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ORIGINAL ARTICLE

Downregulation of the NF-κB protein p65 is a shared phenotype among most anti-aging interventions

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Abstract Many aspects of inflammation increase with aging in mice and humans. