NF-κB inhibition delays DNA damage–induced senescence and aging in mice

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The accumulation of cellular damage, including DNA damage, is thought to contribute to aging-related degenerative changes, but how damage drives aging is unknown. XFE progeroid syndrome is a disease of accelerated aging caused by a defect in DNA repair. NF-κB, a transcription factor activated by cellular damage and stress, has increased activity with aging and aging-related chronic diseases. To determine whether NF-κB drives aging in response to the accumulation of spontaneous, endogenous DNA damage, we measured the activation of NF-κB in WT and progeroid model mice. As both WT and progeroid mice aged, NF-κB was activated stochastically in a variety of cell types. Genetic depletion of one allele of the p65 subunit of NF-κB or treatment with a pharmacological inhibitor of the NF-κB–activating kinase, IKK, delayed the age-related symptoms and pathologies of progeroid mice. Additionally, inhibition of NF-κB reduced oxidative DNA damage and stress and delayed cellular senescence. These results indicate that the mechanism by which DNA damage drives aging is due in part to NF-κB activation. IKK/NF-κB inhibitors are sufficient to attenuate this damage and could provide clinical benefit for degenerative changes associated with accelerated aging disorders and normal aging.

Introduction

Aging is characterized by the inability of tissues to maintain homeostasis (1, 2). This leads to an impaired response to stress and, as a consequence, an increased risk of morbidity and mortality (2). The incidence of numerous debilitating chronic diseases, such as cardiovascular disease, neurodegeneration, diabetes, arthritis, and osteoporosis, increases almost exponentially with age (3). Aging is thought to be driven, at least in part, by the accumulation of stochastic damage in cells. This includes damage to proteins (2), DNA, mitochondria (4), and telomeres (5), which is driven by ROS (6–8) generated through chronic inflammation (9, 10) or aerobic respiration in mitochondria (4). However, the mechanism by which cellular damage drives aging is not known. The simplest model is that damage causes attrition of functional cells. But this is inadequate in light of emerging evidence that aging-related degenerative changes in old and damaged organisms can be delayed or reversed by circulating factors (11–15). These observations point instead toward the cellular response to damage being the key driver of aging.

The transcription factor NF-κB is a central component of the cellular response to damage, stress, and inflammation (16). In mammals, the NF-κB family consists of 5 subunits, RelA or p65, c-Rel, RelB, p50, and p52. NF-κB binds to DNA as a dimer, the most common being the p65p50 heterodimer (17). The p65p50 heterodimer is localized primarily in the cytoplasm, maintained in this inactive state via sequestration by IκB proteins (17). NF-κB activation via the canonical pathway is mediated by the upstream IκB kinase (IKK), a heterotrimer consisting of 2 catalytic subunits, IκKα and IκKβ, and a regulatory subunit termed IκKγ or NF-κB essential modulator (NEMO) (17). In response to a variety of factors, including proinflammatory cytokines, pathogens, oxidative stress, and growth factors, IKK is activated and phosphorylates IκB, leading to its polyubiquitination and subsequent proteasomal degradation (16, 18–20). IκB degradation allows NF-κB to translocate to the nucleus, in which it binds to its cognate DNA sequence as well as coactivators, such as CBP/p300, to regulate gene expression (21).

Numerous studies report increased NF-κB activity with aging. Human fibroblasts from aged individuals and patients with Hutchinson-Gilford progeria syndrome have increased NF-κB activation (22, 23). NF-κB DNA binding is increased in skin, liver, kidney, cerebellum, cardiac muscle, and gastric mucosa of old rodents compared with that in young rodents (24–28). In addition, NF-κB was identified as the transcription factor most associated with mammalian aging, based on patterns of gene expression (23). Furthermore, chronic activation of NF-κB is observed in numerous age-related diseases (29), including muscle atrophy (30, 31), multiple sclerosis (32), atherosclerosis (33), heart disease (34), both type 1 and 2 diabetes (35), osteoarthritis (36), dementia (37), osteoporosis (38), and cancer (39). However, these studies do not demonstrate a causal relationship between NF-κB activation and aging.
NF-κB activation is increased in tissues of old WT and progeroid, DNA repair–deficient mice. Kidney sections from NF-κBEGFP mice were imaged using fluorescent microscopy to detect EGFP expression (green). Nuclei were counterstained with Hoechst dye (blue; original magnification, ×20). (A) Young adult (3-month-old) and old WT NF-κBEGFP (2-year-old) mice. (B) Ercc1Δ/ΔNF-κBEGFP and WT NF-κBEGFP mice at 3 months of age. (C) Ercc1Δ/ΔNF-κBEGFP and WT NF-κBEGFP mice at 21 days of age. (D) Quantification of EGFP expression. The number of EGFP+ cells was counted in 5 random fields of tissue per mouse (n = 6 mice per group). The fold difference in the number of EGFP+ cells relative to the mean value (black bar of the group is reported. Diamond symbols represent individual mice (controls in green and Ercc1Δ/ΔNF-κBEGFP mice in yellow). P values were calculated using a Student’s t test. (E) Ercc1Δ/Δ and WT primary MEFs were passaged 5 times at 20% O2 to promote the onset of senescence (58). The levels of p-p65, IκBα, and p-IκBα in nuclear and cytoplasmic extracts were measured by immunoblot. (F) NF-κB EMSA was performed with a radiolabeled oligonucleotide containing an NF-κB binding site using nuclear extracts from Ercc1Δ/Δ and WT primary MEFs.

Genetic depletion of NF-κB in the skin of transgenic mice reversed age-related gene expression and histologic changes (23), providing support for NF-κB activation playing a causal role in skin aging. Similarly, haploinsufficiency of p65 leads to improved growth and extended life span of Sirt6−/− mice (40). However, Sirt6−/− mice manifest severe colitis, suggesting that chronic inflammation may drive their degenerative phenotypes and that attenuating NF-κB activation delays the onset of senescence (58). The levels of p-p65, IκBα, and p-IκBα in nuclear and cytoplasmic extracts were measured by immunoblot. (F) NF-κB EMSA was performed with a radiolabeled oligonucleotide containing an NF-κB binding site using nuclear extracts from Ercc1Δ/Δ and WT primary MEFs.

To address these gaps in knowledge, we used a mouse model of XFE progeroid syndrome, a disease of accelerated aging caused by mutations in XPF, which encodes the catalytic subunit of the xeroderma pigmentosum group F–excision repair cross-complementing rodent repair deficiency complementation group 1 (XPF-ERCC1) DNA repair endonuclease. The syndrome is characterized by accelerated aging of virtually all organ systems, all driven by failure to repair stochastic endogenous DNA damage. A murine model of XFE progeroid syndrome, Ercc1Δ/Δ mice, have about 10% of the normal amount of ERCC1 protein and spontaneously develop progressive, degenerative changes that correlate strongly with natural aging (43–47). Thus, Ercc1Δ/Δ mice, which have a complete absence of ERCC1 protein, and Ercc1Δ/Δ mice offer a unique opportunity to investigate the mechanism by which one type of cellular damage promotes aging and whether NF-κB plays a pivotal role.

We found that NF-κB is stochastically activated in a variety of cell types with normal and accelerated aging and that genetic or pharmacologic inhibition of NF-κB activation delays the onset of numerous aging-related symptoms and pathologies. Inhibition of IKK/NF-κB activity reduced cellular senescence and oxidative damage, including DNA and protein damage, revealing that cellular stress responses promote further cellular damage. Our findings strongly suggest that inhibitors of the IKK/NF-κB pathway may delay damage and extend healthspan in patients with accelerated aging and chronic degenerative diseases of old age.

Results

NF-κB is activated during normal and accelerated aging. To examine the extent of NF-κB activation associated with aging, NF-κBEGFP knockin mice, with the EGFP reporter under the control of NF-κB regulatory elements (NF-κBEGFP), were used (48). Kidney specimens from 3-month-old and 2-year-old NF-κBEGFP reporter mice were compared (Figure 1A). The older WT mice had more cells expressing EGFP compared with young WT mice, indicative of increased NF-κB activation. EGFP expression was observed primarily in the glomeruli and was stochastic, with many cells showing strong expression while neighboring cells showed none. To determine
whether NF-κB is also upregulated in the progeroid mouse model, kidney specimens from Ercc1−/−NF-κBEGFP mice (life span, 7 months; ref. 49) and WT NF-κBEGFP littermates were isolated at 3 months of age (Figure 1B) and kidney specimens from Ercc1−/− NF-κBEGFP mice (life span, 28 days; ref. 45) and WT NF-κBEGFP littermates were isolated at 21 days of age (Figure 1C) to measure EGFP expression. Similar to natural aging, NF-κB activity was greater in the kidneys of ERCC1-deficient mice, particularly in the glomeruli, compared with that in WT littermates. In addition, EGFP+ cells were detected in the livers, pancreata, spleens, and muscle of Ercc1−/−NF-κBEGFP mice (Supplemental Figure 1; supplemental material available online with this article; doi:10.1172/JCI45785DS1). Among 6 littermate pairs, we detected a significant increase in the fraction of EGFP+ cells in the progeroid mice relative to that in WT littermates: kidney (2.5-fold increase), pancreas (2.5-fold increase), muscle (1.7-fold increase), and liver (4-fold increase) (Figure 1D). In contrast, the percentage of EGFP+ cells was 35% lower in the spleens of Ercc1−/−NF-κBEGFP mice than in those of WT NF-κBEGFP littermates, indicating that NF-κB activation is not exclusively driven by inflammation. These data support earlier reports (22, 23) that there is increased NF-κB activation with mammalian aging and extend this to include a murine model of XFE progeroid syndrome, which is driven by a DNA repair defect.

To confirm that NF-κB activity is increased in cells from progeroid Ercc1−/− mice, the levels of phosphorylated p65 (p-p65) and IκB were measured by immunoblot in nuclear and cytoplasmic extracts from passage 5 WT, Ercc1−/−, Ercc1−/−p65−/−, and Ercc1−/−p65−/− MEFs grown at 20% O2 to measure NF-κB activity after depletion of p65. Similar to natural aging, NF-κB activity was greater in the kidneys of ERCC1-deficient mice, particularly in the glomeruli, compared with that in WT littermates. Increased NF-κB binding activity was also detected in nuclear extracts from Ercc1−/− MEFs by EMSA (Figure 1F). Pretreatment of the nuclear lysates with anti-p50 and, in particular, anti-p65 antibodies reduced this DNA binding activity (Supplemental Figure 2). These data establish increased NF-κB activity in DNA repair–deficient Ercc1−/− mice and suggest that p65 is the predominant subunit contributing to this increased activity.

Figure 2
Genetic depletion of the p65 subunit of NF-κB delays aging symptoms and chronic diseases in progeroid Ercc1−/Δ mice. (A) EMSA on nuclear extracts from passage 5 WT, Ercc1−/Δ, Ercc1−/Δp65−/+ , and Ercc1−/Δp65−/− MEFs grown at 20% O2 to measure NF-κB activity after depletion of p65. (B) Ercc1−/Δ and Ercc1−/Δp65−/+ mice were evaluated biweekly for the onset of spontaneous symptoms associated with aging. The aging score, which represents the fraction of aging symptoms delayed in a particular mouse compared with its sibling, for littermate pairs of Ercc1−/Δ (red) and Ercc1−/Δp65−/+ (orange) mice is a measure of healthspan (11). The mean aging score for each genotype is represented by a black bar. (C) Representative images of Ercc1−/Δ and Ercc1−/Δp65−/+ sex-matched littermates at 15 weeks of age. (D) Histopathologic changes in Ercc1−/Δp65−/+ and Ercc1−/Δ mice. Liver sections from 10-week-old mice were stained with oil red O to detect neutral lipids (hepatic steatosis; original magnification, ×100). Kidney specimens from 15-week-old mice were stained with H&E to detect proteinaceous renal tubular hyaline casts and glomerulosclerosis (original magnification, ×20). Cerebellar sections from 10-week-old mice were immunostained for GFAP (red), a marker of neurodegeneration. Nuclei were counterstained with DAPI (blue; original magnification, ×40). μCT of the vertebrae to assess bone porosity (for quantification, see Supplemental Figure 3A).
Pharmacologic inhibition of NF-κB delays the onset of progeroid symptoms in Ercc1ΔΔ mice. To determine whether pharmacological suppression of IKK/NF-κB signaling also results in an extension of healthspan, a peptide inhibitor of IKK, termed the NEMO-binding domain (NBD), was used. The 11–amino acid NBD peptide, when fused to a protein transduction domain such as Antp or 8K, is efficacious for treating muscular dystrophy (54), inflammatory bowel disease (55), arthritis (56), and Parkinson’s disease (57) in mice. The activity of the peptide was tested in vitro by treating Ercc1−/− primary MEFs with 200 μM 8K-NBD. This led to a reduction in nuclear p-65 (Figure 3A). Ercc1−/− mice were chronically treated with 8K-NBD (10 mg/kg i.p., 3 times per week) beginning at 5 weeks of age, which is prior to the onset of their aging symptoms. Littermate mutant animals were treated with an equivalent dose of an inactive, mutant peptide (8K-mNBD) used as a negative control. Investigators conducting the experiment were blinded to the treatment group. Mice treated with 8K-NBD showed a delay in the onset of the majority of symptoms compared with siblings treated with the mutant peptide (Table 2). Ataxia, sarcopenia, and weight loss were significantly delayed (Table 2 and Supplemental Figure 4B). In addition, the aging score revealed a highly significant difference between treatment groups (P = 0.003; Figure 3B). There also was a visible difference in the appearance of the mice treated with 8K-NBD compared with that of their siblings treated with the mutant peptide at 15 and 19 weeks of age (Figure 3C) as well as improved reflexes, gait, muscle, and eyes.

