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Andria R. Robinson
Matthew J. Yousefzadeh
Tania A. Rozgaja
Jin Wang
Xuesen Li

See next page for additional authors

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Authors
Andria R. Robinson; Matthew J. Yousefzadeh; Tania A. Rozgaja; Jin Wang; Xuesen Li; Jeremy S. Tilstra; Chelsea H. Feldman; Siobhan Q. Gregg; Caroline H. Johnson; Erin M. Skoda; Marie-Celine Frantz; Harris Bell-Temin; Hannah Pope-Varsalona; Aditi U. Gurkar; Luigi A. Nasto; Rena A.S. Robinson; Heike Fuhrmann-Stroissnigg; Jolanta Czerwinska; Sara J. McGowan; Nadiezlda Cantu-Madellin; Jamie B. Harris; Salony Maniar; Mark A. Ross; Christy E. Trussoni; Nicholas F. LaRusso; Eugenia Cifuentes-Pagano; Patrick J. Pagano; Barbara Tudek; Nam V. Vo; Lora H. Rigatti; Patricia L. Opresko; Donna B. Stolz; Simon C. Watkins; Christin E. Burd; Claudette M. St, Croix; Gary Siuzdak; Nathan A. Yates; Paul D. Robbins; Yinsheng Wang; Peter Wipf; Eric E. Kelley; and Laura J. Neidernhofer
Spontaneous DNA damage to the nuclear genome promotes senescence, redox imbalance and aging

Andria R. Robinson¹, Matthew J. Yousefzadeh²,¹, Tania A. Rozgaj³,¹, Jin Wang⁴, Xuesen Li⁵, Jeremy S. Tilstra⁶, Chelsea H. Feldman⁷, Siobhán Q. Gregg⁸,¹, Caroline H. Johnson⁸, Erin M. Skoda⁹, Marie-Céline Frantz¹⁰, Harris Bell-Temin¹¹, Hannah Pope-Varsalona¹², Aditi U. Gurkar¹³, Luigi A. Nastò¹⁴, Renà A.S. Robinson¹⁵, Heike Fuhrmann-Stroissnigg¹⁶, Jolanta Czerwinska¹⁷, Sara J. McGowan¹⁸, Nadiezha Cantu-Medellin¹⁸, Jamie B. Harris¹⁹, Salony Maniaro, Mark A. Rosso, Christy E. Trussoni¹⁹, Nicholas F. LaRussa²⁰, Jolanta Czerwinska¹⁷, Sara J. McGowan¹⁸, Nadiezha Cantu-Medellin¹⁸, Jamie B. Harris¹⁹, Salony Maniaro, Mark A. Rosso, Christy E. Trussoni¹⁹, Nicholas F. LaRussa²⁰, Eugenia Cifuentes-Pagano²¹, Patrick J. Pagano²², Barbara Tudek²³,²⁴, Nam V. Vo²⁵, Lora H. Rigatti²⁶, Patricia L. Opresko²⁶,²⁷, Donna B. Stolz²⁶,²⁷, Simon C. Watkins²⁶,²⁷, Christin E. Burd²⁸, Claudette M. St. Croix²⁹,³⁰, Gary Siuzdak³¹,³², Nathan A. Yates³³,³⁰, Paul D. Robbins³⁴,³⁰,³¹,³²,³³, Yinheng Wang³⁴, Peter Wipf³⁴,³⁵, Eric E. Kelley³⁶,³⁷, Laura J. Niedernhofer³⁸,³⁹, Lora H. Rigattib,³⁴,³⁵,³⁷,³⁸,³⁹

¹ Department of Human Genetics, University of Pittsburgh Graduate School of Public Health, Pittsburgh, PA 15261, USA
² University of Pittsburgh Medical Center, Hillman Cancer Center, Pittsburgh, PA 15232, USA
³ Department of Microbiology and Molecular Genetics, University of Pittsburgh School of Medicine, Pittsburgh, PA 15219, USA
⁴ Department of Molecular Medicine and the Center on Aging, The Scripps Research Institute, Jupiter, FL 33458, USA
⁵ Department of Chemistry, University of California, Riverside, CA 92521, USA
⁶ The Scripps Research Institute California, La Jolla, CA 92037, USA
⁷ Department of Chemistry, University of Pittsburgh, Pittsburgh, PA 15260, USA
⁸ Department of Molecular Genetics and Cell Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA
⁹ Department of Pharmacology and Chemical Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA
¹⁰ Institute of Genetics and Biotechnology, Faculty of Biology, University of Warsaw, Warsaw, Poland
¹¹ Department of Environmental and Occupational Health, University of Pittsburgh, Pittsburgh, PA 15213, USA
¹² Department of Paediatric Orthopaedics, G. Gaslini Children’s Hospital, Genoa, Italy
¹³ Department of Pharmacology and Chemical Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA
¹⁴ Institute of Biochemistry and Biophysics, Polish Academy of Sciences, 02-106 Warsaw, Poland
¹⁵ Vascular Medicine Institute, University of Pittsburgh, Pittsburgh, PA 15213, USA
¹⁶ Center for Biologic Imaging, University of Pittsburgh School of Medicine, Pittsburgh, PA 15261, USA
¹⁷ Division of Gastroenterology and Center for Cell Signaling in Gastroenterology, Mayo Clinic, Rochester, MN 55905, USA
¹⁸ Department of Pharmacology and Chemical Biology, University of Pittsburgh School of Medicine, Pittsburgh, PA 15213, USA
¹⁹ Institute of Genetics and Biotechnology, Faculty of Biology, University of Warsaw, Warsaw, Poland
²⁰ Department of Molecular Genetics, Cancer Biology and Genetics, The Ohio State University, Columbus OH 43210 USA
²¹ Department of Physiology & Pharmacology, West Virginia University, Morgantown, WV 26506, USA

Abstract

Accumulation of senescent cells over time contributes to aging and age-related diseases. However, what drives senescence in vivo is not clear. Here we used a genetic approach to determine if spontaneous nuclear DNA damage is sufficient to initiate senescence in mammals. Ercc1⁻/⁻ mice with reduced expression of ERCC1-XPF endonuclease have impaired capacity to repair the nuclear genome. Ercc1⁻/⁻ mice accumulated spontaneous, oxidative DNA damage more rapidly than wild-type (WT) mice. As a consequence, senescent cells accumulated more rapidly in Ercc1⁻/⁻ mice compared to repair-competent animals. However, the levels of DNA damage and redox imbalance were higher in WT mice. The results indicate that redox imbalance is a consequence of the accumulation of senescent cells in vivo. The mechanisms by which increased redox imbalance and increased levels of DNA damage drive senescence in vivo are not clear.
Aging senescent cells in Ercc1−/α mice never exceeded that observed in old WT mice. Surprisingly, levels of reactive oxygen species (ROS) were increased in tissues of Ercc1−/α mice to an extent identical to naturally-aged WT mice. Increased enzymatic production of ROS and decreased antioxidants contributed to the elevation in oxidative stress in both Ercc1−/α and aged WT mice. Chronic treatment of Ercc1−/α mice with the mitochondrial-targeted radical scavenger XJB-5–131 attenuated oxidative DNA damage, senescence and age-related pathology. Our findings indicate that nuclear genotoxic stress arises, at least in part, due to mitochondrial-derived ROS, and this spontaneous DNA damage is sufficient to drive increased levels of ROS, cellular senescence, and the consequent age-related physiological decline.

