides were allowed to cool down for 30 minutes and were then rinsed in TBS twice for 5 minutes. Tissue sections were blocked with NGS (Agilent-Dako, X090710-8) for 30 minutes at room temperature followed by primary antibody incubation overnight at 4°C. Sections were washed in TBS and incubated with peroxidase block (Agilent-Dako, K500711) for 30 minutes at room temperature. HRP-conjugated secondary antibody was then applied for 30 minutes at room temperature, followed by TBS washes and DAB incubation for 5-10 minutes. Sections were counterstained with hematoxylin according to standard procedure.

Immuno-FISH

FFPE tissue sections were deparaffinized in 100% Histoclear and hydrated through a graded ethanol series of 100, 90 and 70% ethanol (twice for 5 minutes each), and washed twice for 5

minutes in distilled water. Antigen retrieval was performed by placing sections in 0.01 M citrate buffer (pH 6.0) and heating it until boiling for 10 minutes. Sections were allowed to cool down to room temperature and then washed in distilled water for 5 minutes. Blocking was then performed using normal goat serum (1:60) in BSA/PBS for 30 minutes followed by overnight incubation with rabbit monoclonal anti- γ H2AX antibody (1:400, 9718; Cell Signaling) at 4°C. Following three PBS washes, tissues were incubated with a goat anti-rabbit biotinylated secondary antibody (1:200, PK-6101; Vector Labs) for 30 minutes at room temperature. Sections were then washed in PBS three times and incubated with fluorescein avidin DCS (1:500, A-2011; Vector Labs) for 30 minutes at room temperature. Tissues were then washed in PBS three times and incubated in 4% paraformaldehyde in PBS for 20 minutes for cross-linking. Following three PBS washes, sections were dehydrated in graded cold ethanol solutions (70, 90, 100%) for three minutes each and were then allowed to air-dry. Next, 10 μ l of PNA hybridization mix (70% deionized formamide (Sigma), 20 mM MgCl₂, 1 M Tris pH 7.2, 5% blocking reagent (Roche) containing 2.5 μ g/ml Cy-3-labelled telomere-specific (CCCTAA) peptide nucleic acid probe (PANAGENE)) was added to sections and denaturation was allowed to occur for 10 minutes at 80°C. Sections were then incubated in PNA hybridization mix for 2 hours at room temperature in the dark to allow hybridization to occur. Tissues were washed in 70% formamide in 2X SSC for 10 minutes, followed by one wash in 2X SSC for minutes and a PBS wash for 10 minutes. Tissues were mounted using ProLong Gold Antifade Mountant with DAPI (Invitrogen) and imaged using in-depth Z stacking (a minimum of 40 optical slices with 63x objective).

Immunogold Electron Microscopy

Cells were fixed in 0.1% glutaraldehyde and 4% paraformaldehyde in 0.1 mol/L phosphate buffer for 2 hours and were then harvested and centrifuged at 900xg for 5 minutes. For cryoprotection, cells were incubated with 2.3 mol/L sucrose in 0.1 mol/L phosphate buffer overnight and then frozen in liquid nitrogen. Thin cryosections (60 nm) were cut using Leica cryo-microtome. Sections were incubated with primary antibody (1:20) at 4°C overnight. Sections were then incubated with a 10nm anti-mouse IgG gold secondary antibody (Sigma, G7652) for 2 hours at room temperature. Following washes, sections were fixed in 1% glutaraldehyde and embedded in 2% methyl cellulose solution containing 0.3% uranyl acetate. Specimens were imaged using a Jeol 1200 electron microscope (Mayo Clinic Core Microscopy Facility) operating at 60 to 80 kV.

Microscopy

Imaging was performed with the following confocal microscopes: SP8 Leica and LSM780 Zeiss; and super-resolution microscopes: confocal microscopy with AiryScan type detector LSM800 Zeiss AiryScan; Structured Illumination Microscopy (SIM) Zeiss Elyra PS.1 Super Resolution.

Analysis of extra-mitochondria DNA foci and mitochondria positive for BAX(6A7) was performed manually using Fiji software.

For the visualization of cytosolic cytochrome c, CLSM images were subjected to deconvolution using AutoQuant X3 Deconvolution. Pearson's R value was subsequently determined for each cell using Coloc2 ImageJ plugin.

For the 3D visualization of mitochondrial leakage, Imaris 9.6 Image Visualization and Analysis Software was used.

Images for miMOMP analysis were acquired from cells labelled with Cytochrome C and TOM20 antibodies by 3D imaging using z-stacks on the Leica SP8 Confocal microscope. Approximately 30 z-stacks were acquired for each coverslip imaged. Following imaging, micrographs were processed using the Huygens Deconvolution Software. Level of colocalization was assessed using the colocalization tool within the Huygens Deconvolution Software.

Statistical analysis

GraphPad Prism 6.0 was used for statistical analysis, results are considered as statistically significant when $p \leq 0.05$. For normally distributed data, the differences between two groups were tested for statistical significance using an independent samples two-tailed t-test. For data which was normally distributed and there was more than one group a one-way ANOVA was used, a Tukey's comparison post-hoc test was also performed. Where data was not normally distributed a Mann-Whitney U-test was used for determining statistical significance.

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Competing interests

No competing interests

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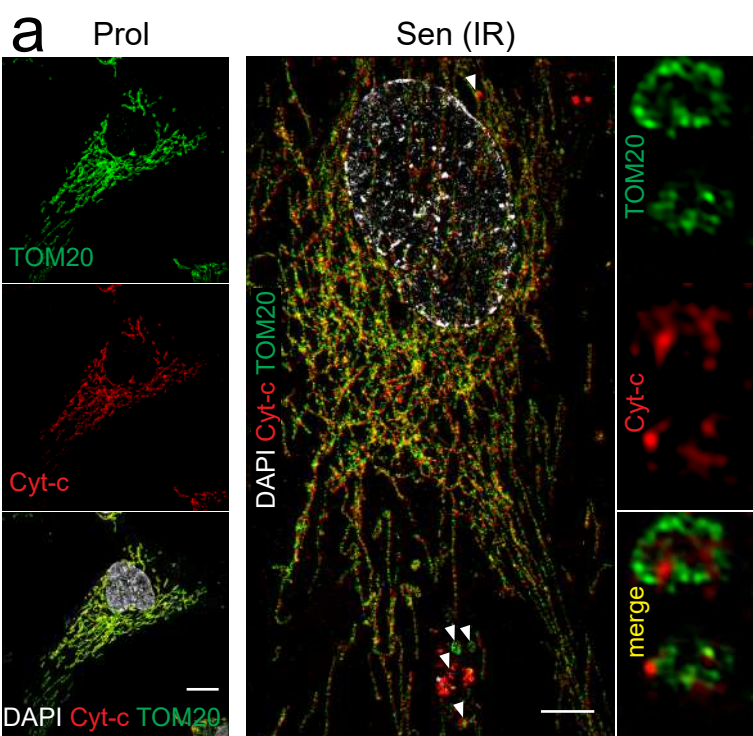
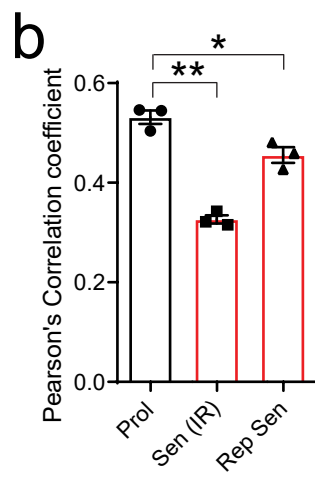
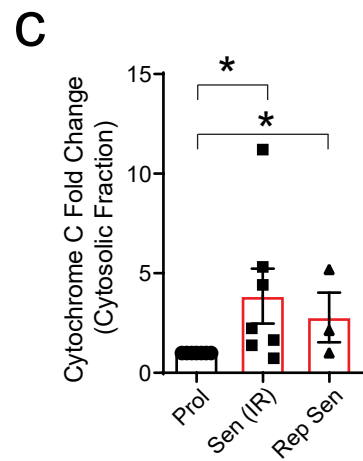
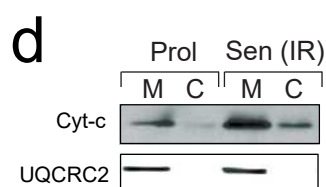
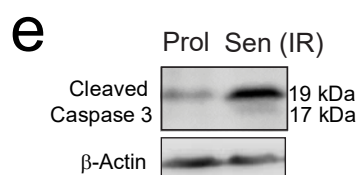
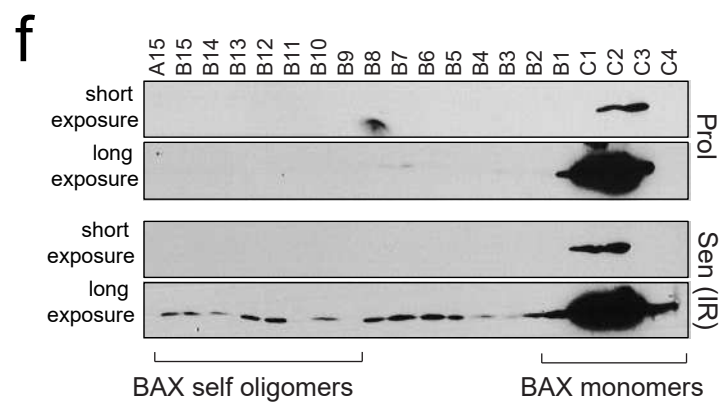
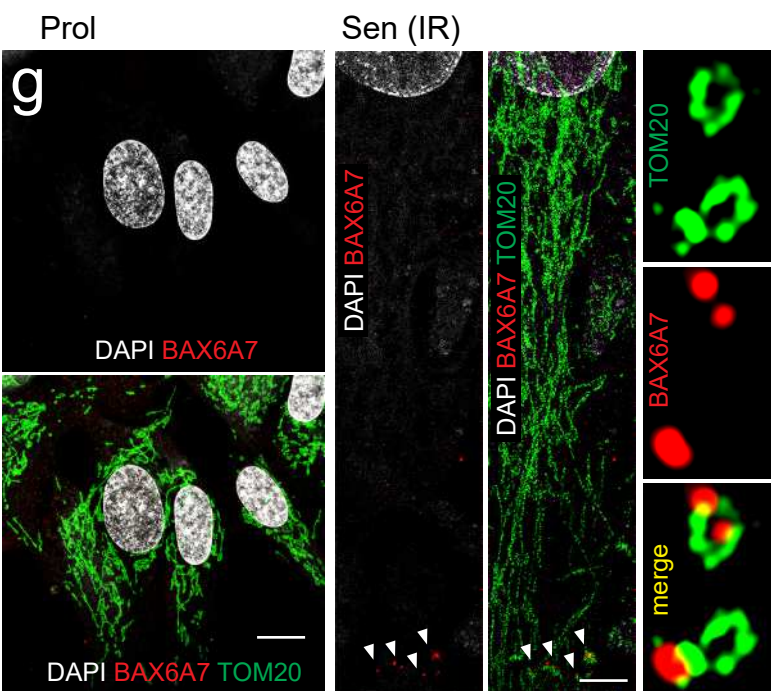
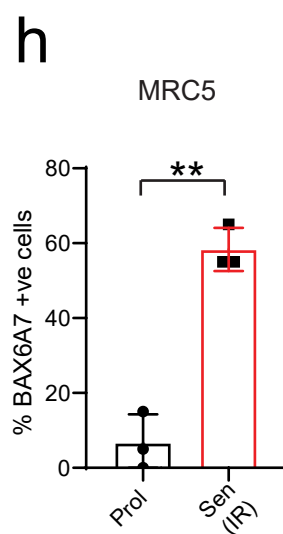
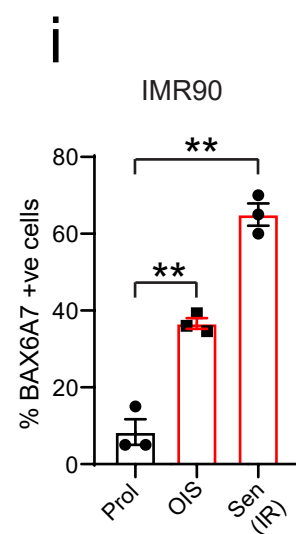


Figure 1 – Sub-lethal mitochondrial apoptotic signaling is a feature of cellular senescence. **(a)** Representative super-resolution SIM microscopy images of cytochrome c (green) and TOM20 (red) in proliferating and stress-induced senescent human fibroblasts (scale bar is 10 μ m). Magnification on the left shows areas where cytochrome c does not co-localize with TOM20 in senescent cells. **(b)** Quantification of the co-localization between cytochrome C and TOM20 using Pearson's correlation coefficient. Data are mean of n=3 independent experiments $\pm$ S.E.M. Statistical significance (one-way ANOVA) is indicated (*P<0.05; **P<0.01). **(c)** Relative levels of cytosolic cytochrome c in stress-induced (IR) and replicative senescent cells expressed as fold change to proliferating control. Data are mean of n=6 (Sen IR) and n=3 (Rep Sen) independent experiments $\pm$ S.E.M. Statistical significance (one-way ANOVA) is indicated (*P<0.05). **(d)** Representative Western blot of **(c)**, showing cytochrome c enriched in cytosolic fraction of senescent cells. UQCRC2 (complex III mitochondria protein) shows cytosolic fraction is devoid of mitochondria. **(e)** Western blot showing the increase in cleaved caspase-3 in stress-induced senescent cells. Data representative of n=4 independent experiments. **(f)** Western blot of BAX done in FPLC fractions of proliferating and senescent cells. Fractions of decreasing protein molecular weight are shown from left to right. Films were exposed for 2 minutes (short exposure) and overnight (long exposure). **(g)** Representative super-resolution SIM microscopy images of BAX(6A7) (activated form of BAX; red) and TOM20 (green) (scale bar is 10 μ m). Quantification of the percentage of **(h)** MRC5 and **(i)** IMR90 fibroblasts positive for BAX(6A7) co-localizing with TOM20. Data are mean of n=3 independent experiments $\pm$ S.E.M. Statistical significance (unpaired t-test and one-way ANOVA, respectively) is indicated (**P<0.01).

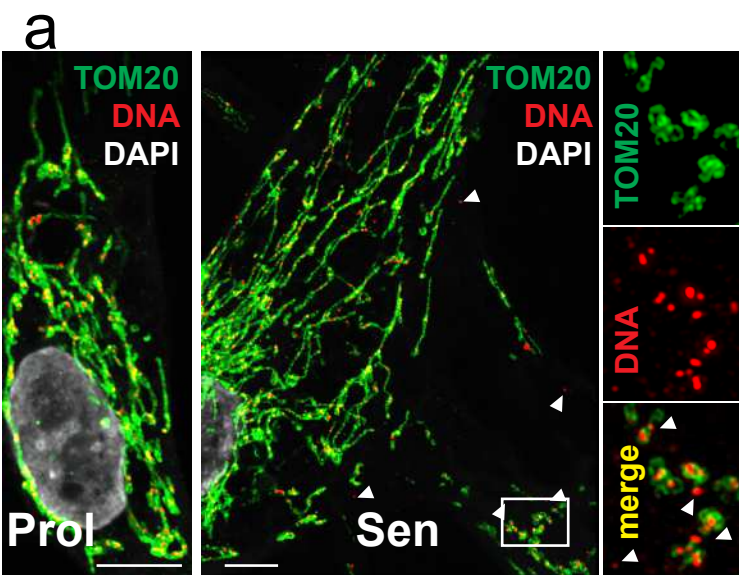
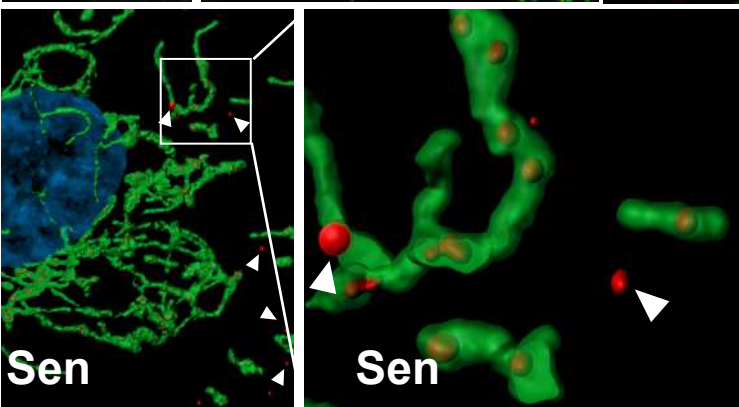
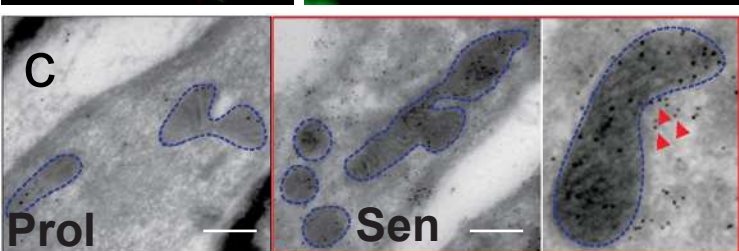
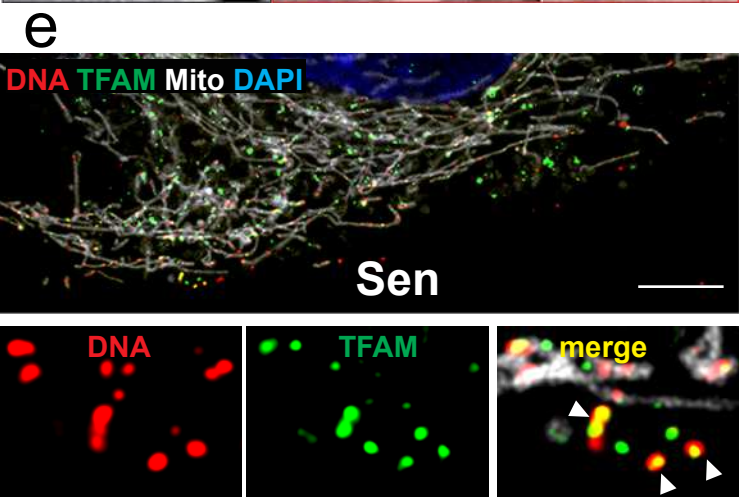
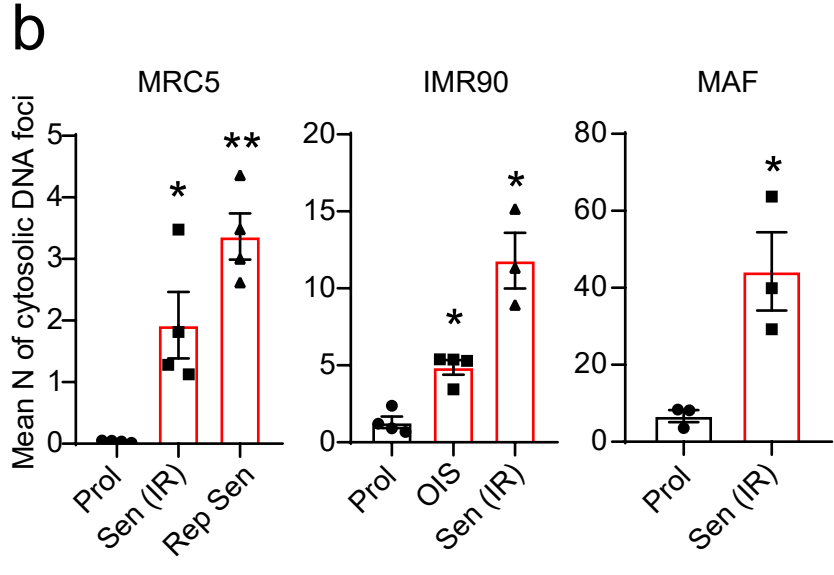
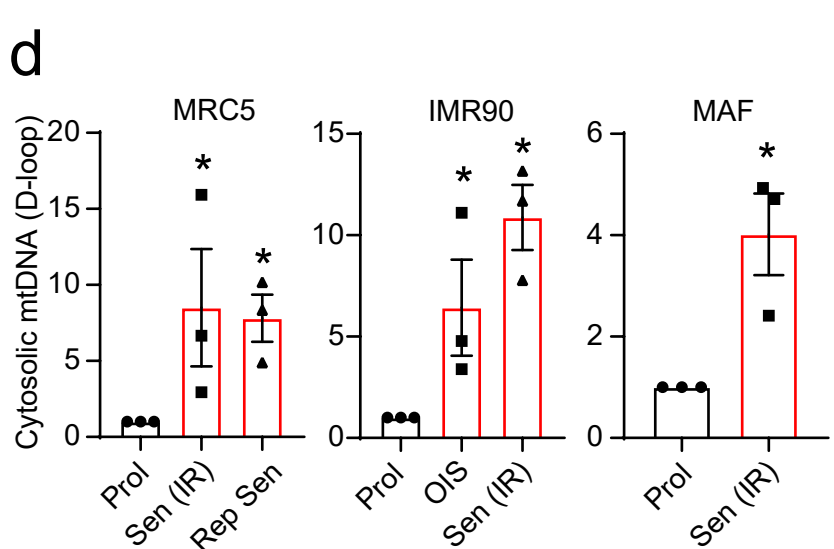
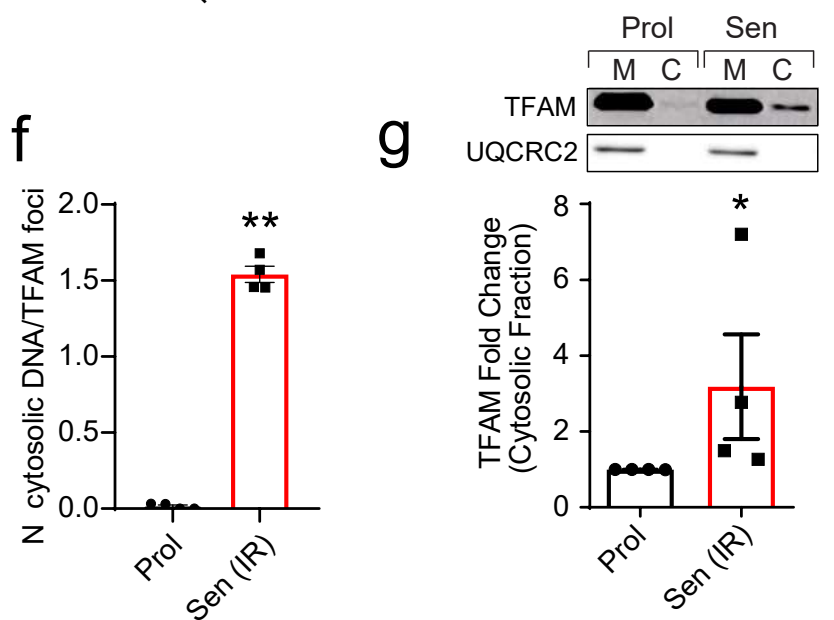


Figure 2 – Senescent cells have increased levels of cytosolic mtDNA. **(a)** Representative super-resolution AiryScan microscopy images of DNA (red) and TOM20 (green) in proliferating and senescent cells (scale bar is 5 μ m). Below are 3D representations of mitochondrial network (green) and DNA (red), showing that most DNA foci are located within mitochondria with some foci in the cytoplasm of senescent cells. **(b)** Quantification of the mean number of DNA foci present in the cytoplasm of proliferating and senescent human MRC5 and IMR90 fibroblasts and MAF. Data are mean of n=4 independent experiments $\pm$ S.E.M. Statistical significance (one-way ANOVA) is indicated (* P <0.05; ** P <0.01). **(c)** Representative electron microscopy images of DNA immuno-gold labelling in proliferating and senescent cells. Representative image of n=2 independent experiments (scale bar is 200 nm). **(d)** qPCR quantification of levels of mtDNA (D-loop region) present in cytosolic fraction of proliferating and senescent human MRC5 and IMR90 fibroblasts and MAF, normalized to the levels of total cellular mtDNA. Data are mean of n=3 independent experiments $\pm$ S.E.M. Statistical significance (one-way ANOVA and unpaired t-test, respectively) is indicated (* P <0.05). **(e)** Representative super-resolution AiryScan microscopy image of DNA (red), TFAM (green) and TOM20 (white) in senescent cells (scale bar is 10 μ m). Magnification shows TFAM co-localizing with DNA outside of the mitochondrial network, representing mtDNA leakage. **(f)** Quantification of the mean number of DNA+TFAM foci present outside of the mitochondrial network in proliferating and senescent cells. Data are mean of n=4 independent experiments $\pm$ S.E.M. Statistical significance (unpaired t-test) is indicated (** P <0.01). **(g)** Representative Western blot (top) and quantification (bottom) of TFAM present in the cytosolic fraction of proliferating and senescent cells. Values are normalized to the mitochondrial protein UQCRC2 and expressed as fold change. Data are mean of n=4 independent experiments $\pm$ S.E.M. Statistical significance (unpaired t-test) is indicated (* P <0.05).