Histologic analysis was performed on tissues of 19-week-old mice to determine whether treatment with 8K-NBD reduced age-related pathology. Similar to that in the Ercc1ΔΔ mice, 8K-NBD treatment resulted in reduced liver steatosis and renal hyaline casts compared with that in control mice (Figure 3D). GFAP staining was also reduced, consistent with the delay in onset of symptoms caused by neurodegeneration (Figure 3D). Microcomputed tomography (μCT) analysis revealed a significant reduction in bone porosity (osteoporosis) in mice treated with 8K-NBD compared with that in untreated Ercc1ΔΔ mice (Figure 3D and Supplemental Figure 3B). Collectively these data demonstrate that pharmacological inhibition of IKK/NF-κB activation leads to attenuation of age-related pathologies.

Unexpectedly, the age at onset of progeroid symptoms was delayed slightly in mutant animals treated with the mutant NBD peptide compared with that in untreated mutant animals (compare Table 2 with Table 1), suggesting that the mutant peptide has residual activity, which was confirmed in cell culture assays (data not shown). Therefore, we also compared the age at onset of symptoms in Ercc1ΔΔ mice treated with 8K-NBD with that of Ercc1ΔΔ mice treated with vehicle only (PBS) (Supplemental Table 1). This comparison revealed a significant delay in the onset of even more symptoms in mice treated with 8K-NBD (treating, ataxia, sarcopenia) and a more significant delay of all symptoms assessed. At 15 weeks of age, Ercc1ΔΔ mice exhibited dystonia and cachexia, and, by 19 weeks of age, incontinence, ocular defects, and sarcopenia were obvious (Supplemental Figure 5). Ercc1ΔΔ mice exhibited dystonia and cachexia, and, by 19 weeks of age, incontinence, ocular defects, and sarcopenia were obvious (Supplemental Figure 5). Ercc1ΔΔ mice exhibited dystonia and cachexia, and, by 19 weeks of age, incontinence, ocular defects, and sarcopenia were obvious (Supplemental Figure 5).
mice treated with 8K-NBD were largely spared these symptoms. These data demonstrate that pharmacologic inhibition of NF-κB activation can be used to simultaneously delay the onset of symptoms associated with multiple, common, age-related chronic degenerative diseases.

8K-NBD alters NF-κB signaling in vivo. To determine whether 8K-NBD indeed affects NF-κB-regulated gene expression in vivo, the gene expression profile of livers of 19-week-old Ercc1–/– mice chronically exposed to 8K-NBD was compared with that of littermate mutant animals treated with 8K-mNBD. A full mouse genome array revealed 1,269 genes (~5% of all genes) with significantly changed expression patterns when comparing 8K-NBD–treated and 8K-mNBD–treated mice (P ≤ 0.05, 1.2-fold change upregulated or downregulated). Of the 29 genes with known NF-κB regulatory elements that significantly differed between treatment groups, 26 were significantly downregulated in mice treated with 8K-NBD (Table 3), demonstrating that 8K-NBD treatment reduces NF-κB activity in vivo.

All genes with significantly altered expression were grouped according to their known or predicted biological function into gene ontology (GO) categories. Significantly altered biological processes were identified as those with a disproportionate number of genes having altered expression relative to those printed on the Affymetrix chip. Five major biological processes were significantly suppressed in response to inhibition of NF-κB. These processes, ranked by their relative enrichment score, included immune responses, cell cycle regulation, apoptosis, stress and DNA damage responses, and growth hormone signaling (Figure 4A). Of note, NF-κB is known to regulate many of these processes, and NF-κB activation was one of the GO categories identified as suppressed in response to inhibition of NF-κB.
### Table 2

Pharmacologic suppression of IKK/NF-κB activation attenuates progeroid symptoms and pathologies of progeroid *Ercc1<sup>Δ/Δ</sup>* mice

<table>
<thead>
<tr>
<th>Symptoms</th>
<th>Age at onset (wk)</th>
<th>Change of onset (wk)</th>
<th>No. of <em>Ercc1&lt;sup&gt;Δ/Δ&lt;/sup&gt;</em> mice (mNBD, NBD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dystonia</td>
<td>9.2</td>
<td>–0.1</td>
<td>12, 17</td>
</tr>
<tr>
<td>Trembling</td>
<td>10.7</td>
<td>–0.9</td>
<td>12, 17</td>
</tr>
<tr>
<td>Kyphosis</td>
<td>12.9</td>
<td>–0.7</td>
<td>11, 15</td>
</tr>
<tr>
<td>Ataxia&lt;sup&gt;a&lt;/sup&gt;</td>
<td>14.5</td>
<td>1.7</td>
<td>10, 13</td>
</tr>
<tr>
<td>Sarcopenia&lt;sup&gt;a&lt;/sup&gt;</td>
<td>15.1</td>
<td>2.1</td>
<td>8.12</td>
</tr>
<tr>
<td>Spontaneous activity</td>
<td>20.4</td>
<td>0.3</td>
<td>3.2</td>
</tr>
<tr>
<td>Urinary incontinence</td>
<td>17.5</td>
<td>2.2</td>
<td>5.3</td>
</tr>
</tbody>
</table>

Sibling, sex-matched pairs of *Ercc1<sup>Δ/Δ</sup>* mice were treated with 10 mg/kg 8K-NBD or 8K-mNBD i.p.; 3 times per week, beginning at 5 weeks of age and continuing throughout their life span. The average age at onset of characteristic progeroid symptoms in treated *Ercc1<sup>Δ/Δ</sup>* mice and the difference between the group averages is shown. Symptoms delayed in mice treated with the NF-κB inhibitor 8K-NBD compared with those in mice treated with 8K-mNBD are indicated in bold. <sup>a</sup>Significant delay (P < 0.05; Student’s t test).

The resolution afforded by the reporter construct revealed that endogenous DNA damage is sufficient to drive NF-κB expression in kidneys, skeletal muscle, pancreata, and livers (Figure 1 and Supplemental Figure 1). There was not significantly greater NF-κB expression in the spleen, suggesting that inflammatory cells are not the primary driver of NF-κB activation. These data strongly support the conclusion that spontaneous, endogenous DNA damage is sufficient to drive NF-κB activation in vivo. The resolution afforded by the reporter construct revealed that age-related activation of NF-κB is stochastic, meaning that there is activation in one cell while none is detected in adjacent cells.

### Discussion

Time-dependent accumulation of damage to cells and macromolecules is thought to drive aging (2). DNA damage is one type of damage implicated in aging based on the fact that mutations affecting a diverse array of DNA repair mechanisms lead to accelerated aging of one or more tissues (66). However, what is not known is the mechanism by which, for instance, damage to the nuclear genome drives aging. The mechanism could be via loss of functional cells once a threshold of damage is reached. Alternatively, activation of conserved stress response pathways may promote aging. Herein, to decipher how damage drives aging, we used a well-defined murine system: mice that spontaneously age rapidly as a consequence of failure to repair endogenous DNA damage (44, 49, 67).

The NF-κB family consists of transcription factors activated in response to a diverse array of cellular stressors (68). NF-κB activity increases with chronologic age in a variety of tissues of mammals (24–28). Thus, NF-κB activation could drive aging in response to time-dependent accumulation of cell damage. However, prior studies do not demonstrate a causal relationship between NF-κB activation and aging; neither do they reveal what drives NF-κB activation with aging.

Using a knockin NF-κBEGFP reporter system, we discovered a significant increase in the percentage of cells in which NF-κB was activated in old and DNA repair-deficient, progeroid mice relative to that in young WT mice. The progeroid mice had increased EGFP expression in kidneys, skeletal muscle, pancreata, and livers (Figure 1 and Supplemental Figure 1). There was not significantly greater NF-κB activation in the spleen, suggesting that inflammatory cells are not the primary driver of NF-κB activation. These data strongly support the conclusion that spontaneous, endogenous DNA damage is sufficient to drive NF-κB activation in vivo.

The resolution afforded by the reporter construct revealed that age-related activation of NF-κB is stochastic, meaning that there is activation in one cell while none is detected in adjacent cells,
The NF-κB κB inhibition was the immune response, which was not surprisingly downregulated. In addition, many processes previously demonstrated to be altered in progeroid or old WT mice, including suppression of the growth hormone/IGF-1 axis, inhibition of cell cycle progression, activation of pro-apoptotic mechanisms, and DNA damage/stress response (45), were at least partially corrected by NF-κB inhibition. This provides experimental evidence that NF-κB is indeed a master regulator of aging-related transcriptional reprogramming.

Gene expression analysis also confirmed the efficacy of inhibition of IKK/NF-κB by 8K-NBD, demonstrating decreased expression of numerous genes with known NF-κB promoter sequences, including Gadd45b, Cnd2, Cnd3, Bcl2, Apod, and Prkcd (77). The NF-κB-regulated genes that were most downregulated by 8K-NBD were Apod and Gadd45b, both of which have been shown to have roles in cellular senescence and age-related disease (78, 79). Similarly, a number of cytokines and other proinflammatory genes expressed by senescent cells (80) are downregulated rather than pan-activation throughout a tissue. This is consistent with the stochastic theory of aging, which posits that cellular damage occurs randomly in a fraction of cells (2).