1. Introduction

Aging is the primary risk factor for the majority of chronic diseases; hence, aging is now being considered as a therapeutic target [1]. However, this remains a challenge as the precise molecular mechanisms underpinning aging are not well defined. Cellular senescence was recently established to play a causal role in aging [2] and many age-related diseases [3–8]. Senescence is a programmed cell fate characterized by growth arrest, a metabolic shift, resistance to apoptosis and often a secretary phenotype [9]. The senescent cell burden increases with age in virtually all vertebrates [10–12]. In replicating human cells, shortened telomeres drive senescence [13]. It has become increasingly clear that non-replicating cells also undergo senescence [14]. However, in non-dividing cells, which are the majority of cells in mammalian organisms, the cause of senescence is not clear.

A variety of cellular stressors including genotoxic, proteotoxic, inflammatory and oxidative have been implicated in driving senescence [9,15]. However, senescence itself is associated with many of these cellular stressors [16], making it very difficult to decipher cause and effect. For example, DNA damaging agents definitively cause increased senescence (e.g. in cancer patients) [17]. Yet senescent cells are defined by persistent activation of the DNA damage response [18], increased expression of surrogate markers of DNA damage [19] and are able to trigger genotoxic stress in neighboring cells [16]. Therefore, in vivo, the importance of DNA damage as a driver of senescence and aging is debated [20].

Even less is known about endogenous DNA damage as a potential driver of senescence and aging. The vast majority of evidence implicating DNA damage in senescence comes from experiments implementing very high doses of environmental genotoxins such as ionizing radiation, doxorubicin, etoposide or cisplatin [19,21,22]. Also of note, all genotoxins damage not only DNA, but also all cellular nucleophiles including phospholipids, proteins and RNA. Thus, it remains unknown whether physiological levels of spontaneous DNA damage is sufficient to drive cellular senescence.

A major source of endogenous DNA damage is reactive oxygen species (ROS) produced during mitochondrial-based aerobic metabolism (e.g. the superoxide anion (O2•−) and the hydroxyl radical (‘OH) produced from O2 or H2O2 via the Fe3+ -dependent Fenton or Haber-Weiss reaction) [23]. The DNA lesions caused by ROS include oxidized bases, abasic sites, single-strand breaks and lipid peroxidation-induced adducts such as interstrand crosslinks [24]. Some mitochondrial-derived ROS, such as H2O2, can diffuse throughout the cell, resulting in oxidative damage to lipids, proteins, RNA and DNA [25]. Thus, mitochondrial dysfunction, which leads to an increase in ROS production, was proposed to be central to the aging process [26,27]. However, this too remains controversial [28].

To address these gaps in knowledge, we utilized a genetic approach to increase endogenous nuclear DNA damage in mice. Ercc1-XPF is an endonuclease complex required for nucleotide excision repair, interstrand crosslink repair and the repair of a subset of DNA double-strand breaks [29]. Mutations that mediate reduced expression of this enzyme cause accelerated aging in humans and mice [29]. Ercc1 is required to stabilize XPF in vivo [30]. Therefore, Ercc1−/α mice, with one knock-out and one hypomorphic allele of Ercc1 have 5–10% of the normal complement of ERCC1-XPF [31]. Genetic depletion of DNA repair mechanisms does not increase the amount of damage incurred, it simply accelerates the pace at which damage triggers a demonstrable physiological impact, affording an opportunity to investigate the role of endogenous nuclear DNA damage in driving senescence.

Here, we demonstrate that Ercc1−/α mice accumulate oxidative DNA damage and senescent cells more rapidly than age-matched wild-type (WT) controls, yet comparable to WT mice over two years of age. Surprisingly, we found that Ercc1−/α mice are also under increased oxidative stress. Increased ROS production and decreased antioxidant buffering capacity contributed to the oxidative stress, which was also observed in aged WT mice. Treatment of Ercc1−/α mice with a mitochondrial-targeted radical scavenger (XJB-5–131) was sufficient to suppress oxidative DNA damage, senescence and age-related pathologies. These data demonstrate that damage of the nuclear genome arising spontaneously in vivo is sufficient to drive cellular senescence.

Our data also demonstrate that endogenous DNA damage, as a primary insult, is able to trigger increased reactive oxygen species (ROS) and further oxidative damage in vivo.

2. Methods

2.1. Chemicals and reagents

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. Primary antibodies used for immunoblotting were purchased from Abcam (Cambridge, MA) unless indicated.

2.2. Animal care and experimentation

All animal studies were conducted in compliance with the U.S. Department of Health and Human Services Guide for the Care and Use of Laboratory Animals, and were approved by the Scripps Florida or University of Pittsburgh Institutional Animal Care and Use Committee. Ercc1−/α mice were bred and genotyped as previously described [32]. P16-luciferase reporter mice were obtained from Ohio State University [10] and bred to create Albino C57BL/6 p16Luc/+;Ercc1−/− and FVB/n p16Luc/+;Ercc1+/

Δ mice. These mice were further crossed to create f1 p16Luc/+;Ercc1−/Δ mice with white fur for imaging. All animals were genotyped from an ear punch by TransnetYX (Cordova, TN).

2.3. DNA extraction and measurement of cyclopurine DNA lesions

DNA was isolated using a high-salt extraction method [33] from cultured MEFs or liver tissue, which was pulverized with a mortar and pestle under liquid nitrogen. Cyclopurine lesions were measured by LC-MS/MS/MS using an LTQ linear ion trap mass spectrometer using our recently described conditions with some modifications [34]. Nuclease P1 (0.1 U/μg DNA), phosphodiesterase 2 (0.000125 U/μg DNA), 20 nmol of erythros-9-(2-hydroxy-3-nonyl) adenine EHNA and a 20-μL solution containing 300 mM sodium acetate (pH 5.6) and 10 mM zinc chloride were added to isolated nuclear DNA. In this context, EHNA served as an inhibitor for deamination of 2'-deoxyadenosine to 2'-deoxyinosine induced by adenine deaminase [34]. The above digestion mixture was incubated at 37°C for 48 h. To this mixture were then
added alkaline phosphatase (0.05 U/µg DNA), phosphodiesterase 1 (0.00025 U/µg DNA) and 40 µL of 0.5 M Tris-HCl buffer (pH 8.9). The digestion was continued at 37°C for 2 h and subsequently neutralized by addition of formic acid. To the mixture were then added appropriate amounts of uniformly 15N-labeled standard lesions, which included R-cdG, S-cdG, R-cdA and S-cdA. The digestion mixture was subsequently extracted twice with chloroform. The resulting aqueous layer was subjected to off-line high performance liquid chromatography (HPLC) separation for the enrichment of the lesions under study, following our previously described procedures [34]. The LC-MS/MS/MS experiments were conducted using an LTQ linear ion trap mass spectrometer using our recently described conditions with some modifications [34]. Briefly, a 0.5 × 150 mm Zorbax SB-C18 column (particle size, 5 µm, Agilent) was used for the separation of the above-enriched lesion fractions, and the flow rate was 4.0 µL/min. A solution of 0.1% (v/v) formic acid in water (solution A) and a solution of 0.1% (v/v) formic acid in methanol (solution B) were used as mobile phases for the analyses of all four cyclopurine lesions, i.e. the (5’S) and (5’R) diastereomers of cdA and cdG, after HPLC enrichment, and a gradient of 5 min 0–20% B, 30 min 20–80% B, and 5 min 80% B was employed for the separation.

2.4. Fluorescence in situ hybridization for telomere-specific γH2AX foci

Primary murine embryonic fibroblasts (MEFs) were fixed with 2% paraformaldehyde for 15 min followed by permeabilization (0.2% Triton X-100 in PBS) for 15 min. Cells were then blocked with 5% BSA, 20% goat serum in PBS (for 2 h. Cells were immuno-stained with mouse anti-γH2AX monocolonal antibody (1:500; Upstate, Billerica, MA) overnight and goat anti-mouse 594 secondary antibody (1:1000) for 1 h. Cells were then fixed in 2% paraformaldehyde for 5 min. Samples were dehydrated in 70%, 95%, 100% ethanol (5 min each) and then denatured for 10 min at 80°C in hybridization solution (70% denatured formamide, 10% NEN blocking reagent [Roche], 0.1 M Tris-HCl [pH 7.4], MgCl2 buffer [82 mM NaH2PO4, 9 mM citric acid, 20 mM MgCl2]), and 0.5 µg/mL Cy3-OO-(CCCTAA)3 PNA probe (Panagene, South Korea). After 2 h hybridization at room temperature, the samples were washed twice with 70% deionized formamide in 10 mM Tris-HCl, pH 7.2. Samples were counterstained with DAPI, mounted onto slides with Gelvatol and images were acquired with a Nikon A1 confocal microscope (Nikon Instruments, Inc.).

2.5. Senescence-associated β-galactosidase (SA-β-gal) staining of tissue

Fresh tissues were fixed in 10% neutral buffered formalin (NBF) for 3–4 h and then transferred to 30% sucrose overnight. Tissues were then embedded in cryo-embedding media (OCT) and cryosectioned at 6 µm for SA-β-gal staining (pH 5.8) at 37°C for 16–24 h in SA-β-gal staining solution (pH 6.0; 40 mM citric acid in sodium phosphate buffer, 5 mM K4[Fe(CN)6]·3H2O, 5 mM K3[Fe(CN)6], 150 mM sodium chloride, 2 mM magnesium chloride and 1 mg/mL X-gal dissolved in N,N-di-methylformamide).

2.6. IVIS in vivo imaging detection of luciferase activity

Isoflurane-anesthetized mice were injected intraperitoneally with Dulciferin substrate (Caliper Life Sciences, Hopkinton, MA; 15 mg/mL in PBS) and were imaged by using an IVIS Lumina (Caliper Life Sciences) as previously described [10].

2.7. RNA isolation and qPCR

Tissues were harvested from euthanized animals and snap frozen in liquid nitrogen. Tissues were homogenized using FastPrep-24 homogenizer (MP Biomedicals, Solon, OH) and total RNA was isolated using Trizol, according to manufacturer’s specifications (Thermo Fisher, Waltham, MA). Total RNA was quantified using a Nanodrop spectrophotometer (Thermo Fisher) and 1 µg of total RNA was used to generate cDNA with the Transcriptor First Strand cDNA synthesis kit (Roche, Basel Switzerland) according to the manufacturer’s specification. Gene expression changes in p16 was quantified by qPCR reactions using 20 µL reaction volumes using a StepOne thermocycler (Thermo Fisher) with input of 100 ng cDNA per reaction. Reactions were performed in duplicate (n = 4–12 mice per group). Data was analyzed by ΔΔCT method and expression was normalized to Gapdh. Primer sequences are as follows: Cdk2a (p16) Fwd 5′- CGGAAGAGGCTGCACTGGA-3′; Cdk2a (p16) Rev 5′-GAGGAAGAGGCTGTAAGTGA-3′; Gapdh Fwd 5′-AGGTATCC AGAGGCTGAA-3′; Gapdh Rev 5′-CTGGCTTACCACTCCCTTGA-3′.

2.8. Biochemical detection of superoxide

Fresh murine tissue slices were incubated in a 30 µM solution of hydroethidine (HE) in PBS for 45 min at 37°C in the dark. The slices were washed with iced cold PBS and placed into a 1.5 mL Eppendorf tube and immediately frozen by immersion in liquid nitrogen. Superoxide levels were measured by the presence of 2-hydroxyethidium (2-OH-•’E−) using a HPLC system equipped with electrochemical detector as previously reported [35,36]. Briefly, the separation of the oxidized products of hydroethidine (HE) was performed using an ether-linked phenyl column (Phenomenex, 100 × 4.6 mm, 2.6 µm) and a gradient elution method using two mobile phases with an increasing fraction of acetonitrile (from 25% to 60% over 10 min). The presence of superoxide was also confirmed by electron paramagnetic resonance (EPR) spectroscopy spin-trapping of a 1-hydroxy-3-methoxy-carbonyl-2,2,5,5-tetramethylpyrrolidine (CMH) (Noxygen Science Transfer and Diagnostics, Elzach, Germany) superoxide-sensitive probe and analyzed using a temperature- and O2-controlled Bruker EPR (Mllerca, MA) at 37°C as described [37]; n = 3–9 mice per genotype.

2.9. Immuno-spin trapping of biomolecular free radicals

Briefly, mice were injected with 500 mg/kg 5,5-dimethyl-1-pyrroline N-oxide (DMPO, Dojindo, Japan) at 24, 12 and 6 h prior to euthanasia. Tissues were fixed in 2% PFA in PBS for 1 h then submerged in 30% sucrose for 24 h, with several solution exchanges. Tissues were cryopreserved in 2-methylbutane then sectioned on a cryostat (Leica Biosystems, Richmond, IL). Sections were stained with polyclonal anti-DMPO (ALX-210–530-R050; Enzo Life Sciences) followed by secondary antibody (Alexa Fluor 488 anti-rabbit IgG; Life Technologies). Tissues were counterstained with DAPI to detect nuclei and for actin (fluor-conjugated phallolidin) to reveal tissue architecture. For liver, 9×9 image sections were stitches together from multiple images with 10% stitching overlap using the Nikon NIS-Elements software. 3–5 mice were used per group.

2.10. Lipid peroxidation

4-Hydroxynonenal-protein adducts, which are by-products of lipid peroxidation, were measured in murine liver using the OxiSelect HNE Adduct Competitive ELISA kit (Cell Biolabs, San Diego, CA). Livers lysates were prepared in RIPA buffer and normalized based on protein concentration. µg of total protein was used for each assay. Four liver samples were measured in duplicate for each group except old WT mice (n = 3–4). Measurements were taken using an EnVision plate reader (Perkin Elmer, Waltham, MA).

2.11. Xanthine oxidase activity

Xanthine oxidase activity was measured as previously described [38]. Briefly, liver samples (50 mg) from 7 to 9 mice per group were homogenized in ice-cold potassium phosphate buffer (50 mM, pH 7.4) and incubated in the presence of 200 µM xanthine and 100 µM oxonic acid.
acid for 60 min at 37°C (with and without 200 μM allopurinol). Accumulation of uric acid over this time (above that observed in the presence of allopurinol) was assessed via reverse phase HPLC coupled to an electrochemical detector (ESA CoulArray, Chelmsford, MA), (1 Unit = 1 μmole urate/min). Similarly, XO activity was measured from the serum of mice (n = 3–9).

2.12. NADPH oxidase activity

Tissue O$_2^-$ production was calculated from the initial linear rate of SOD-sensitive cytochrome c reduction quantified at λ = 550 nm. Briefly, homogenates of frozen liver samples were resuspended in Oxidase Assay Buffer (65 mM sodium phosphate buffer (pH 7.0), 1 mM EGTA, 10 μM FAD, 1 mM MgCl$_2$, 2 mM Na$_2$S$_2$O$_7$, 300 U/mL catalase, and 0.2 mM cytochrome c), in the presence or absence of superoxide dismutase (150 U/mL). After 5 min baseline measurement, NADPH (180 μM) was added and O$_2^-$ production was measured at 550 nm using a Biotech Synergy 4 hybrid multimode microplate reader. 6–13 mice per group were used. Data are expressed as fold change from WT.

2.13. Mitochondrial respiration

Mitochondrial respiration in isolated liver mitochondria was measured by the oxygen consumption rate (OCR) using a Seahorse XF96 Extracellular Flux Analyzer (Agilent Seahorse, Santa Clara, CA). Liver mitochondria were isolated as described [39]. 150 μL suspension of liver mitochondria (6 μg protein/well) was plated on a pre-chilled Seahorse PS 96-well microplate reader. The plate was centrifuged at 3220 × g for 50 min at 4 °C, subsequently incubated in 37 °C (without CO$_2$) for 15 min then transferred to the XF flux analyzer for respiration measurement. The measurement cycle consisted of a 3 min mixing time and a 4 min time point. After three basal measurements in the presence of complex I substrate pyruvate (5 mM), 150 μM ADP, 2 μg/mL oligomycin (inhibitor of ATP synthase), 4 μM carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP; an optimized concentration to give maximum respiratory capacity), 2 μM rotenone and 2 μg/mL antimycin A were auto-injected into the experimental wells, and another three measurement cycles were performed. Each experimental point is an average of a minimum of three replicate wells on four mice per group. State III and maximal respiration were calculated as described [40].

2.14. Metabolite extraction

Liver samples were weighed (10 mg) from 12-week-old WT (n = 7), old WT (120–136-week-old) (n = 7) and 12-week-old Ercc1$^{-/-}$ (n = 6) mice. Samples were homogenized in 400 μL methanol/water (80/20 v/v) with 1 mm glass beads (Biospec, Bartlesville, OK, USA) in 1.5 mL glass vials. A Minilys homogenizer (Bertin Technologies, Montigny le Bretonneux, France) was used for 30 s at 3000 rpm. The samples were sonicated for 5 min, centrifuged for 15 min at 15,000 × g for 4 min at 4°C. The supernatant was transferred to 1.5 mL glass vials and stored at -20°C until use. The samples were dried down in a centrifugal concentrator with inline cold trap (Burdick & Jackson, Muskegon, MI).

Mass spectrometric raw files were translated and analyzed using the CHORUS cloud computing label free quantitation analysis suite (chorusproject.org). Briefly, chromatographic peaks, features, are separated from noise and placed into appropriate isotope groups before alignment across all samples and quantification using label free differential mass spectrometry [48,49]. Identification is performed using the Comet and Percolator MS/MS identification engines compared to the Uniprot reference data set for Mus musculus, generating identification and
Statistical analysis was performed on all identified features by rejecting any feature not found in at least 75% of samples following outlier removal. Feature level data was brought to protein level by taking the median level of all unmodified, unique peptides of a given protein per sample, with a minimum of two unique peptides per protein. A two-tailed Student's t-test was performed to establish statistical significance for all proteins identified.
2.17. Catalase activity

Catalase activity in liver tissue was determined as previously reported [52]. 50 μg of liver lysate from each mouse were used and analyzed in duplicate. Detection of peroxidase (Fisher Scientific, Pittsburgh, PA) at 240 nm was performed using a Cary 300 Bio UV–VIS spectrophotometer (Varian, Palo Alto, CA) at 30 s intervals for a total of 1 min. Catalase activity per milligram of protein (k/mg) was quantified using the following formula: k/mg = (3 ln (Abs_initial/abs_final)) / [milligrams of protein × time]. Values represent the mean ± standard deviation. p values were calculated using a two-way ANOVA. ***p < 0.001, ****p < 0.0001, * over the black dots indicate significant differences between the WT and Ercc1Δ/Δ mice. * over the black bars indicate a significant difference between Ercc1Δ/Δ of different age groups. (f) qPCR detection of p16expression in liver (n = 6–12), kidney (n = 4–6) and spleen (n = 7–10) of Ercc1Δ/Δ mice (blue), age-matched WT mice (red) and old WT mice (green). Values represent the mean ± SD, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001 determined by one-way ANOVA with Tukey’s test.

2.18. Superoxide dismutase activity

SOD activity (mitochondrial and cytoplasmic) was quantified using the Superoxide Dismutase Assay Kit (Cayman) per the manufacturer’s instructions. All liver samples were normalized based on protein concentration with n = 3 per group.

2.19. Glutathione analysis

Livers were harvested from euthanized mice, fixed in 5% sulfo-salicylic acid and extracts were prepared by homogenization in MES buffer (0.2 M 2-(N-morpholino) ethanesulphonic acid, 0.05 M phosphate and 1 mM EDTA, pH 6.0) to prevent post-mortem oxidation of glutathione [53]. Samples were normalized by protein concentration and analyzed for concentration of total GSH and GSSG using a Glutathione Assay Kit (Cayman Chemicals, Ann Arbor, MI) per the manufacturer’s specifications. Sample absorbance was measured at 405 nm using a plate reader. Equation to determine reduced GSH was [Reduced GSH] = [Total GSH] – [GSSG], and ratio was reported as [Reduced GSH]/[GSSG] [54]. n = 3–14 mice per age/genotype.

2.20. Immunoblotting

Liver and kidney samples from 18-week-old Ercc1Δ/Δ and WT mice (n = 5) were homogenized in RIPA buffer (Pierce, Rockford, IL). with protease inhibitor cocktail (Roche, Indianapolis, IN). Mitochondrial extracts were prepared using Mitochondria Isolation Kit (Pierce) per the manufacturer’s specifications. Samples were separated on 4–20% polyacrylamide gel (Bio-Rad, Hercules, CA), transferred to nitrocellulose membrane, blocked and blotted with anti-PCNA (PC10, Santa Cruz Biotechnology, Santa Cruz, CA), anti-ERCC1 (D-10, Santa Cruz Biotechnology), anti-COXIV (Abcam, Cambridge, MA), anti-XPF (SPM228, Novus Biologicals, Littleton, CO) or anti-GAPDH, anti-MnSOD, anti-CuZnSOD, anti-catalase (3H3L29), anti-NO, and anti-rabbit secondary (all from Life Technologies, Carlsbad, CA) then visualized with ECL reagent (Pierce). Films exposed to membrane were imaged with ImageJ (NIH, Bethesda, MD). GAPDH was used as a loading control.

2.21. Chronic treatment of mice with XJB-5–131

The Ercc1Δ/Δ mice were given intraperitoneal injections of 2 mg/kg XJB dissolved in sunflower oil (SS007 Sigma-Aldrich, St. Louis, MO) or an equal volume of vehicle only (sunflower oil) three times per week, beginning at five weeks of age, by an investigator blinded to the treatment group. Whenever possible, littermate pairs of Ercc1Δ/Δ mice were used, with one mouse in each treatment group, to minimize variability. The mice were weighed twice a week and monitored for the onset of age-related symptoms, including dystonia, trembling, ataxia, priapism and urinary incontinence (neurodegenerative symptoms), hind-limb muscle wasting, lethargy (reduced spontaneous activity) and kyphosis (hunched posture). Data from littermate pairs were evaluated to determine the fraction of symptoms delayed in the mouse treated with XJB vs. its sibling treated with vehicle only using a paired Student’s t-test. All mice were euthanized at 20 weeks of age and their tissues were isolated for pathological analysis.

2.22. Micro-computed tomography measurement of bone density

µCT of spines was acquired as previously described [55] using a VivaCT 40 (Scanco USA Inc.) with 15-μm isotropic voxel size resolution, 55 kVp of energy, and 145 μA of current. After the acquisition of transverse 2-dimensional image slices, 3-dimensional reconstruction of the lumbar vertebrae was performed using a constant threshold value of 235, which was selected manually for the bone voxels by visually matching the threshold areas to the gray-scale images.

2.23. Statistics

The mean and standard deviation or standard error of the mean were calculated for all experimental groups and analyzed using unpaired two-tailed Student’s t-tests, or one-way or two-way ANOVA or Tukey’s test for multiple comparisons using GraphPad Prism 6.

3. Results

3.1. Ercc1Δ/Δ mice have accelerated accumulation of spontaneous oxidative DNA damage

To test conventional wisdom that nucleotide excision repair (NER) is exclusively nuclear and not a mitochondrial DNA repair mechanism [56], ERCC1 and XPF protein levels were measured in fractionated murine liver lysates (Fig. 1A). Both proteins were detected in the nuclear but not mitochondrial fractions, establishing their role in protecting the nuclear genome, exclusively. Cyclopurines (cPus) are DNA lesions generated by endogenous reactive oxygen species [57], which are repaired by NER [58]. Thus, cPus are expected to be increased in Ercc1Δ/Δ mice compared to age-matched WT animals. LC-MS/MS/MS was used to measure the four cPus lesions (R-cdG, S-cdG, R-cdA and S-cdA) in kidney tissue of mice (Supplemental Fig. 1) [59]. At two months of age, the levels of cPu were not elevated in Ercc1Δ/Δ mice (Fig. 1B). By five months of age, however, all four lesions were significantly increased in Ercc1Δ/Δ compared to WT mice. Notably, S-cdG, R-cdA and S-cdA also were significantly increased in old WT mice compared to young animals. Furthermore, adduct levels were equivalent in 5-month-old Ercc1Δ/Δ and 3-year-old WT mice. This indicates that Ercc1Δ/Δ mice have an increased burden of endogenous DNA damage than age-
A. Oxidative DNA Lesions

B. Kidney

C. Kidney

D. Lipid Peroxidation

(caption on next page)
matched repair-proficient animals and that they accumulate spontaneous DNA damage faster than WT animals.

3.2. Ercc1Δ/Δ mice have accelerated accumulation of senescent cells

To determine if endogenous DNA damage is sufficient to drive cellular senescence in vivo, multiple markers of senescence were measured in tissues of Ercc1Δ/Δ and WT mice of various ages. Senescence-associated β-galactosidase (SA-β-gal) activity was increased in 5-month-old Ercc1Δ/Δ mouse kidney and liver compared to WT littermates (Fig. 1C). Two-year-old WT mice also had increased SA-β-gal activity in these tissues relative to young animals. The level of p16Ink4a expression was measured using a p16Ink4a-Luciferase transgenic reporter (Fig. 1D) [10]. Total p16Ink4a-Luciferase expression was modestly but significantly increased in mutant animals at weaning (Fig. 1E). The signal increased steadily as the mutant animals aged, in particular, after 8 weeks of age. Notably, the signal level seen in the DNA repair-deficient mice did not exceed that of older WT mice. As both WT and Ercc1Δ/Δ mice aged, the heterogeneity in signal between animals increased dramatically, as previously reported for WT mice in a different genetic background [10].

Increased p16Ink4a expression was validated by qPCR (Fig. 1F). p16Ink4a mRNA expression was significantly greater in the liver, kidney and spleen of Ercc1Δ/Δ mice compared to WT age-matched controls. p16Ink4a expression in 3–4 month-old Ercc1Δ/Δ mice was comparable to that of 2-year-old WT mice. Taken together, these data document the premature accumulation of senescent cells in DNA repair-deficient Ercc1Δ/Δ mice. Importantly, Ercc1-XP-F-deficient human and murine cells do not show accelerated telomere attrition [60]. To confirm the absence of telomere dysfunction, we measured telomere damage-induced foci [61] in Ercc1Δ/Δ and WT mouse embryonic fibroblasts (Supplemental Fig. 2).

Notably, the number of γH2AX foci was significantly increased in Ercc1Δ/Δ cells compared to WT, as expected for DNA repair-deficient cells. However, γH2AX foci at telomeric DNA was significantly lower in Ercc1Δ/Δ cells, confirming prior studies [60]. These data rule-out telomere dysfunction as the driver of senescence in the absence of ERCC1-XPF. This suggests that it is unrepaird, endogenous DNA damage that drives cellular senescence in mammalian tissues.

3.3. Ercc1Δ/Δ mice demonstrate elevated ROS abundance

The presumption is that Ercc1Δ/Δ mice have increased oxidative DNA damage because of their defect in NER. However, it is also possible that Ercc1Δ/Δ mice are under increased oxidative stress. To test this, we measured cPus in liver tissue of age-matched Ercc1Δ/Δ, WT and XpaΔ/Δ mice. The latter are completely deficient in nucleotide excision repair of cyclopropine (cPu) lesions. XpaΔ/Δ mice also show no signs of accelerated aging [62]. Notably, all four cPus were significantly elevated in liver of Ercc1Δ/Δ mice compared to WT, but cPus were not elevated in XpaΔ/Δ mouse liver (Fig. 2A). This indicates that lack of DNA repair does not adequately explain the increased oxidative DNA damage in Ercc1Δ/Δ mice.

To determine if the Ercc1Δ/Δ mice are under increased oxidative stress, superoxide anion (O2−) production was measured in fresh renal and liver tissue by quantification of 2-OH-E+ by HPLC/electrochemical analysis in DHE-treated kidney (n = 3–7) and liver (n = 6–9 animals per genotype/age). (c) Representative images from immuno-spin trapping of endogenous, biomolecular free radicals with 5,5-dimethyl-1-pyrroline-N-oxide (DMPO). The level of oxidant stress was determined by immunodetection of DMPO-adducted biomolecules in renal and liver sections. DMPO staining is illustrated in red, acting in green to illustrate tissue architecture and DAPI in blue to highlight cell nuclei. (d) Lipid peroxidation as measured by quantitation of 4-hydroxynonenal protein adducts via ELISA (n = 3–4 mice per group).

For all panels, values represent the mean ± SD, *p < 0.05, **p < 0.01, ***p < 0.001 determined by one-way ANOVA with Tukey’s test.
metabolomics on liver extracts comparing 3-month-old WT and Ercc1−/Δ mice and old WT mice revealed 6812 aligned features, of which 69 were significantly changed between the two groups. Comparison of liver tissues from 3-month-old and 2.5-year-old WT mice revealed 6812 aligned features, of which 69 were significantly changed between the two groups. Metabolites were identified from both analyses using a combination of MS/MS with spectrum matching on the METLIN database, and confirmed using authentic standards. One of the key nodes identified by metabolomics as significantly altered with accelerated and normal aging was glutathione metabolism (Fig. 4A), a key antioxidant and index of oxidative stress.61

Differential MS was used for proteomics analysis to identify redox-related proteins significantly altered in the livers of 3–4 month-old progeroid Ercc1−/Δ mice and old WT mice (>2 years-old) vs. adult WT mice. Expression of catalase, SOD1 (CuZnSOD) and SOD2 (MnSOD) were significantly reduced in Ercc1−/Δ and old WT mice compared to young adult WT mice (Fig. 4B). In fact, numerous proteins affecting redox status were identified as altered in mutant mice, including aconitase 1, cytochrome c oxidase, ATP citrate lyase and microsomal glutathione s-transferase 1 (Supplemental Fig. 4). A very similar pattern of expression changes occurred in old WT mice, relative to younger animals. The MS data were validated by immunodetection of several antioxidants by immunoblot (Supplemental Fig. 5).

To validate the predictions arising from the omics studies, activity of key antioxidants was measured. In liver tissue, catalase activity was significantly decreased in Ercc1−/Δ mice compared to age-matched controls (Fig. 4C). Interestingly, in young mutant animals (1 month-old), catalase activity was normal, but then declined progressively over the rest of their lifespan, reaching a level in the 4–6 month-old Ercc1−/Δ mice comparable to that of 2.5-year-old WT mice. SOD1 (CuZnSOD-cytosolic) and SOD2 (MnSOD-mitochondrial) activity in Ercc1−/Δ was similar to WT mice until they reached 4–5 months of age whereby the enzymatic activity of CuSOD and MnSOD were significantly lower than that of age-matched controls (Fig. 4D–E). Similarly, MnSOD activity was lower in 2.5-year-old WT animals (Fig. 4E).

The reduced form of glutathione (GSH) is the active antioxidant and becomes oxidized to glutathione disulfide (GSSG). A decreased ratio of GSH/GSSG is indicative of a state of oxidative stress as well as antioxidant depletion. The GSH/GSSG was significantly reduced in 2-month-old Ercc1−/Δ mice compared to age-matched controls and increased further by 5 months of age (Fig. 4F). In WT mice, the GSH/GSSG ratio was significantly decreased at one year of age compared to younger animals, then diminished further by 2 years of age, as previously reported in numerous tissues of rodents.72 These data provide multiple lines of evidence indicating that, in addition to increased ROS production, there is a significant decline in antioxidant buffering capacity in Ercc1−/Δ mice, likely contributing to the enhanced levels of O2− detected in liver and kidney of the progeroid mice. The parallels between the Ercc1−/Δ mice and aged WT mice suggest a role for spontaneous DNA damage and ROS in normal aging as well.

3.6. A mitochondrial-targeted radical scavenger suppresses senescence

To determine if the increased oxidative stress plays a causal role in driving senescence, Ercc1−/Δ mice were treated with the mitochondrial-targeted free radical scavenger XJB-5–131. XJB-5–131 is a conjugate between the nitrooxide TEMPO (2,2,6,6-tetramethylpiperidine-1-oxyl) and the mitochondrial-targeting moiety gramicidin S (Supplemental Fig. 6A).73 TEMPO, a stable free radical, is a potent antioxidant due to its proclivity for mimicking SOD in vitro. XJB-5–131 has several advantages over other classes of antioxidants, including its capacity to recycle (Supplemental Fig. 6B), direct acceptance of electrons from the mitochondria respiratory complexes to prevent production of ROS, plus SOD-, catalase- and peroxidase-mimetic activities to neutralize existing ROS.
ROS [72–75]. XJB-5–131 localizes to mitochondria within 1 h in primary cell cultures [76] and is enriched 600-fold in the mitochondria fraction of cells over the cytosol [77, 78].

Ercc1Δ/Δ mice were treated with 2 mg/kg XJB-5–131 or vehicle control, delivered by intraperitoneal injection beginning at 5 weeks of age (Fig. 5A). Chronic exposure of Ercc1Δ/Δ mice to XJB-5–131 significantly reduced the levels of cPus oxidative DNA lesions in liver tissue (Fig. 5B). XJB-5–131 also suppressed the accumulation of senescent cells. SA-β-gal staining was reduced in the liver of Ercc1Δ/Δ mice treated with XJB-5–131 (Fig. 5C). Similarly, XJB-5–131 significantly reduced the luciferase signal in Ercc1Δ/Δ p16INK4a-luciferase reporter mice (Fig. 5D). Taken together, these results implicate mitochondria-derived ROS as driving endogenous DNA damage and senescence in vivo.

3.7. A mitochondrial-targeted radical scavenger suppresses aging symptoms and pathology

XJB-5–131-treated mice were monitored daily for the onset of progeroid symptoms by an investigator blinded to the treatment groups. Mice treated with XJB-5–131 exhibited a significant delay in the onset of dystonia and ataxia, as well as kyphosis, reduced spontaneous activity and hind-limb muscle wasting (Table 1 and Fig. 5E). Seventy percent of the age-related symptoms measured were significantly reduced by XJB-5–131 compared to vehicle-treated controls. A sixth symptom (urinary incontinence) was not observed in the 6 male mice in the treatment group, but was seen in 2 of 5 males in the vehicle-only group. XJB-5–131 treatment delayed the onset of symptoms by 1–2 weeks in Ercc1Δ/Δ mice which is equivalent to 5–8 years in humans, based on a median lifespan of 84 years.

Mice from both treatment groups were euthanized at 20 wks of age for histopathological analyses. At this age, Ercc1Δ/Δ mice display significant levels of age-related hepatic lesions including necrosis and ballooning degeneration [79]. Both lesions were reduced in Ercc1Δ/Δ mice treated with XJB-5–131 compared to those treated with vehicle only (Fig. 5F). Age-related changes in the kidney, including hyaline casts, glomerular and tubule-interstitial inflammation and fibrosis were attenuated by XJB-5–131. The drug also delayed the loss of pancreatic islets in Ercc1Δ/Δ mice. The brains of XJB-5–131 treated mice showed reduced staining for glial fibrillary acidic protein (GFAP), a marker of neurodegeneration (Fig. 5G). Finally, microcomputed tomography of the spine revealed a significant reduction in osteoprotic changes in Ercc1Δ/Δ mice treated with XJB-5–131 (Fig. 5H and Supplemental Fig. 7). This demonstrates that a mitochondrial-targeted radical scavenger is sufficient to attenuate endogenous oxidative DNA damage, cellular senescence and aging.

4. Discussion

Although cellular senescence has been demonstrated to drive aging [2], it is not known what endogenous processes are primarily responsible for causing cellular senescence in mammals, particularly in post-mitotic tissues. Here, we used mice in which DNA repair was attenuated genetically. By definition, the primary insult in untreated Ercc1Δ/Δ mice is unrepaired endogenous DNA damage to the nuclear genome. Not surprisingly, the Ercc1Δ/Δ mice accumulate senescent cells more rapidly than WT mice. This formally demonstrates that physiologically-relevant types and levels of endogenous DNA damage are able to trigger the time-dependent accumulation of senescent cells.

The surprising discovery is that there is increased ROS in tissues of the Ercc1Δ/Δ mice. This reveals that spontaneous, endogenous nuclear DNA damage can instigate oxidative stress. We found that elevated ROS is likely due, at least in part, to increased enzymatic production by xanthine oxidase (XO) and NADPH oxidase (NOX), altered mitochondrial metabolism, as well as an attrition of the expression and activity of several key antioxidants, catalase, MnSOD and glutathione. Similar events were found in aged WT mice, consistent with prior studies [80–83]. The dramatic parallels between the progeroid and naturally aged mice suggest that oxidative stress is an important common denominator in aging.

To determine if this oxidative stress is pathological, we suppressed it pharmacologically in Ercc1Δ/Δ mice with the mitochondrial-targeted radical scavenger XJB-5–131. Chronic administration XJB-5–131 significantly reduced both oxidative DNA damage and senescence (Fig. 5). The reduced level of senescent cells corresponded to a reduction in age-related morbidity. This is consistent with numerous recent studies demonstrating that genetic or pharmacologic elimination of senescent cells slows age-related decline [2, 4, 7, 8, 84–86]. The observation that suppressing oxidant production is sufficient to decrease senescence indicates that reactive species are required to ultimately cause or maintain senescence in response to genotoxic stress.

Analogous to our work, recent studies demonstrated increased NOX activity in cells from patients with genome instability disorders such as ataxia telangiectasia (AT) and Nijmegen breakage syndrome [87], as well as AtmΔ mice [88] that model AT. Interestingly, in worms, NOX triggers transcriptional activation of stress response mechanisms [89]. Indeed, increased ROS in AtmΔ mice appears to be pathogenic [88, 90] as well as a critical signaling mechanism both up and downstream of ATM [91–93]. Increased ROS has also been reported in cells from xeroderma pigmentosum and Cockayne syndrome patients [94, 95], two diseases caused by a defect in nucleotide excision repair, but the source of ROS is unclear.

Our results are consistent with the oxidative stress theory of aging originally proposed by Denham Harman [26], and the notion that a vicious cycle of ROS generation and oxidative damage is the ultimate driver of aging [27]. Our data also indicate that endogenous nuclear DNA damage is able to trigger this cycle of escalating ROS abundance, oxidative damage, senescent cell accumulation and age-related pathology.

Numerous studies counter the oxidative stress theory of aging. Notably, overexpression of MnSOD, which detoxifies O2•−, does not extend the lifespan of mice [96, 97]. This appears at odds with our data indicating that XJB-5–131 improves the health of Ercc1Δ/Δ mice. However, XJB-5–131 is able to both prevent ROS production and neutralize existing ROS. The nitrooxide radical form of XJB-5–131 can be reduced to a hydroxylamine by accepting an electron from the electron transport chain and subsequent protonation (Supplemental Fig. 6B), thereby preventing electron transfer to O2 and resultant ROS production [98]. Hydroxylamines act as robust reducing agents by hydrogen atom transfer to free radicals and non-radicals such as O = NOO, resulting in a significant diminution of oxidative/nitrosative stress. While admittedly, reaction of hydroxylamine with O2•− will result in H2O2 generation, it will neutralize O = NOO- and by default, this process...
Thus, in the presence of elevated levels of mitochondrial O2•−/MS/MS/MS detection of cyclopurine adducts (catalase expression and activity (Fig. 4B-C and Supplemental Fig. 5).

Polθ−/− mice treated with 8 mg/kg XJB-5–131, i.p., 3X per week for 4.5 weeks and plotted relative to the signal in Ercc1−/− mice treated with vehicle only. Dots represent individual animals. Graphed is the mean ± SD; p < 0.05 determined by an unpaired two-tailed Student’s t-test.

Table 1 Chronic administration of XJB-5–131 delays the onset of age-related functional decline in Ercc1−/− mice.

| Symptom                     | Ercc1−/− + vehicle n=6 | Ercc1−/− + XJB-5–131 n=6 |
|-----------------------------|------------------------|--------------------------|
| Dystonia                    | 7.4                    | 8                        | 8.6                      | 10 |
| Trembling                   | 7.7                    | 8                        | 8.9                      | 10 |
| Kyphosis                    | 11.7                   | 8                        | 12.2                     | 10 |
| Ataxia                      | 14.5                   | 8                        | 16.3                     | 10 |
| Hind-limb wasting           | 14.0                   | 8                        | 16.0                     | 10 |
| Reduced spontaneous activity| 17.5                   | 5                        | 22.0                     | 5 |
| Urinary incontinence        | 12.8                   | 3                        | 12.8                     | 0 |
| Fraction of symptoms delayed| 10%                    | 5                        | 8                        | 66%| 8 |

a Ercc1−/− mice + vehicle; n = 8 mice, 5 ♀; 2 ♂; n indicates the number of mice showing that symptom.

b Ercc1−/− mice + XJB-5–131; n = 10 mice, 6 ♀; 4 ♂.

c Individual symptoms that were significantly delayed in mice treated with XJB-5–131; p < 0.05 one-tailed Student’s t-test.

gerenegrates the nitroxide form of XJB-5–131, thus recycling the radical scavenger [75]. In contrast, SOD solely reduces O2•− levels, yet generates H2O2 in the process (2O2•− → H2O2 + O2) which is likely not well-neutralized in older organisms due to the significant reduction in catalase expression and activity (Fig. 4B-C and Supplemental Fig. 5). Thus, in the presence of elevated levels of mitochondrial O2•−, over-expression of MnSOD may yield increased formation of H2O2; an oxidation than SOD alone [100].

Another argument against the oxidative stress theory of aging is based on the Polθfl/fl mice, which age rapidly due to increased mutations in the mitochondrial genome caused by inactivation of the exonuclease domain of the mitochondrial polymerase γ [101]. Unlike the progeroid Ercc1−/− mice, ROS is not increased in tissues of Polθfl/fl mice, countering the notion that oxidative stress drives aging. However, mutagenesis in the Polθfl/fl mice is stochastic and there are thousands of mitochondrial genomes per cell, making it plausible that phenotypes are less consistent in Polθfl/fl mice than in Ercc1−/− mice where every cell is affected. Therefore, the lack of increased ROS in Polθfl/fl mice was not considered to counter the mitochondrial theory of aging [101].

In conclusion, we demonstrate that spontaneous, endogenous, nuclear DNA damage leads to an accelerated accumulation of senescent cells in vivo. In addition, we provide novel evidence supporting the oxidative stress theory of aging. In a mammalian system where spontaneous endogenous DNA damage is the primary insult, cellular senescence and ROS abundance is increased, leading to further damage and senescence. Attenuating mitochondrial-ROS defuses this cycle and suppresses age-related decline, implicating it as causative. Taken together, this supports the potential of radical scavengers as a treatment for age-related co-morbidities.

Acknowledgements

This work was supported by the National Institutes of Health [grant numbers P01-AG043376, ES016114, P20 GM109098, K99-AG049126, R00AG036817, CA076541, CA101864, AG044376, AI068021, P30AG024827, 5P20GM109096 and P30CA047904]. LJN had additional funding from the Ellison Medical Foundation (AG-NS-0303-05).

Conflict of interest

The authors declare no conflicts of interest.

Author contributions

SQG, AUG, HFS and SM contributed data that led to this manuscript. ARR, CHF, JST, MJY and SJM conducted the in vivo experiments. JW and YW measured cyclopurine adducts. EEEK, MJY, PDR and LJN oversaw various aspects of the experiments. JC, BT, RASR and MJY measured oxidative damage. NVV, CMS, DBS, CEB, PW, YW, PDR and LJN measured species that were significantly delayed in mice treated with XJB-5–131; p < 0.05 one-tailed Student’s t-test.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.redox.2018.04.007.

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