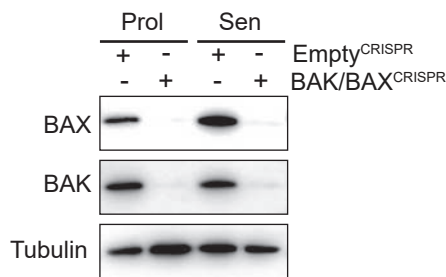
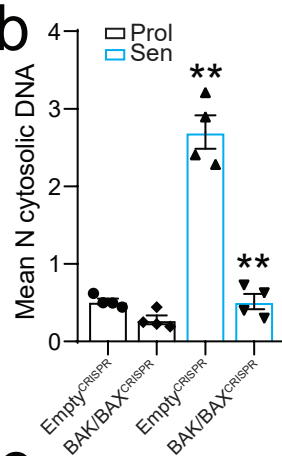
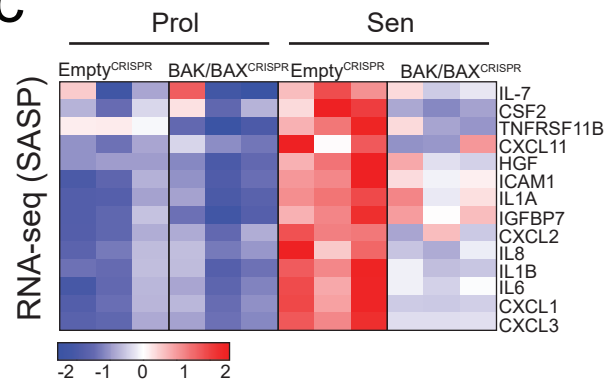
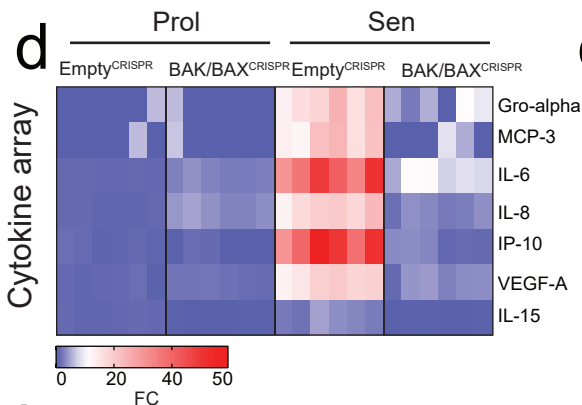
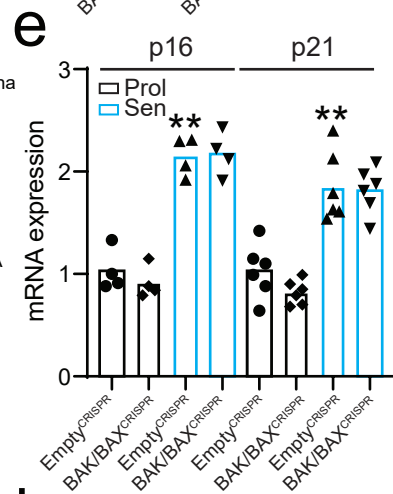
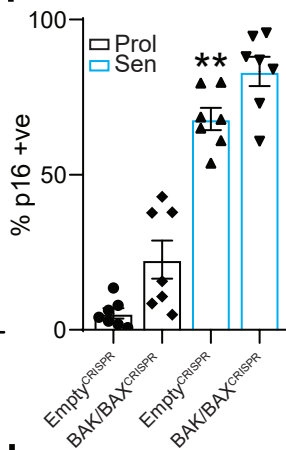
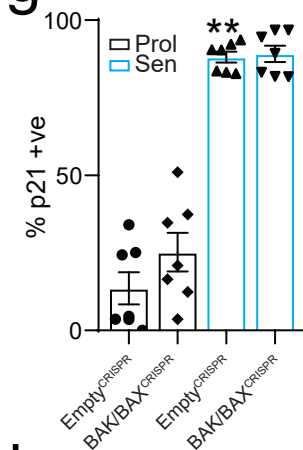
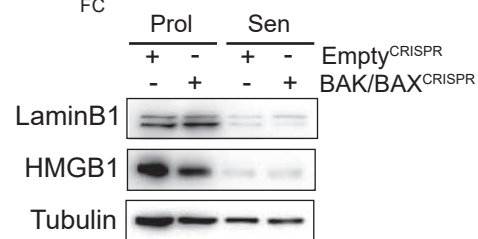
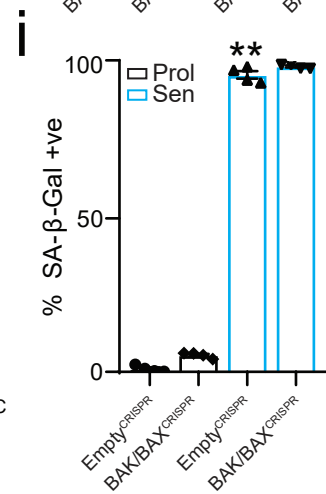
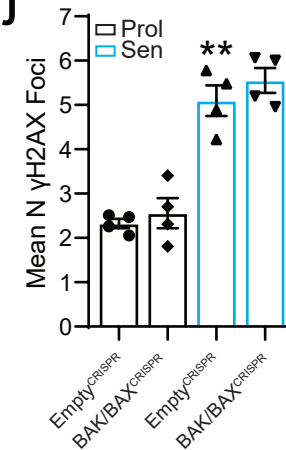
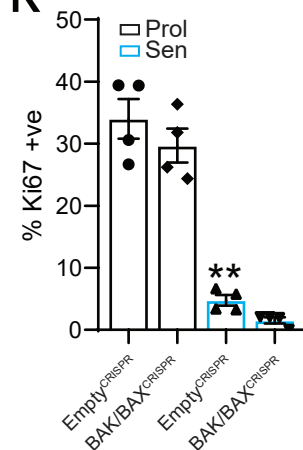
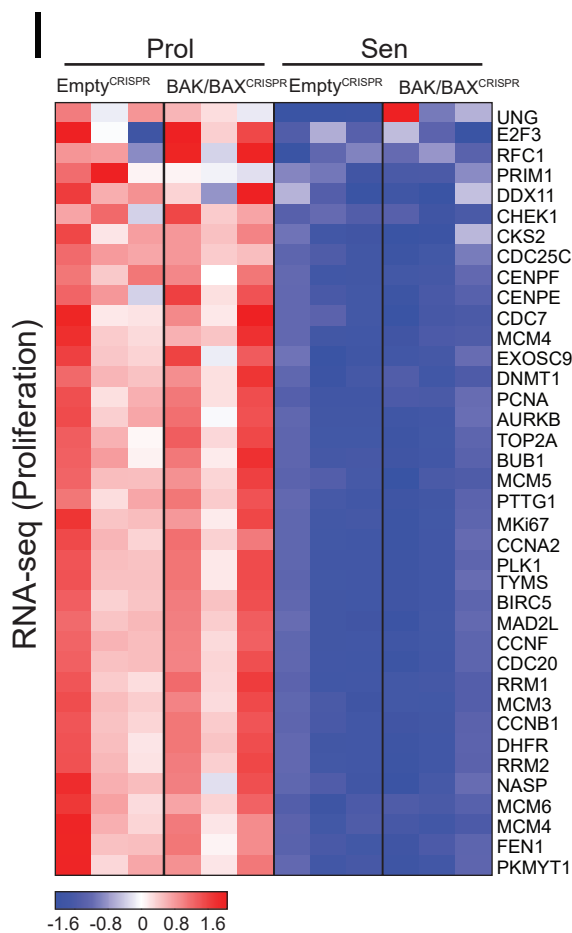
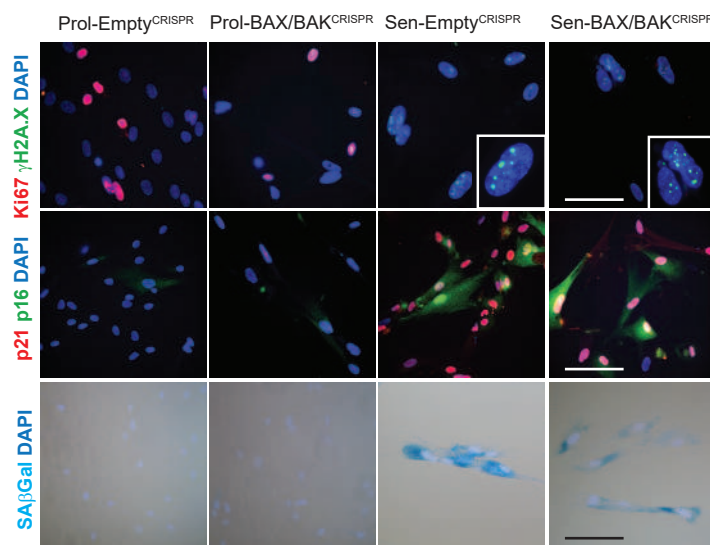
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Figure 3 – BAX and BAK macropores mediate mtDNA release and the SASP in senescent cells. (a) Representative Western blot showing successful CRISPR/Cas9-mediated deletion of BAX and BAK in proliferating and senescent cells. (b) Quantification of the mean number of cytosolic DNA foci in proliferating and senescent BAX/BAK^{-/-} cells. Data are mean of n=4 independent experiments $\pm$ S.E.M. Statistical significance (one-way ANOVA) is indicated (**P<0.01). (c) Column clustered heatmap of SASP genes that are differentially expressed in senescence and rescued by deletion of BAX and BAK. The color intensity represents column Z-score, where red and blue indicate high and low expression, respectively. (d) Heatmap showing levels of secreted cytokines in proliferating and senescent empty vector (EV) and BAX/BAK^{-/-} cells. Data are n=6 independent experiments. (e) Quantification of mRNA levels of p16 and p21 in proliferating and senescent EV and BAX/BAK^{-/-} cells. Data are mean of n=4-6 independent experiments $\pm$ S.E.M. Statistical significance (one-way ANOVA) is indicated (**P<0.01). Quantification of the percentage (f) p16-positive and (g) p21-positive proliferating and senescent EV and BAX/BAK^{-/-} cells. Data are mean of n=7 independent experiments $\pm$ S.E.M. Statistical significance (one-way ANOVA) is indicated (**P<0.01). (h) Representative Western blot showing protein levels of LaminB1 and HMGB1 in proliferating and senescent EV and BAX/BAK^{-/-} cells. Representation of n=3 independent experiments. Quantification of (i) the percentage of Sen- β -Gal positive cells, (j) the mean number of γ H2AX foci and (k) the percentage of Ki67-positive proliferating and senescent EV and BAX/BAK^{-/-} cells. Data are mean of n=4 independent experiments $\pm$ S.E.M. Statistical significance (one-way ANOVA) is indicated (**P<0.01). (l) Column clustered heatmap of proliferation genes that are differentially expressed in senescent cells and are not rescued by BAX/BAK deletion. The color intensity represents column Z-score, where red and blue indicate high and low expression, respectively. (m) Representative microscopy images of: (top panel) Ki67 (red) and γ H2AX (green); (middle panel) p21 (red) and p16 (green) (DAPI is shown in blue); (bottom panel): Sen- β -Gal in proliferating and senescent EV and BAX/BAK^{-/-} cells.

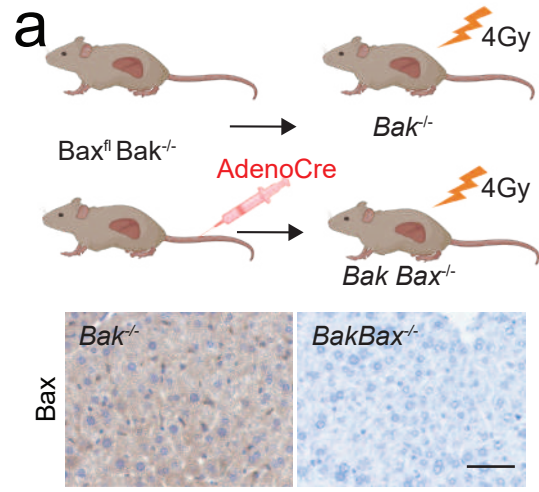
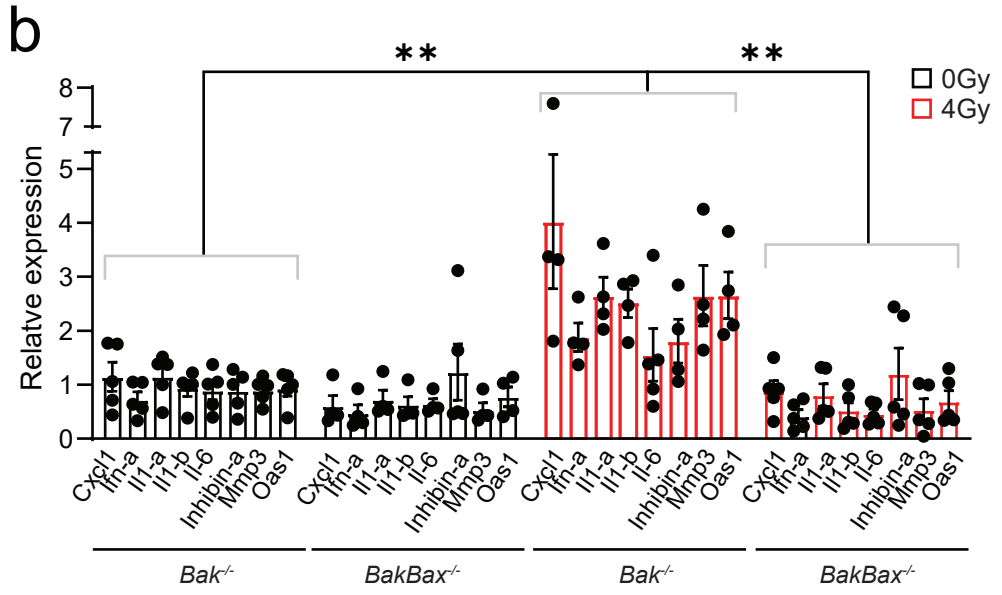
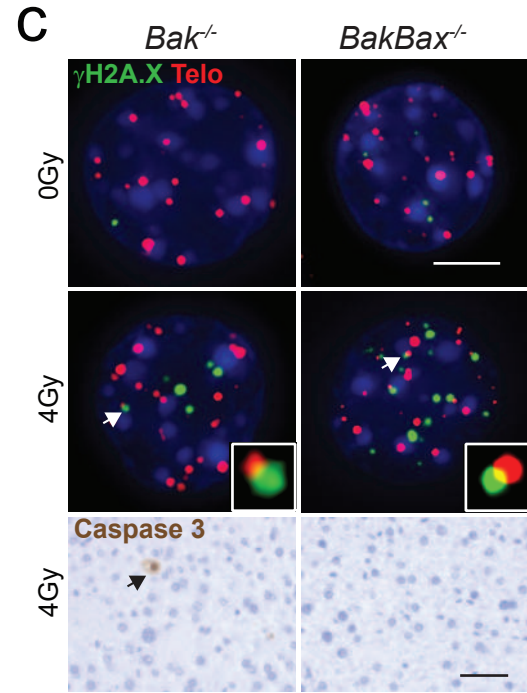
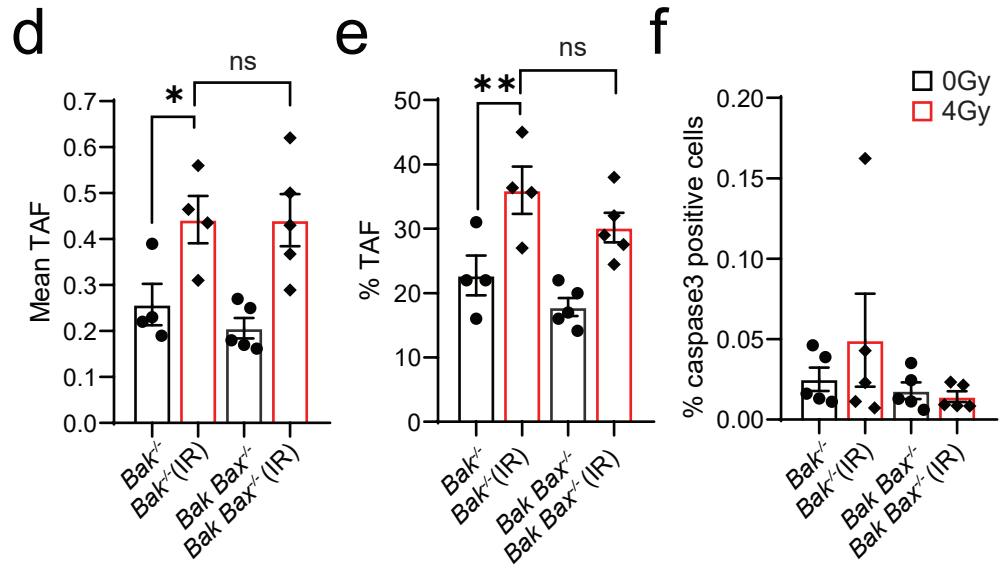


