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Dysregulation of DAF-16/FOXO3A-mediated stress responses accelerates oxidative DNA damage induced aging

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ABSTRACT

DNA damage is presumed to be one type of stochastic macromolecular damage that contributes to aging, yet little is known about the precise mechanism by which DNA damage drives aging. Here, we attempt to address this gap in knowledge using DNA repair-deficient *C. elegans* and mice. ERCC1-XPF is a nuclear endonuclease required for genomic stability and loss of ERCC1 in humans and mice accelerates the incidence of age-related pathologies. Like mice, *ercc-1* worms are UV sensitive, shorter lived, display premature functional decline and they accumulate spontaneous oxidative DNA lesions (cyclopurines) more rapidly than wild-type worms. We found that *ercc-1* worms displayed early activation of DAF-16 relative to wild-type worms, which conferred resistance to multiple stressors and was important for maximal longevity of the mutant worms. However, DAF-16 activity was not maintained over the lifespan of *ercc-1* animals and this decline in DAF-16 activation corresponded with a loss of stress resistance, a rise in oxidant levels and increased morbidity, all of which were *cep-1/ p53* dependent. A similar early activation of FOXO3A (the mammalian homolog of DAF-16), with increased resistance to oxidative stress, followed by a decline in FOXO3A activity and an increase in oxidant abundance was observed in *Ercc1*$^{-/-}$ primary mouse embryonic fibroblasts. Likewise, *in vivo*, ERCC1-deficient mice had transient activation of FOXO3A in early adulthood as did middle-aged wild-type mice, followed by a late life decline. The healthspan and mean lifespan of ERCC1 deficient mice was rescued by inactivation of *p53*. These data indicate that activation of DAF-16/FOXO3A is a highly conserved response to genotoxic stress that is important for suppressing consequent oxidative stress. Correspondingly, dysregulation of DAF-16/FOXO3A appears to underpin shortened healthspan and lifespan, rather than the increased DNA damage burden itself.

1. Introduction

Aging is the principal risk factor for numerous chronic diseases [1] and macromolecular damage is implicated as a key driver [2]. There is evidence that DNA damage is one type of macromolecular damage that drives aging. For example, humans with inherited mutations in genes required for the repair of DNA damage, have accelerated aging or progeroid syndromes [3,4]. Furthermore, age-related debilitating diseases occur decades earlier in cancer survivors treated with genotoxic agents compared to their untreated siblings [5]. In addition, levels of endogenous DNA lesions appear higher in older or diseased individuals compared to younger, healthier subjects [6]. However, the mechanism(s) by which DNA damage contributes to aging and disease is not fully elucidated.

In replicating cells, DNA damage activates a robust DNA damage response (DDR) program that leads to cell cycle arrest to prevent

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mutagenesis caused by replicating a damaged genome [7]. If the DNA damage is extensive or irreparable, apoptosis ensues. Apoptosis can contribute to aging by depleting stem cell function and tissue regeneration [8]. Alternatively, the cell cycle arrest can become essentially irreversible causing the cell to senesce [9]. Senescent cells contribute to aging and age-related diseases [10] at least in part *via* the secretion of proinflammatory factors and metalloproteinases [11]. Many cell types in the body replicate slowly, if at all [12] and recent evidence suggests that DNA damage may also play a prominent role in degenerative disease in post-mitotic tissues. For example, elevated levels of DNA damage (oxidative lesions and strand breaks) are observed in Alzheimer's patients compared to age-matched controls [13,14]. The genotoxic stress is detected before neurodegeneration is evident, suggesting a causative role, at least in mice [15]. Yet, the mechanism by which DNA damage promotes age-related disease in non-replicating tissues is unknown.

To address this gap in knowledge, we exploited *Caenorhabditis elegans (C. elegans)*, an organism that has yielded a wealth of information on mechanisms that regulate lifespan [16]. Genetic studies in *C. elegans* led to the identification of numerous molecular pathways that regulate longevity, including nutrient signaling, proteostasis, mitochondrial function and regulation of insulin/IGF signaling. DNA repair pathways are well conserved in *C. elegans,* but contradictory studies exist about their impact on lifespan [17–23]. Moreover, there is no consensus as to the longevity pathways that are dysregulated by DNA damage [24].

ERCC1-XPF is a structure-specific endonuclease involved in at least three DNA repair pathways that protect the nuclear genome [3]. Mutations in either gene that result in a reduction of ERCC1-XPF expression result in accelerated accumulation of endogenous oxidative DNA damage [25] and accelerated aging in humans and mice [26,27]. Here we demonstrate for the first time that *ercc-1* mutant worms recapitulate many of the phenotypes of ERCC1-XPF deficient mice, including increased oxidative DNA damage and reduced healthspan/lifespan. Then we asked which stress response mechanisms were activated in response to endogenous DNA damage. We found the DAF-16/FOXO3A transcription factor was activated in *ercc-1* worms, leading to increased stress resistance. However, DAF-16 activation was not sustained throughout the lifespan in *ercc-1* worms. Decline in DAF-16 activation was *cep-1* dependent and was associated with a decrease in stress resistance and a concomitant rise in reactive oxygen species (ROS). Importantly, these events were recapitulated in ERCC1-XPF deficient mammalian cells and mice, as well as naturally aged mice. Thus, we propose that it is not DNA damage *per se* that drives aging, but rather it is the cellular stress response to this damage that leads to aging phenotypes.

2. Results

Mutations causing reduced expression of the nuclear DNA repair endonuclease ERCC1-XPF cause an accelerated accumulation of spontaneous DNA damage, the same lesions that accumulate over time with normal aging [28]. It is presumed that it is the increased damage burden that drives the shortened healthspan and lifespan in ERCC1-depleted humans and mice [26], yet the precise mechanism remains enigmatic. Here, we used the nematode *C. elegans* to identify conserved mechanisms that might explain the effects of ERCC1-XPF deficiency across species. In worms, the *ercc-1(tm1981)* mutation generates a 619 bp in-frame deletion, whereas, *xpf-1*(*tm2842*) generates a 343 bp deletion, and both are considered null alleles [29]. We found *ercc-1* worms were hypersensitive to UV irradiation (Fig. 1A), had an increased fraction of unhatched eggs, higher frequency of males and delayed growth (Fig S1), all phenotypes associated with genomic instability in nematodes [20,29–35]. Similar phenotypes have been previously shown for the *xpf-1* mutant [17,29]. Furthermore, *ercc-1* worms accumulated spontaneous oxidative DNA lesions more rapidly than N2 (Fig. 1B). These observations clearly establish *ercc-1 / xpf-1* worms as nucleotide excision repair-deficient.

Consistent with the accelerated aging observed in DNA repair mutant mice and humans harboring mutations in *ERCC1* or *XPF* [26,27,36–38], we found significantly reduced pharyngeal pumping and thrashing frequency in *ercc-1* and *xpf-1* mutants compared with N2 at day 5 and 9 of adulthood, but not day 1 (Fig. 1C, D, S2A & B), Additionally, backcrossed *ercc-1* and *xpf-1* mutants exhibited a reproducible reduction in lifespan (Fig. 1E and Fig S2C) compared to N2. Moreover, we observed a reduction in lifespan in the homozygous mutant progeny from a genetically balanced *ercc-1* strain (Fig S2D), indicating that these phenotypes are a consequence of *ercc-1* deletion. Collectively, these data establish *ercc-1* worms DNA repair deficient and prematurely aged, analogous to ERCC1-XPF deficient mammals.

2.1. DNA damage activates DAF-16/FOXO and confers stress resistance

Having confirmed the validity of the worm as an accurate representation of mammalian systems, we used the *ercc-1* background to identify pathways activated by DNA damage that may drive aging. We introduced the *ercc-1* deletion into multiple transgenic GFP strains that act as reporters of pathways involved in modulating lifespan [39] to identify which were altered by endogenous DNA damage. The screen included reporters for the unfolded protein response (UPR) in endoplasmic reticulum (UPR^{ER}–*hsp-4*::GFP), cytoplasmic heat shock response (*hsp-16.2::GFP*), the Nrf2 (*skn-1::GFP*) and FOXO3A (*daf-16::GFP*) stress defense pathways. Under basal conditions, DAF-16 is maintained in the cytoplasm, but upon exposure to stress, post-translational modifications of DAF-16 leads to its nuclear localization and activation of transcriptional programs that mediate stress resistance and promote longevity [40,41]. In wild-type worms, DAF-16::GFP was primarily cytoplasmic in D1 adults (Fig. 2A). In contrast, in D1 *ercc-1* animals, DAF-16::GFP was nuclear (Fig. 2A). This suggests that this stress defense pathway is activated in adult *ercc-1* animals in response to unrepaired, spontaneous, endogenous DNA damage, as previously reported for larvae exposed to UV irradiation [20].

As a functional readout of DAF-16 activation, we measured survival after administration of various exogenous stressors in *ercc-1* mutants. At D1 of adulthood, *ercc-1* mutants were more resistant to paraquat than N2 (Fig. 2B). *ercc-1* mutants also showed a significant increase in survival following exposure to rotenone, a mitochondrial complex I inhibitor (Fig. 2C), antimycin A, a mitochondrial complex III inhibitor (Fig. 2D), as well as in response to heat stress (Fig. 2E). Importantly, *daf-16* RNAi completely abrogated the rotenone resistance of *ercc-1* worms (Fig. 2F). Surprisingly, although DAF-16 confers stress resistance in *ercc-1* worms, it did not significantly impact lifespan of the animals, since knock-down of *daf-16* dramatically shortened lifespan of N2 worms, but did not further shorten *ercc-1* lifespan (Fig. 2G).

2.2. Loss of DAF-16 activation is associated with organismal decline

Despite DAF-16 activation in early adulthood, *ercc-1* mutants are not long-lived. This is surprising because typically DAF-16 activation and an elevated stress response correlate with increased longevity [42,43]. We therefore carried out a temporal analysis of DAF-16 activation in N2 worms and *ercc-1* mutants. In N2, DAF-16::GFP was predominantly cytoplasmic until D5, after which nuclear localization became evident (Fig. 3A), consistent with previous reports [20,44]. In contrast, in *ercc-1* worms, nuclear localization of DAF-16::GFP was evident at D1 and D3 of adulthood, but by D5, DAF-16::GFP was primarily in the cytoplasm (Fig. 3A & S3). The antioxidant GST-4 is a downstream target of the transcription factor, *daf-16*. A GFP reporter for *gst-4* [41,45] was robustly induced in *ercc-1* mutant animals at D3 compared to N2 worms, but was significantly reduced by D5, and further suppressed by D7 and D9 (Fig. 3B). A similar pattern of DAF-16 nuclear localization was observed in the *xpf-1* mutant background (Fig. S4). Thus, DAF-16 and *daf-16*-modulated antioxidant program is only

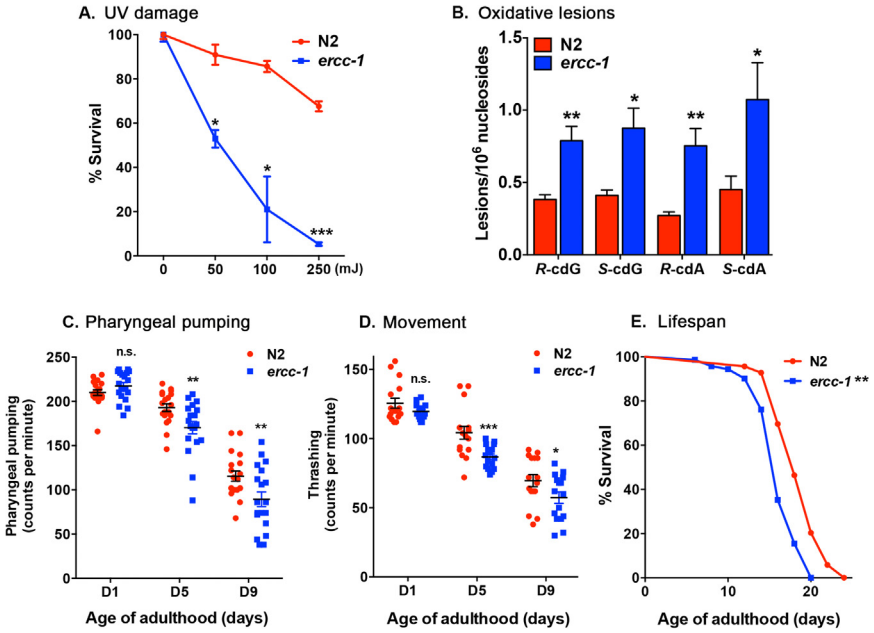

Fig. 1. Characterization of DNA repair deficient *ercc-1* worms. (A) Quantification of eggs laid by N2 and *ercc-1* adult worms after UV irradiation at various doses relative to mock treatment (0 mJ UV, set at 100% survival). Mean ± S.D. of n = 50–60 worms/genotype; 4 biological replicates. Student's two-tailed *t*-test *p < 0.05, * **p < 0.001. (B) Levels of endogenous oxidative DNA lesions (*R*-cdG, *S*-cdG, *R*-cdA, and *S*-cdA cyclopurines) in N2 and *ercc-1* worms at day 9 of adulthood (mean ± S.D. from DNA isolated from 1500 to 2000 worms; n = 3 independent populations per genotype). Student's two-tailed *t*-test *p < 0.05 and * *p < 0.01. (C) Number of pharyngeal contractions and (D) thrashing in liquid per minute for D1, 5 and 9 adult N2 and *ercc-1* worms. Mean ± S.D, n = 15–20 worms/group; 3 replicas performed. Student's two-tailed *t*-test *p < 0.05, * *p < 0.01, * **p < 0.001, n.s. not significant. (E) The lifespan of N2 and *ercc-1* mutant worms. Kaplan-Meier survival curves were calculated from populations of 40–70 animals/genotype. Log-rank (Mantel-Cox) test * *p < 0.05.

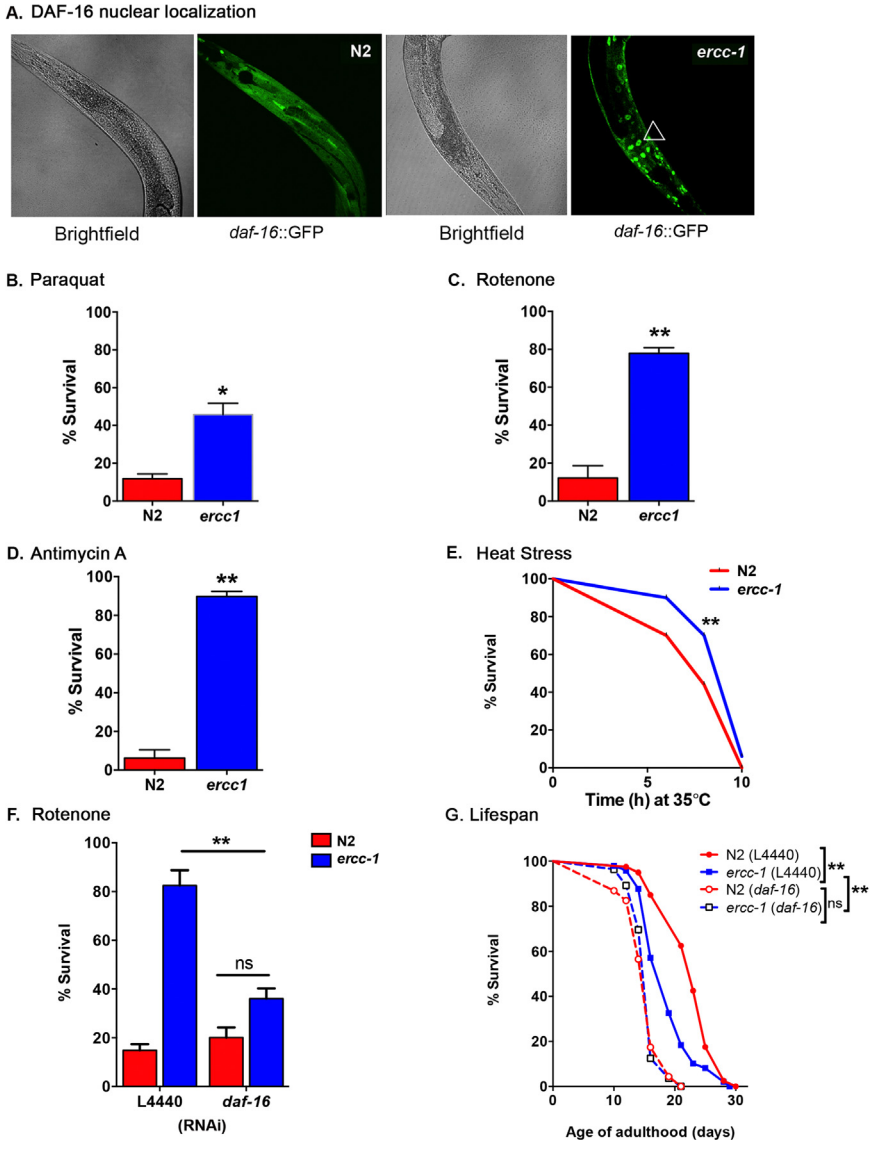

Fig. 2. DAF-16 activation in *ercc-1* worms leads to multi-stress resistance. Representative images of D1 adult N2 and *ercc-1* worms expressing the *daf-16*::*GFP* reporter. (B) Viability of day 1 adult N2 and *ercc-1* worms 12 h after treatment with 100 mM paraquat (mean ± S.E.M., n = 8 groups of 40–60 worms/genotype). Student's two-tailed *t*-test *p < 0.05. (C) Viability of day 1 adult worms 16 h after treatment with 12.5 μM rotenone (mean ± S.E.M., n = 6 groups of 40–60 worms/genotype). Student's two-tailed *t*-test * *p < 0.01. (D) Viability of day 1 adult N2 and *ercc-1* worms 12 h after treatment with 25 μM antimycin A (mean ± S.E.M., n = 6 groups of 40–60 worms/genotype). Student's two-tailed *t*-test * *p < 0.01. (E) Survival of day 1 adult N2 and *ercc-1* under acute thermal stress at 35 °C; 3 replicas. Kaplan-Meier survival curves were calculated. Log-rank (Mantel-Cox) test * *p < 0.01. (F) Stress resistance of day 1 adult N2 and *ercc-1* worms fed RNAi against control (L4440) or *daf-16*, 16 h after treatment with 12.5 μM rotenone (mean viability ± S.E.M., n = 4 groups of 40–60 worms/genotype). Student's two-tailed *t*-test * *p < 0.01; ns not significant. (G) Lifespan of N2 and *ercc-1* worms after knockdown of *daf-16* with RNAi. Kaplan-Meier survival curves were calculated from populations of 40–70 animals/genotype. Log-rank (Mantel-Cox) test * *p < 0.01; ns not significant.

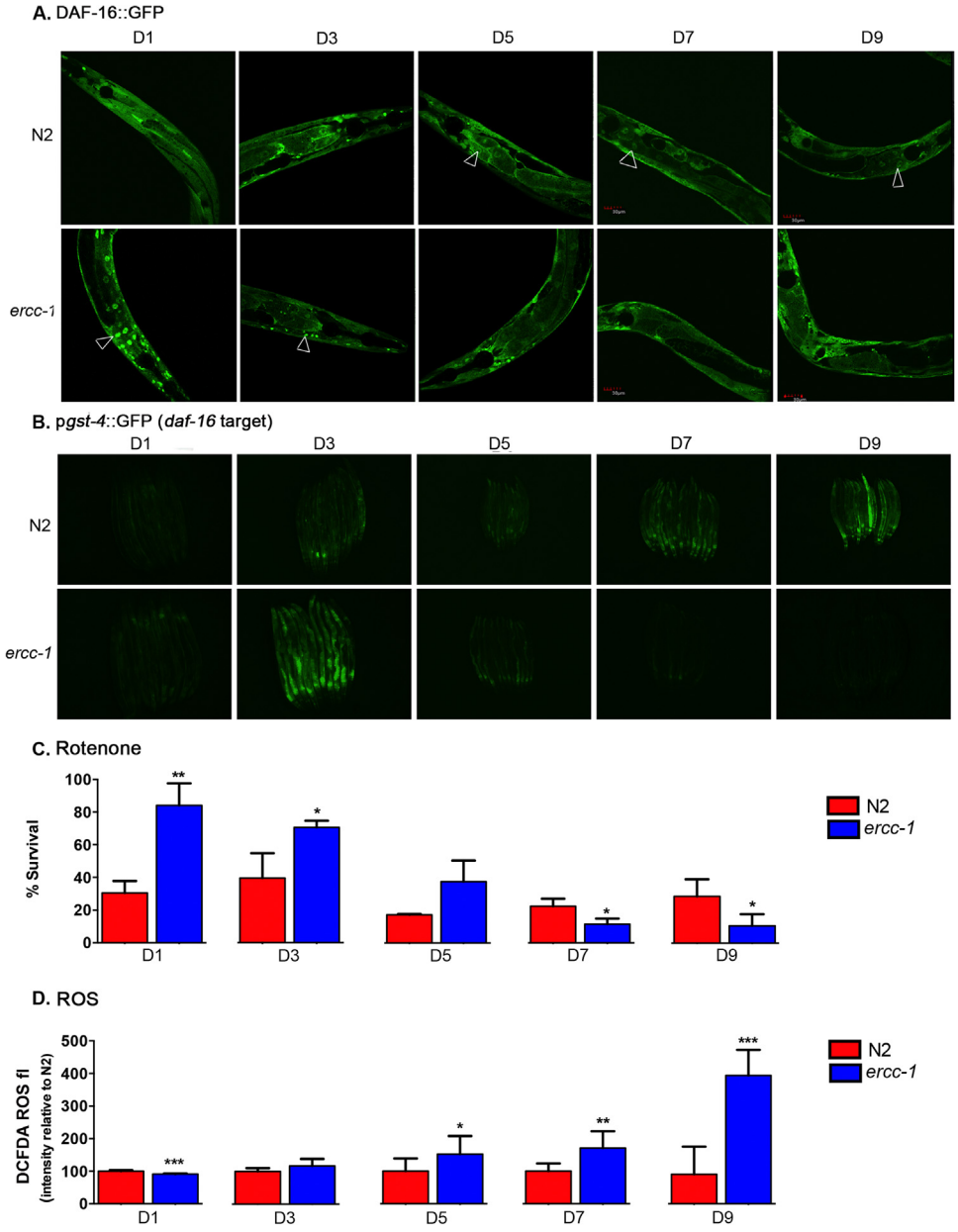

Fig. 3. Loss of DAF-16-dependent stress resistance in DNA repair deficient *ercc-1* worms. (A) Representative images of expression of the *daf-16::GFP* reporter in N2 and *ercc-1* worms at D1, 3, 5, 7 and 9 of adulthood. Arrows indicate nuclear localization of DAF-16::GFP. (B) Representative images of the expression of *gst-4::GFP*, a reporter of DAF-16 activity, in N2 and *ercc-1* worms with age. (C) Viability of N2 and *ercc-1* worms 16 h after treatment with 12.5 μM rotenone measured at multiple ages (mean ± S.E.M., n = 3 groups of 40–60 worms/genotype). Student's two-tailed *t*-test *p < 0.05, **p < 0.01. (D) Nonspecific oxidant levels measured by staining with H_2-DCFDA in N2 and *ercc-1* worms at multiple ages (mean ± S.D. of n = 3 groups of 8–15 worms/genotype). Student's one-tailed *t*-test *p < 0.05, **p < 0.01, ***p < 0.001.

transiently activated in response to endogenous DNA damage. Interestingly, while UV irradiation of D9 *ercc-1* mutants did not restore DAF-16::GFP nuclear localization (Fig S5A), a brief period of starvation did (Fig S5B), demonstrating that older adult *ercc-1* worms are able to activate DAF-16 in response to other forms of stress, but no longer in response to genotoxic stress.

Since DAF-16 activation declines with age, we predicted that this should coincide with an attrition of stress resistance in *ercc-1* worms. We therefore systematically analyzed stress response with age, in N2 and *ercc-1* worms exposed to rotenone, a mitochondrial complex I inhibitor that induces oxidative stress, in D 1, 3, 5, 7 and 9 adults at a dose that leads to ~30–40% survival of N2. In this paradigm, we found *ercc-1* mutants were significantly more resistant to rotenone than N2 as D1 and 3 adults (Fig. 3C). However, by D5, this resistance was lost, and with increasing age, the *ercc-1* worms became hypersensitive to rotenone compared to N2. These results were confirmed in *ercc-1* mutant worms derived from a balancer strain, as well as in *xpf-1* mutants (Fig S6).

The loss of DAF-16 nuclear localization and reduced expression of *gst*-4 in the *ercc-1* worms was correlated with an increase in endogenous oxidant abundance as assessed by the nonspecific and potentially metal-catalyzed, one-electron oxidation of DCFH to DCF (Fig. 3D). *ercc-1* mutants had lower oxidant levels as D1 adults than N2 worms, but by D5 of adulthood there was significantly more oxidants in the *ercc-1* worms and this became exaggerated with increasing age of the worms (Fig. 3D). By D9, when *ercc-1* mutants were stress sensitive, there was an increase in oxidants (~3.5 fold higher) (Fig. 3D) compared to wild-type worms. Taken together, these data invoke a scenario in which endogenous DNA damage initially triggers activation of a well-described stress response/longevity assurance mechanism, but that activation declines over time, at which point increased oxidant levels, damage, and functional decline ensue.

2.3. *cep-1* mediates the effect of DNA damage on DAF-16 dynamics

To begin to understand the mechanism by which DNA damage leads to activation of DAF-16, we performed a targeted RNAi screen for suppressors of stress resistance in *ercc-1* mutant worms. We selected 50

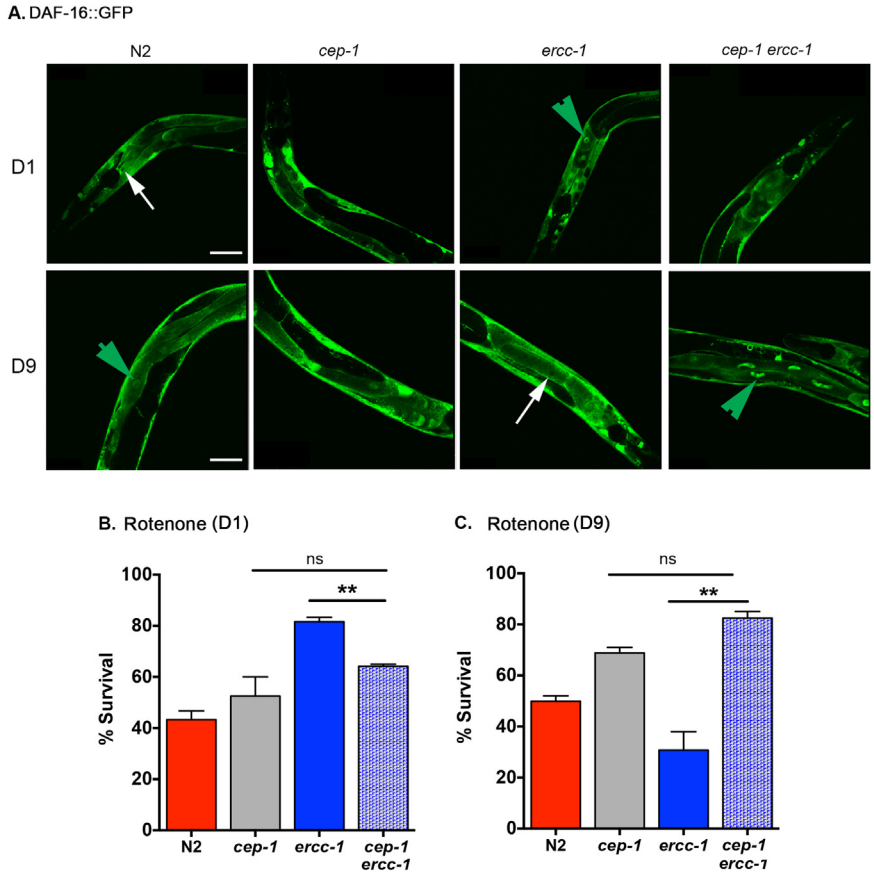

Fig. 4. Temporal dynamics of DAF-16 is *cep-1* dependent. (A) Representative images of DAF-16::GFP in N2, *cep-1*, *ercc-1* and *cep-1 ercc-1* worms at D1 and D9 of adulthood. Green arrows indicate nuclear localization of DAF-16::GFP and white arrows indicate lack thereof. (B-C) Percent survival of D1 or D9 adult N2, *cep-1*, *ercc-1* and *cep-1 ercc-1* worms 16 h after treatment with rotenone (mean ± S.E.M., n = 4 groups of 40–60 worms/genotype; 3 replicas). Student's two-tailed *t*-test * *p < 0.01; ns not significant.

candidate genes on the basis of their involvement in processes such as DNA repair signaling, mitochondrial function, metabolism, autophagy and signal transduction. From this screen, we observed that knockdown of *cep-1* led to a significant suppression of paraquat resistance in *ercc-1* mutants (Fig S7A). *cep-1* is the ortholog of the tumor suppressor gene, p53 [46] which is known to be activated in response to DNA damage and activates FOXO3A in response to genotoxic stress [47]. We therefore generated a *cep-1 ercc-1* double mutant carrying the *daf-16::GFP* transgene to examine the effect of *cep-1* deletion on DAF-16 activation.

Unlike *ercc-1* worms, the *cep-1 ercc-1* D1 adults did not have nuclear localization of DAF-16::GFP, but there was strong nuclear localization of DAF-16::GFP in the double mutants at D9, effectively reverting the *ercc-1* worms to a WT phenotype in terms of *daf-16* activation (Fig. 4A & S7B & C). Consistent with these changes in DAF-16 localization, *cep-1* deletion in an *ercc-1* background suppressed stress resistance in D1 animals (Fig. 4B & S7D & E). But at D9, *cep-1 ercc-1* double mutants were stress resistant compared to the *ercc-1* single mutant (Fig. 4C). These results confirm a role for *cep-1* in regulating the stress response elicited by endogenous DNA damage. Surprisingly, this also included deactivating DAF-16 in older organisms.

2.4. Nuclear DNA damage prematurely activates FOXO3A in mammalian cells

We next turned to mammalian systems to determine if this response is conserved by examining the dynamics of FOXO3A, the mammalian homolog of DAF-16 [48]. We found early passage 3 (P3) *Ercc1*$^{-/-}$ mouse embryonic fibroblasts (MEFs) were resistant to paraquat and rotenone compared to WT cells (Fig. 5A-C) and, like worms, there was increased nuclear localization of FOXO3A. However, by P5, FOXO3A was no longer observed in the nucleus of cells (Fig. 5D and S8), while increased H_2-DCFDA fluorescence was observed (Fig. 5E). Specifically, superoxide ($O_2^{\bullet-}$) levels were increased (Fig. 5F). These data demonstrate that in primary mammalian cells, like in nematodes, unrepaired spontaneous DNA damage activates FOXO3A stress response programs, but that this activation is not sustained over time, leading to a rise in oxidative stress.

2.5. Nuclear DNA damage regulates timing of FOXO3A activation in mammals

To determine whether the FOXO3A response to endogenous DNA damage occurred *in vivo*, we next examined liver tissue from DNA repair deficient *Ercc1*$^{-/\Delta}$ mice. FOXO3A was increased in the nuclear fraction of livers of young adult [8 weeks - median lifespan 20 weeks [27]] and reduced in older *Ercc1*$^{-/\Delta}$ mice [16 weeks] compared to same-age WT controls (Fig. 6A & B). Additionally, analysis of the transcriptome from the liver of 16-week-old *Ercc1*$^{-/\Delta}$ mice revealed reduced expression of numerous FOXO3A transcriptional targets compared to WT mice (Fig S9 A). This was validated by qRT-PCR in liver from young adult (8 weeks) but old (16 weeks) *Ercc1*$^{-/\Delta}$ animals. Expression of FOXO3A targets *catalase*, *MsrA*, *Sod2* and *Txrnd2* were increased in *Ercc1*$^{-/\Delta}$ mice at 8 weeks compared to WT, but significantly reduced in older mutant animals (Fig. 6C & D). The antioxidant methionine sulfoxide reductase A (MSRA) is a direct target of FOXO3A, while MSRB is not [49]. Correspondingly, MSRA, but not MSRB, activity was significantly reduced in livers of 16-week-old *Ercc1*$^{-/\Delta}$ mice compared to WT animals (Fig S9 B-C). Collectively, these data demonstrate that the activation of FOXO3A in response to endogenous DNA damage is conserved in mammals, as is the inability to sustain its activation.

Since WT mice accumulate oxidative DNA lesions as they age [25], we hypothesized that FOXO3A activation and subsequent inactivation should be evident in normal mice with aging, albeit with a less compressed time course than ERCC1-deficient mice. Indeed, compared to young adult animals (16 weeks), expression of FOXO3A targets was

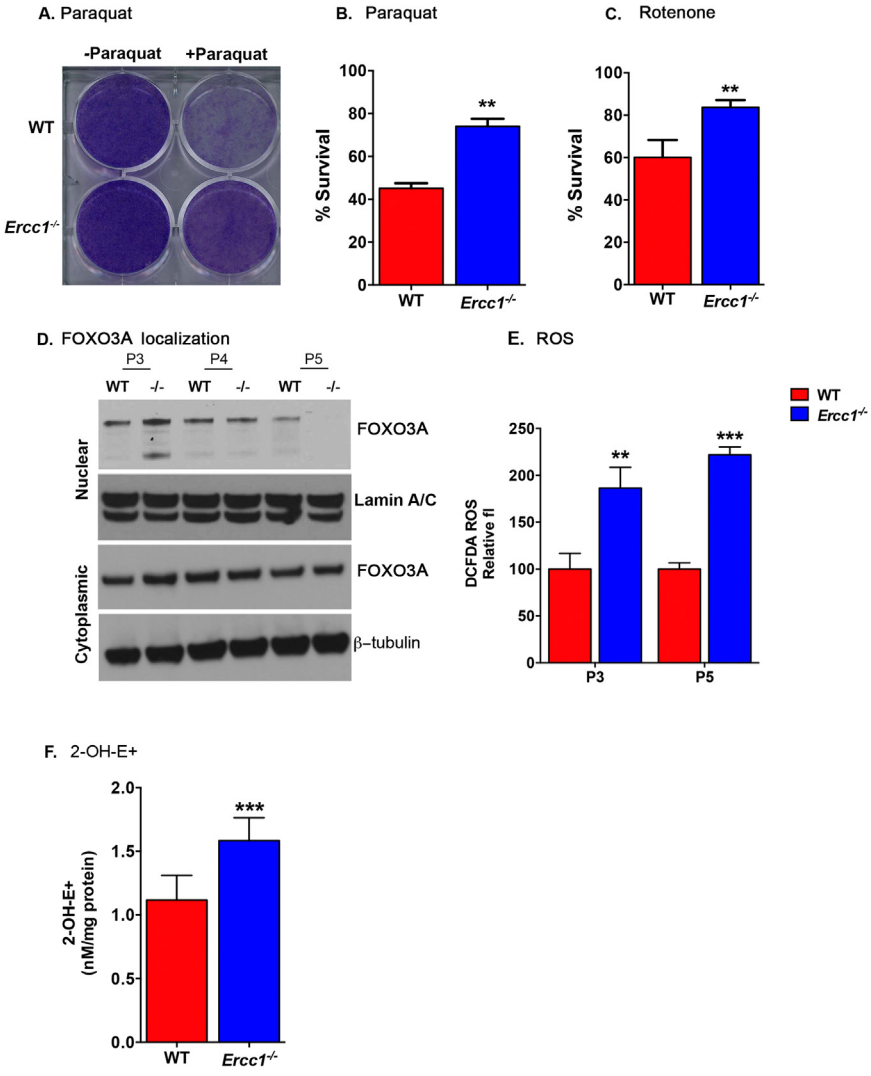

Fig. 5. Activation of FOXO3A in *Ercc1*$^{-/-}$ mammalian cells mimics worms. (A) Representative image of crystal violet staining of cultures of passage 3 WT and *Ercc1*$^{-/-}$ primary MEFs 24 h after treatment with 0.5 mM paraquat. (B) Quantification of cell viability 24 h after treatment of passage 4 WT and *Ercc1*$^{-/-}$ MEFs with 0.5 mM paraquat (mean percent survival compared to untreated cells ± S.E.M.; n = 5 independent cultures/genotype). Student's two-tailed *t*-test * *p < 0.01. (C) Quantification of cell viability 24 h after treatment of passage 3 WT and *Ercc1*$^{-/-}$ MEFs with 1 μM rotenone (mean percent survival compared to untreated cells ± S.E.M. n = 5 replicas). Student's two-tailed *t*-test, * *p < 0.01. (D) Immunodetection of FOXO3A in nuclear (top) or cytosolic fractions (bottom) of primary MEFs at increasing passage number. -/- indicates *Ercc1*$^{-/-}$ cells. Lamin A/C is used as a loading control for nuclear proteins; β-tubulin is used for cytosolic. (E) Nonspecific oxidant levels in WT and *Ercc1*$^{-/-}$ MEFs measured with 2',7'-dichlorofluorescein (H_2-DCFDA) dye and flow cytometry at early (P3) and late (P5) passage of cells (n = 5 independent cell lines; data are represented as mean ± S.D.). Two-tailed Student's *t*-test * *p < 0.01, * **p < 0.05. (F) Detection of 2-hydroxyethidium (2-OH-E$^+$) in WT and *Ercc1*$^{-/-}$ primary MEFs at passage 5 (mean ± S.D. of n = 8 independent cell pellets/genotype). Student's two tailed *t*-test ***p < 0.001.

significantly higher in middle-aged WT mice (50 weeks) (Fig. 6E) but significantly reduced in liver of old WT mice (2 years-old; Fig. 6F). These data demonstrate that an age-related decline in FOXO3A activation is part of the normal aging process in mammals.

Finally, as we observed that deletion of p53 homolog, *cep-1,* rescued loss of DAF-16 nuclear localization in older *ercc-1* worms (Fig. 4), we hypothesized that suppression of *p53* in *Ercc1*$^{-/\Delta}$ mice would improve healthspan and lifespan. *Ercc1*$^{-/\Delta}$*p53*$^{-/-}$ mice were born at sub-Mendelian frequency. However, *p53* heterozygosity delayed the onset of several aging symptoms in *Ercc1*$^{-/\Delta}$ mice (Fig. 7A). Moreover, the median lifespan of *Ercc1*$^{-/\Delta}$*p53*$^{+/-}$ mice was significantly increased compared to *Ercc1*$^{-/\Delta}$ animals (Fig. 7B) indicative of increased healthspan [10]. Taken together, these results demonstrate that genetic reduction of *p53* delays aging in DNA repair deficient mice, consistent with its role in attenuating stress resistance late in life.

3. Discussion

DNA damage has long been implicated as a driver of aging, although the mechanism(s) are poorly defined. In part, this is because deciphering exactly how endogenous DNA damage affects post-mitotic cells and contributes to organismal aging is challenging in mammalian systems. Here, we set out to elucidate this mechanism using nematodes and then confirm is evolutionary conservation using mice in which ERCC1-XPF endonuclease, an enzyme required for nucleotide excision, interstrand crosslink and double-strand break repair, is genetically depleted. In these DNA repair-deficient organisms, the type and amount of DNA damage that is incurred is unchanged compared with wild type animals. However, since DNA repair is compromised, there is an accelerated accumulation of the same DNA lesions that accumulate over the lifespan of wild-type, repair proficient organisms [25]. This enables study of the mechanisms that are triggered in response to physiologically relevant types and levels of genotoxic stress.

Most mammalian DNA repair genes are maintained in *C. elegans,* although lifespan data in some of these mutants has yielded contradictory results. For example, another NER mutant, *xpa-1* has been reported to have a shortened lifespan while others have observed no differences [17,19,23] Previous studies had demonstrated that UV-induced DNA damage leads to activation of DAF-16/FOXO3A [20]. Our results build on this observation to demonstrate that endogenous DNA damage also induces DAF-16/FOXO3A activation, and a corresponding increase in stress resistance, not only in adult worms, but also in murine cells and tissues. The activation of DAF-16/FOXO3A in ERCC1-deficient cells and tissues, suggests a coping mechanism in response to endogenous damage to the nuclear genome. We also found that the FOXO3A stress response pathway is activated in DNA repair-proficient mice, albeit at a later age when damage has accumulated [25].

The activation of DAF-16/FOXO3A is typically associated with longevity. Indeed, FOXO3A variants are linked to longevity in humans, but the functional implications of the sequence variants are not known [50–53]. Our results add to existing evidence that DAF-16/FOXO3A activity is critical for suppressing oxidative stress [54]. Unexpectedly,

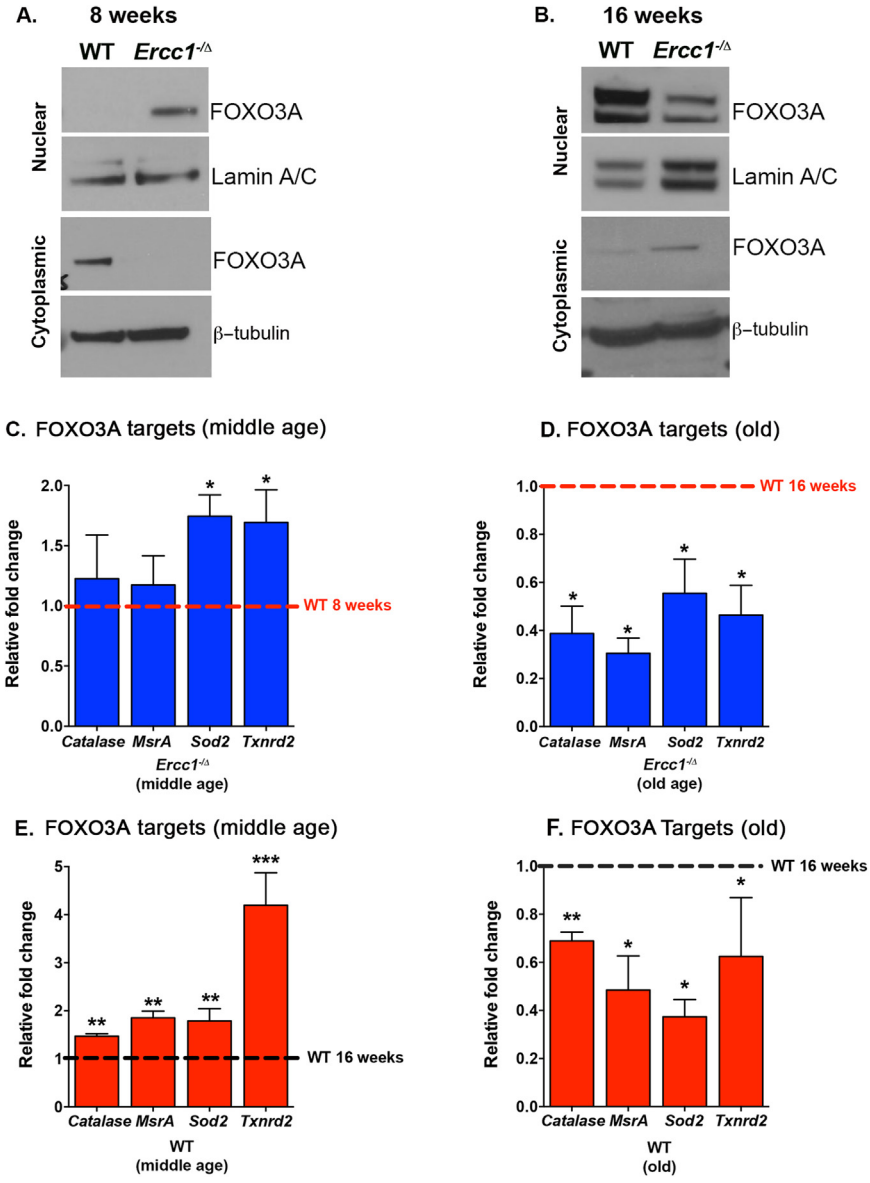

Fig. 6. Activation of FOXO3A is accelerated in DNA repair deficient *Ercc1*$^{-/\Delta}$ mice. Immunoblot detection of FOXO3A in nuclear (top) and cytosolic (bottom) extracts from liver lysates isolated from 8 (A) and (B) 16-week-old mice WT or ERCC1-deficient mice. (C-D) Expression of FOXO3A-regulated genes measured by qRT-PCR in livers of WT and *Ercc1*$^{-/\Delta}$ mice at 8 weeks (middle-age) and 16 weeks of age (old). WT at the same age is set as 1; red dashed line. n = 6–8 per age; Student's one-tailed *t*-test, *p < 0.05. (E-F) Expression of FOXO3A-regulated genes measured by qRT-PCR in liver of WT mice at 50 weeks (middle-age, E) and 110–120 weeks of age (old, F). WT at 16 weeks of age is set at 1; black dashed line. n = 4–6 per age; Student's one-tailed *t*-test, *p < 0.05, **p < 0.01, ***p < 0.001.

we found that oxidant abundance becomes elevated in response to genotoxic stress creating a vicious cycle of escalating damage [55]. Here, we establish that loss of FOXO3A activation likely plays a role in triggering this vicious cycle of increased oxidative stress and damage.

Suppression of FOXO3A activity promotes senescence *in vitro* [56] and *in vivo* [57], and leads to changes in cell morphology, reduced proliferation, an increase in senescence associated-β-galactosidase staining and oxidant abundance. *Ercc1*$^{-/-}$ MEFs senesce prematurely [58], while *Ercc1*$^{-/\Delta}$ mice accumulate senescent cells in multiple tissues [59]. We have previously demontrated that the increase in senescence in *Ercc1*$^{-/\Delta}$ mice is due to elevated oxidative stress. However the mechanism that leads to the surge in oxidants upon endogenous DNA damage was unknown. Similarly, suppression of *msra-1*, a key downstream target of DAF-16/FOXO3A, leads to sensitivity to oxidative stress and ~30% decrease in median lifespan in *C. elegans* [60], whereas overexpression of MsrA in cells lowers oxidant levels [61] and

A.

		p53$^{+/-}$ *Ercc1*$^{-/\Delta}$		*Ercc1*$^{-/\Delta}$	
	Aging symptoms	Age (weeks)	n	Age (weeks)	n
1	Dystonia	10.5	11	8.9	13
2	Trembling	9.6	9	11.7	13
3	Kyphosis	15 *	11	12.6	13
4	Ataxia	15.8	11	15.4	11
5	Rt Eye	16.9	6	14	4
6	Lt Eye	17.6	5	15.8	5
7	Incontinence	20.1 *	4	14.4	5

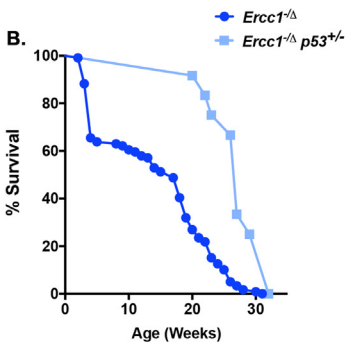

Fig. 7. Genetic depletion of p53 in DNA repair deficient *Ercc1*$^{-/\Delta}$ mice improves healthspan and lifespan. (A) Average age (weeks) of onset for several aging symptoms in *Ercc1*$^{-/\Delta}$ and *Ercc1*$^{-/\Delta}$ *p53*$^{+/-}$ mice. n = 11 *Ercc1*$^{-/\Delta}$ *p53*$^{+/-}$ mice and n = 13 *Ercc1*$^{-/\Delta}$ mice. (B) Kaplan-Meier survival curves of *Ercc1*$^{-/\Delta}$ (n = 119) and *Ercc1*$^{-/\Delta}$ *p53*$^{+/-}$ (n = 11) mice were calculated. Log-rank (Mantel-Cox) test ***p < 0.001.

increases lifespan in *D. melanogaster* and yeast [62,63]. Taken together, our observations suggest that it is the temporal response of the DAF-16/FOXO pathway to unrepaired DNA damage that contributes to deleterious effects on healthspan and lifespan.

What remains unclear is why DAF-16/FOXO3A activity wanes in the face of chronic genotoxic stress. Chronic nuclear localization of DAF-16 is observed in several long-lived mutants [64], indicating that sustained activation of this pathway is not only possible, but is also beneficial. Although the regulation of FOXO3A is not fully elucidated [65,66], *cep-1*/p53, ATM, IGF-1, sirtuins and IKK all contribute to it's regulation [47,67,68], all of which are dysregulated in *Ercc1* mutants [26,69]. We found that in younger worms, *cep-1* promotes DAF-16 activation to invoke a protective stress response. However, upon chronic genotoxic stress in older animals, there is a loss of DAF-16/FOXO3A nuclear retention that is *cep-1* dependent. Under conditions of oxidative stress, p53 is modified and inhibits FOXO3A activity [70,71]. Although, the role of *cep*-1/p53 in healthspan and lifespan is complex [72–74], our results indicate that its suppression attenuates age-related decline. Thus, therapeutic strategies aimed at maintaining DAF-16/FOXO3A activity could provide a means of ameliorating the effects of DNA damage-induced accelerated aging, such as occurs in cancer patients [5]. In this respect, it is noteworthy that starvation of aged *ercc-1* worms leads to increased DAF-16::GFP nuclear localization (Fig S7B). This is in accordance with a recent report demonstrating that caloric restriction extends the healthspan and lifespan of *Ercc1*$^{-/\Delta}$ mice [75]. It will therefore be important to determine whether such interventions are effective in other systems.

4. Experimental procedures

*Detailed experimental procedures in supplemental data

4.1. C. elegans strains

Wild-type (N2), TG1663 [*ercc-1(tm1981)*], TG1660 [*xpf-1(tm2842)*], TJ1 [*cep-1(gk138)*], TJ356 [zIs356(*daf-16p*::*daf-16a/b*::GFP + rol-6), CL2166 [dvIs19((pAF15)*gst-4*p::GFP::NLS)], XMN617 [hT2/+ [bli-4(e937) let-?(q782) qIs48] (I;III); muIs32 (P*mec-7*::GFP)].

C. elegans strains were cultured at 20 °C on Nematode Growth Media (NGM) agar plates seeded with *Escherichia coli* strain OP50 unless stated otherwise [76]. Strains were provided by the *Caenorhabditis* Genetics Center (University of Minnesota).

4.2. Cells and mice

Primary mouse embryonic fibroblasts (MEFs) were isolated on embryonic day 12.5–13.5. In brief, mouse embryos were isolated from yolk sac followed by removal of viscera, lung and heart. Embryos were then minced into fine chunks, covered with media, cultured at 3% oxygen to reduce stresses and serially passaged. MEFs were grown in 1:1 of Dulbecco's Modification of Eagles Medium (with 4.5 g/L glucose and L-glutamine) and Ham's F10 medium, supplemented with 10% fetal bovine serum, penicillin and streptomycin and non-essential amino acids. To induce oxidative stress and oxidative DNA damage, MEFs were switched to 20% oxygen at passage 3 *Ercc1*$^{+/-}$ and *Ercc1*$^{+/\Delta}$ mice from C57BL/6 J and FVB/NJ backgrounds were crossed to generate *Ercc1*$^{-/\Delta}$ F1 hybrid mice. *p53*$^{+/-}$ mice were crossed to *Ercc1*$^{+/-}$ from C57BL/6 J background to generate *Ercc1*$^{+/-}$*p53*$^{+/-}$ mice, which were then bred with *Ercc1*$^{+/\Delta}$ mice from FVB/NJ background to generate F1 *Ercc1*$^{-/\Delta}$ *p53*$^{+/-}$ mice. Breeders were backcrossed for ten generations yielding F1 mice that are genetically identical. All animal studies were conducted in compliance with the US Department of Health and Human Services Guide for the Care and Use of Laboratory Animals and were approved by The Scripps Research Institute (TSRI) and University of Pittsburgh Institutional Animal Care and Use Committee.

4.3. Confocal microscopy and images processing

Worms were immobilized with 6 mM tetramisole hydrochloride (Sigma) in M9 and mounted on 6% agarose pads on glass slides. Images were acquired using Zeiss LSM 700 Upright confocal microscope (Carl Zeiss AG).

4.4. Statistics

Survival analyses were performed using the Kaplan-Meier method and the significance of differences between survival curves calculated using the log rank test. Other analyses are listed in figure legends.

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Author contributions

A.U.G. and L.J.N. conceived the study. *C. elegans* studies were designed and performed with the help of M.S.G. A.U.G. designed and performed most of the experiments with the help of A.W. and M.M. Y.C. and Y.W. measured cyclopurine adducts on DNA. A.U.G., C.M.S. performed IF experiments. C.M.S. performed quantitative image analysis. MsrA and MsrB studies contributed by S.K.A. and H.W. A.U.G., L.J.N., and M.S.G. wrote the manuscript with contributions from other authors and H.W. edited the final manuscript. L.J.N. and M.S.G. supervised the research.

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.redox.2018.06.005.

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