We also demonstrate a key causal role for NF-κB in driving multiple age-related pathologies. Inhibition of the IKK/NF-κB pathway genetically, through deletion of one copy of p65, or pharmacologically, using an IKK inhibitory peptide, delayed the onset and severity of aging-related pathologies in the musculoskeletal, hepatobiliary, renal, and nervous systems (Figure 2D and Figure 3D). Aging-related symptoms caused by these pathologies were also delayed or attenuated (Tables 1 and 2). This provides strong experimental evidence that an increase of IKK/NF-κB activity plays a causal role in aging.

We further demonstrate that genetic reduction of NF-κB reduced the amount of mitochondrial-derived ROS (Figure 6A). This could be mediated through upregulation of antioxidant enzymes. Expression of catalase and targets of NRF2 was significantly increased in mice chronically treated with the IKK inhibitor 8K-NBD (Figure 4) compared with that in mice treated with an inactive mutant peptide. In further support of this, oxidative damage to lipids (lipofuscin; Figure 6B) and DNA (Figure 6, C and D) was significantly reduced in Ercc1Δ/–p65Δ/– mice and Ercc1Δ/– mice chronically treated with the IKK inhibitor 8K-NBD. This corresponded with a reduction in multiple markers of cellular senescence, including reduced proliferation of primary cells, increased γH2AX foci, and senescence-associated β-galactosidase (SA β-gal) activity. These data support a mechanism by which accumulated cellular damage (in particular DNA damage) with aging leads to activation of NF-κB. This in turn drives increased ROS production and even more cellular damage. Inhibiting NF-κB activation in response to stress is sufficient to attenuate damage and extend healthspan of a murine model of accelerated aging.

Interestingly, our data indicate that treating Ercc1Δ/– mice with 8K-NBD, beginning at 5 weeks of age, has a greater beneficial effect than genetic depletion of p65 from conception. The delay in aging symptoms, the attenuation of osteoporosis, and the maintenance of weight (Supplemental Figure 4) were greater in mice in which NF-κB activity was inhibited pharmacologically compared with those with genetic inhibition. This could be because p65/NF-κB has a positive role during development and/or early in life. Also, p65 was heterozygous in our mice, and therefore it is possible that the remaining copy of p65 is sufficient to initiate a stress response. Alternatively, 8K-NBD may be more effective at inhibiting NF-κB in response to stress, possibly because inhibition of IKK would act upstream of p65 and could lead to the cytoplasmic sequestration of more than just the p65 subunit of NF-κB. Targeting IKK also may affect other pathways in addition to NF-κB. For example, IKK phosphorylates BCL-10, β-catenin, cyclin D1, FOXO3A, p53, ERα, mTOR, and HIF-1α in addition to NF-κB (69–72).

The magnitude of the effect on healthspan elicited by NF-κB inhibition has only been observed in outbred mice treated with rapamycin (73), which targets the mTOR pathway, or by genetic deletion of S6K1, a downstream target of mTOR (74). Interestingly, mTOR has been shown to activate NF-κB via interaction with IKK (75). In contrast to the results with rapamycin and our results with NBD, treatment with resveratrol and other SIRT agonists appears only able to extend healthspan and life span in mice on high-fat diets (76).

Interestingly, chronic inhibition of NF-κB with the 8K-NBD peptide caused a dramatic change in gene expression compared with that in Ercc1Δ/– littermates treated with a less active control peptide. Expression of 5% of all genes was significantly altered. Based on ontology analysis of these genes, the biological process most significantly affected by NF-κB inhibition was the immune response, which was not surprisingly downregulated. In addition, many processes previously demonstrated to be altered in progeroid or old WT mice, including suppression of the growth hormone/IGF-1 axis, inhibition of cell cycle progression, activation of pro-apoptotic mechanisms, and DNA damage/stress response (45), were at least partially corrected by NF-κB inhibition. This provides experimental evidence that NF-κB is indeed a master regulator of aging-related transcriptional reprogramming.

<table>
<thead>
<tr>
<th>Gene symbol</th>
<th>Fold change</th>
<th>P value</th>
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<td>Csf3</td>
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<tr>
<td>Ighg1A</td>
<td>−2.4</td>
<td>0.03</td>
</tr>
<tr>
<td>PtX3A</td>
<td>−2.4</td>
<td>0.01</td>
</tr>
<tr>
<td>Lamb2B</td>
<td>−2.5</td>
<td>0.009</td>
</tr>
<tr>
<td>Gadd45bA</td>
<td>−3.5</td>
<td>0.02</td>
</tr>
<tr>
<td>ApodA</td>
<td>−4.4</td>
<td>0.01</td>
</tr>
</tbody>
</table>

RNA was isolated from the livers of 18- to 19-week-old Ercc1Δ/– mice chronically treated with 8K-NBD or 8K-mNBD (n = 4 per group). Differences in gene expression were analyzed using total genome Affymetrix arrays. Genes with known NF-κB regulatory elements that were significantly altered in Ercc1Δ/– mice treated with 8K-NBD compared with those in mice treated with 8K-mNBD are shown. Genes implicated in inflammation. Genes implicated in cell survival and cell cycle control.
in Ercc1−/− mice chronically treated with 8K-NBD, notably Il16, Il17ra, Il20, Il15ra, Il1r1, and Il6st. 8K-NBD treatment also reduced liver expression of p16 at both the mRNA and protein level. Moreover, chronic 8K-NBD treatment significantly upregulated catalase, genes regulated by NRF2, and genes involved in mitochondrial respiration, all important in regulating ROS levels. These observations are consistent with the recent demonstration of a key role for p16-expressing senescent cells in driving aging, suggesting that 8K-NBD treatment can reduce senescence (81). 8K-NBD treatment also suppressed expression of chemokines known to regulate the trafficking of immune cells during inflammation. Overall, the expression data demonstrate that inhibition of IKK/NF-κB leads to suppression of numerous processes that are known to modulate healthspan, including inflammation and cellular senescence.

In conclusion, these studies demonstrate that spontaneous, endogenous DNA damage can activate NF-κB. Activation of NF-κB is stochastic, occurring only in a subset of cells. Chronic inhibition of IKK/NF-κB activation is sufficient to delay the onset of aging symptoms and chronic aging-related diseases that arise spontaneously in DNA repair–deficient Ercc1−/− mice that model a human progeroid syndrome. Moreover, inhibiting NF-κB activation reduces ROS production and oxidative damage to lipids and DNA. This demonstrates a direct causal role for NF-κB in driving aging-related changes in response to cellular damage by promoting continued damage. Inhibition of NF-κB offers what we believe to be a novel strategy for simultaneously delaying and/or attenuating multiple chronic degenerative diseases in patients with progeroid syndromes and potentially in old age.
Mice. Ercc1+/− and Ercc1+/mice were generated in an F1 hybrid background by crossing heterozygous Ercc1+/− and Ercc1+/mice from two different inbred C37BL/6j and FVB/n backgrounds to obtain genetically identical mice, without strain-specific pathology. The mice were genotyped using PCR as previously described (82). Ercc1−/−NF-κBEGFP mice were generated by crossing Ercc1−/−C57BL/6j mice with NF-κBEGFP mice (provided by Christian Jobin, University of North Carolina, Chapel Hill, North Carolina, USA) (83). These mice were then bred with Ercc1−/− FVB/n mice to generate Ercc1−/−NF-κBEGFP mice. p65−/−mice were bred with Ercc1−/−C57BL/6j mice. These were then bred with Ercc1−/− FVB/n mice to generate Ercc1−/−p65−/−mice.

Isolation and treatment of MEFs. Double heterozygous mice were bred to yield WT, Ercc1+/−, Ercc1−/−p65−/−, and Ercc1−/−p65−/− pups for isolation of primary MEFs as previously described (45). The cells were grown in 1:1 DMEM/Ham’s F10 supplemented with 10% FBS, 1% penicillin and streptomycin, and 1% nonessential amino acids. They were passaged 5 times at 20% oxygen prior to fractionation using the NE-PER Cytoplasmic and Nuclear Extraction Reagent Kit (ThermoFisher Scientific) for Western blot analysis or EMSA. For 8K-NBD treatment, cells were incubated with 200 μM 8K-NBD for 3 hours prior to collection and immunoblotting for p-p65.

Nuclear extracts and Western blotting. Immunodetection of activated NF-κB and IκB in nuclear and cytoplasmic cell fractions was performed as previously described (84) using anti-p-p65 (93H1; Cell Signaling Technology), anti-p-IκB (SA5; Cell Signaling Technology), and anti-IκBα (sc371; Santa Cruz Biotechnology Inc.). Anti-lamin A/C (sc20681; Santa Cruz Biotechnology Inc.) and anti-β-actin (Abcam) antibodies were used as loading controls for the nuclear and cytoplasmic fractions, respectively. Immunodetection of p16INK4a from liver extracts was performed using anti-p16INK4a antibody (sc1207; Santa Cruz Biotechnology Inc.) with anti-tubulin (ab4074, Abcam) as a loading control. All primary antibodies were used at a 1:1,000 dilution and an overnight incubation at 4°C.

EMSA. EMSA was completed based on a modified protocol described in ref. 85. Briefly, nuclear cellular fractions were extracted from MEFs using the NE-PER Cytoplasmic and Nuclear Extraction Reagent Kit (ThermoFisher Scientific). Five μg of each extract was mixed with 2 μl of 5X Gel Shift Binding Buffer (Promega) and nuclease-free distilled water in a 9 μl final volume. This was followed by incubation with a 12P-deoxycytodine triphosphate–radiolabeled DNA probe containing the NF-κB-binding domain (MP Biomedical). The design of the NF-κB probe was described previously (86). The oligonucleotide sequences are as follows, with the DNA binding sequence underlined: NF-κB B template, 5′-CAGGGGTCCGGGATTCCATCTCCACAGTTTACATCC-3′; NF-κB annealing, 5′-GAGTGAACAGCTGTTG-3′ (Integrated DNA Technologies Inc.). dNTPs used to fill the overhangs were added using DNA Polymerase I, Large (Klenow) Fragment (Invitrogen), and the reaction was purified using illustra MicroSpin G50 Columns (GE Healthcare). Probes were added at a count per minute of approximately 150,000 in 1 μl, followed by separation on a 6% nondenaturing polyacrylamide gel. For competition assays, 2 μl of 100 μg/ml antibodies against p65/RelA and p50 were added to nuclear extracts and incubated for 20 minutes, prior to the addition of the duplex oligonucleotide.

Fluorescent microscopy. To quantify NF-κB activity, EGFP expression was measured in tissues, using MetaMorph software (MDS Analytical Technologies). Five images (×20 magnification) were taken of each tissue analyzed for each mouse (n = 6 per genotype at each age), and the percentage of cells expressing EGFP was quantified based on tissue area. This was reported as the fold increase in the number of EGFP+ cells above the average value for controls. Anti-GFAP (13-0300; Invitrogen) was used at a 1:250 dilution and incubated overnight at 4°C. Anti-p-H2AX (05-636 Millipore) was used at a 1:500 dilution and incubated overnight at 4°C. For lipofuscin analysis, 5 images (×40 magnification) were taken of liver sections from...
Inhibition of NF-κB reduces oxidative stress and damage in vitro and in vivo. (A) Ercc1Δ/Δ and Ercc1Δ/p65+/– passage 6 primary MEFs grown at 20% O2 were stained with DiOC6 (green) to mark mitochondria and MitoSOX (red) to detect mitochondrial superoxide anion (original magnification, ×40). (B) Liver sections from 10-week-old Ercc1Δ/Δ and Ercc1Δ/p65+/– mice imaged for lipofuscin fluorescence (original magnification, ×20). The histogram indicates the total fluorescent area for 5 images from 3 different mice per genotype calculated using MetaMorph software. (C) The levels of the (S)R and (S)S diastereomers of cdG and cdA in nuclear DNA isolated from the livers of 10-week-old control, p65Δ/Δ, Ercc1Δ/Δ, and Ercc1Δ/p65+/– mice. (D) The levels of cdG and cdA in nuclear DNA isolated from the livers of 19-week-old control, untreated Ercc1Δ/Δ, and 8K-NBD-treated Ercc1Δ/Δ mice.* P < 0.05, Tukey-Kramer test. Values denote the mean ± SEM (n = 3 per group).
the symptoms occurred simultaneously in both mice, both mice received symptoms associated with elevated NF-kappaB activity.

18. Wullaert A, Heyninck K, Beyaert R. Mechanisms of nuclear factor-kappaB signaling in the regulation of transcription factor nucleo-


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research article

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Microarray. Genome-wide expression was measured in mice treated with 8K-NBD or 8k-mNBD as previously described (45, 47). All significant gene entries were subjected to GO classification (http://www.geneontology.org/). Significant overrepresentation of pathways and gene networks was determined by DAVID (http://david.abcc.ncifcrf.gov/summary.jsp; through BBID, BIOCARDATA, and KEGG annotations) as well as by the ingenuity pathway analysis software (http://www.ingenuity.com/). The microarray data are available on ArrayExpress (E-MEXP-3615).

Statistics. To determine significance, a 2-tailed Student’s t test was used, with P values of less than 0.05 considered significant. In addition, higher levels of significance (P < 0.01; P < 0.001) are indicated in Supplemental Table 1. For analysis of experiments involving more than 2 groups, ANOVA with a post-hoc Tukey-Kramer test was performed to determine significance (90). For the microarray data, significant overrepresentation of pathways and gene networks was determined by DAVID (http://david.abcc.ncifcrf.gov/summary.jsp) through BBID, BIOCARDATA, and KEGG annotations, as well as by the ingenuity pathway analysis software (http://www.ingenuity.com/).

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