Figure 4 – Liver-specific BAX/BAK deletion reduces the SASP *in vivo*. **(a)** (Top) Scheme showing experimental procedure. (Bottom) Representative immunohistochemical image of Bax showing successful deletion of BAX in the liver following AAV injection. **(b)** Quantification of mRNA levels of the SASP genes indicated in livers from Sham- and 4Gy-irradiated Bak^{-/-} and BakBax^{-/-} mice. Values are expressed as fold change compared to Sham-irradiated Bak^{-/-} animals. Data are mean of n=4-5 animals $\pm$ S.E.M. Statistical significance (one-way ANOVA) is indicated (**P<0.01). **(c)** (Top) Representative immuno-FISH image in liver sections from Sham- and 4Gy-irradiated Bak^{-/-} and BakBax^{-/-} mice. γ H2AX is shown in green, telomeres are in red, and co-localization between the two (TAF) is indicated by arrows and amplified at the bottom. (Bottom) Representative immunohistochemical image of caspase-3 in liver sections from irradiated Bak^{-/-} and BakBax^{-/-} mice. Quantification of **(d)** mean number of TAF, **(e)** percentage of hepatocytes containing TAF and **(f)** percentage of cells positive for caspase-3 in livers from Bak^{-/-} and BakBax^{-/-} mice in the conditions indicated. Data are mean of n=4-5 animals $\pm$ S.E.M. Statistical significance (one-way ANOVA) is indicated (*P<0.05; **P<0.01).

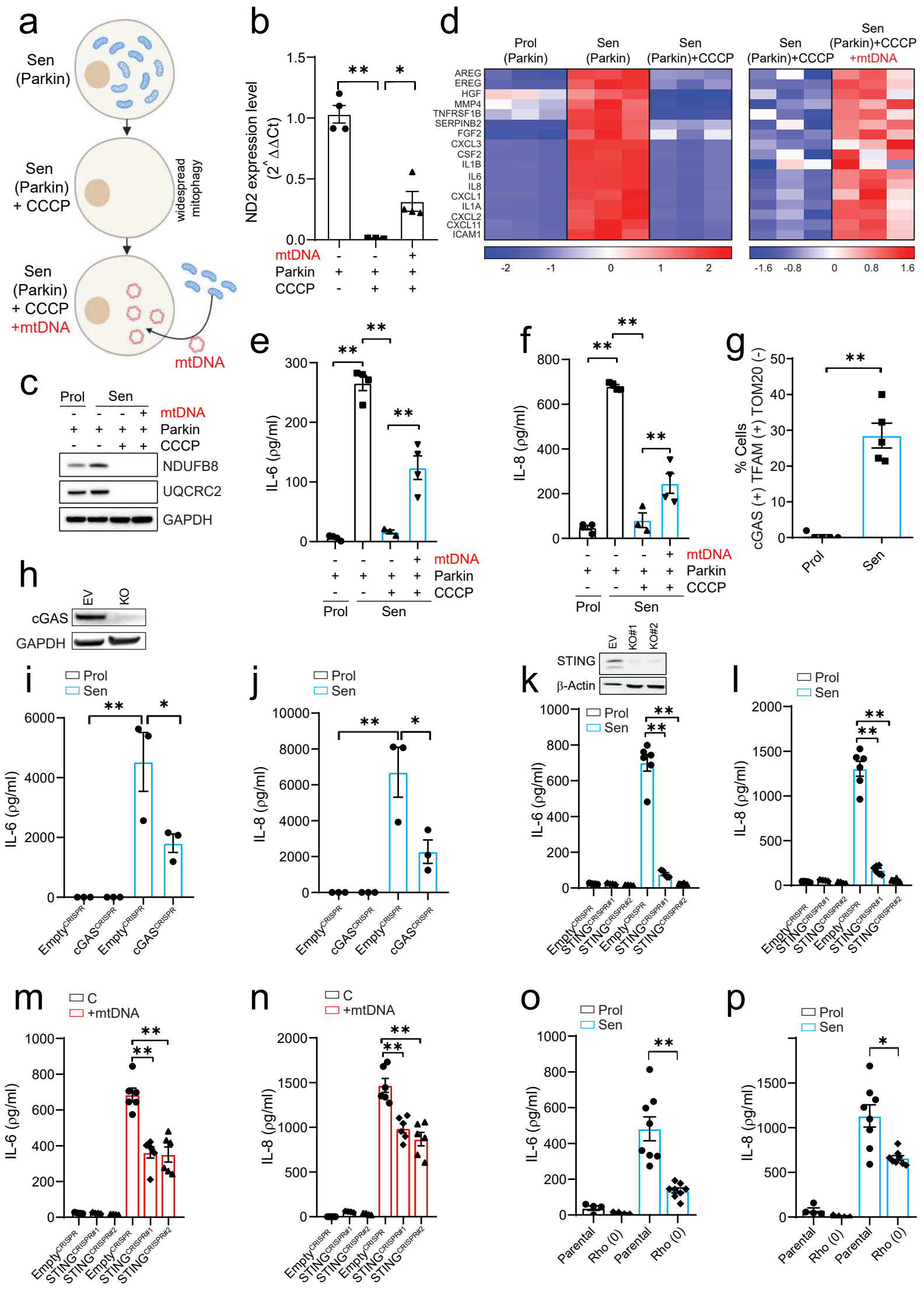


Figure 5 – Cytosolic mtDNA acts via the cGAS-STING pathway to drive the SASP in senescent cells. (a) Scheme representing the experimental approach. (b) Quantification of qPCR showing levels of the mitochondrial gene ND2 in Parkin cells following widespread mitophagy and upon mtDNA transfection. Data are mean of n=4 independent experiments $\pm$ S.E.M. Statistical significance (one-way ANOVA) is indicated (*P<0.05; **P<0.01). (c) Column clustered heatmap of SASP genes that are differentially expressed in senescent cells, down-regulated upon mitochondria clearance and are rescued by mtDNA transfection. The color intensity represents column Z-score, where red and blue indicate high and low expression, respectively. (d) Western blot showing expression levels of mitochondrial proteins NDUFB8 and UQCRC2 demonstrating that mitochondria are not restored upon mtDNA transfection. Secretion levels of (e) IL-6 and (f) IL-8 in Parkin cells following mitochondria clearance and upon mtDNA transfection. Data are mean of n=3-4 independent experiments $\pm$ S.E.M. Statistical significance (one-way ANOVA) is indicated (**P<0.01). (g) Quantification of the percentage of proliferating and senescent cells containing cGAS co-localizing with cytosolic TFAM foci. Data are mean of n=5 independent experiments $\pm$ S.E.M. Statistical significance (unpaired t-test) is indicated (**P<0.01). (h) Representative Western blot showing the level of cGAS upon CRISPR/Cas9-mediated cGAS deletion. Secreted levels of (i) IL-6 and (j) IL-8 in proliferating and senescent EV and cGAS-deficient cells. (k) Representative Western blot showing the level of STING upon CRISPR/Cas9-mediated STING deletion using two different gRNAs (top). Secreted levels of (k) IL-6 (bottom) and (l) IL-8 in proliferating and senescent EV and STING-deficient cells. Secreted levels of (m) IL-6 and (n) IL-8 in EV and STING-deficient cells upon mtDNA transfection, and of (o) IL-6 and (p) IL-8 in proliferating and senescent Rho0 cells. Data are mean of n=3-8 independent experiments $\pm$ S.E.M. Statistical significance (one-way ANOVA) is indicated (*P<0.05; **P<0.01).

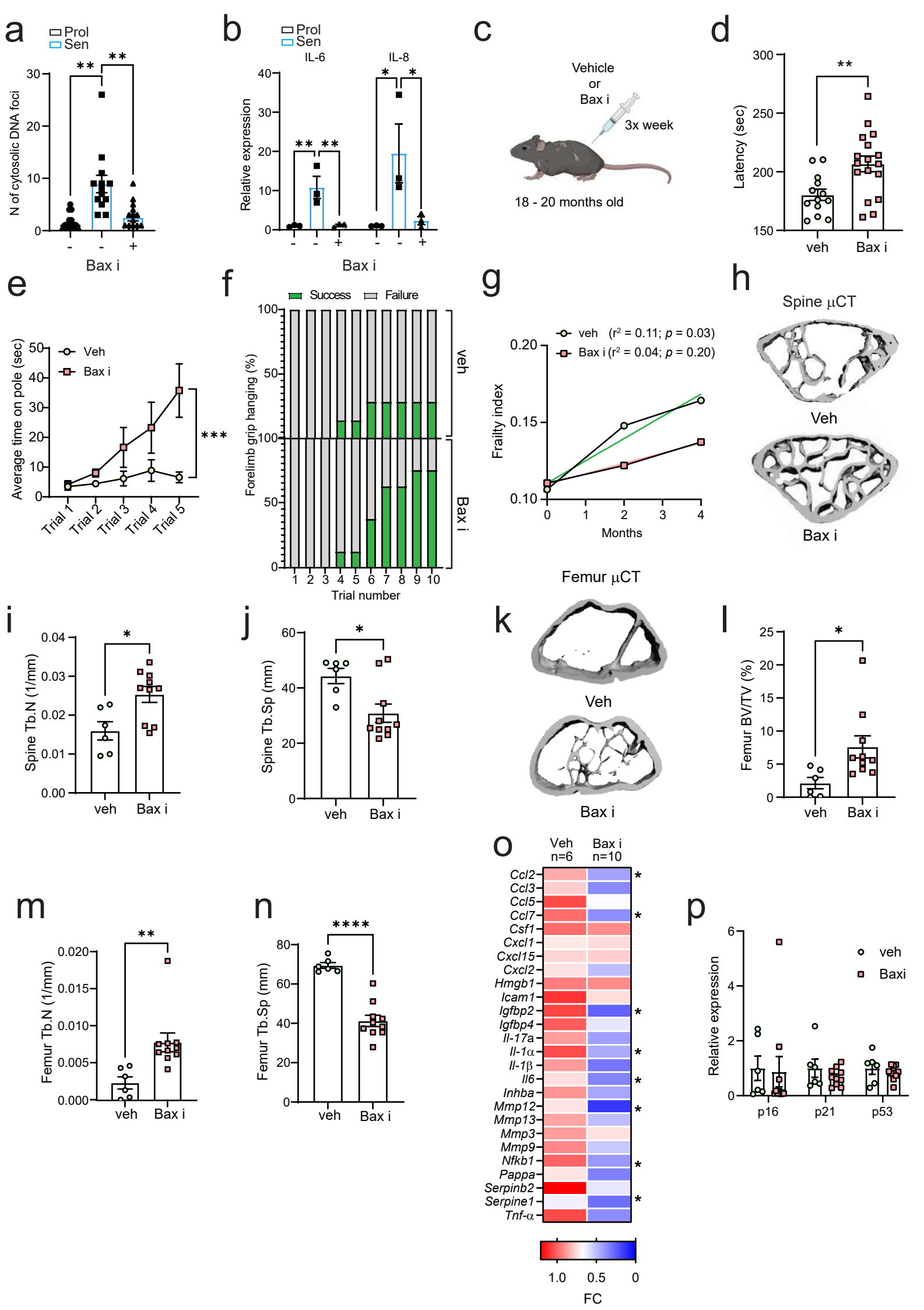


Figure 6 – Pharmacological inhibition of Bax improves healthspan in aged mice. (a) Mean number of cytosolic DNA foci, and (b) Quantification of the mRNA levels of IL-6 and IL-8 in proliferating and senescent cells with or without treatment with a Bax inhibitor. Data are mean of $n=10 \pm$ S.E.M. Statistical significance (one-way ANOVA) is indicated (* $P<0.05$; ** $P<0.01$). (c) Scheme representing the experimental design. (d) Rotarod latency ($n=13-17$ per group), (e) average time spent on the pole ($n=7-8$ per group), and (f) forelimb grip strength assessed as the number of trials required to remain hanging for a total of 90 s (% success in green; $n=7-8$ per group) in aged animals treated with vehicle or Bax inhibitor for 3 months. Data are mean $\pm$ S.E.M. Statistical significance (unpaired t-test) is indicated (** $P<0.01$; *** $P<0.001$). (g) Frailty index of mice at 0, 2 and 4 months of treatment with vehicle- or Bax inhibitor ($n=14-15$ per group). Linear regression of the mean frailty index at each time point is shown. (h) Representative microcomputed tomography (μ CT) images of bone microarchitecture at the lumbar spine of vehicle- and Bax inhibitor-treated animals ($n=6-10$ per group). Quantification of μ CT-derived (i) trabecular number (Tb.N; 1/mm) and (j) trabecular separation (Tb.Sp; mm) at the lumbar spine. (k) Representative microcomputed tomography (μ CT) images of bone microarchitecture at the femur of vehicle- and Bax inhibitor-treated animals ($n=6-10$ per group). Quantification of μ CT-derived (l) bone volume fraction (BV/TV; %) (m) trabecular number (Tb.N; 1/mm) and (n) trabecular separation (Tb.Sp; mm) at the femur. (o) Heatmap showing the mRNA expression of SASP genes assessed by RT-qPCR in femur from mice treated with vehicle or Bax inhibitor ($n=6-10$ per group). Values are expressed as fold change to vehicle mice. Red indicated high expression and blue denotes low expression. (p) Relative expression levels of p16, p21 and p53 assessed by qPCR in femur from mice treated with vehicle or Bax inhibitor ($n=6-10$ per group). Data are mean $\pm$ S.E.M. Statistical significance (unpaired t-test) is indicated (* $P<0.05$; ** $P<0.01$; *** $P<0.001$).

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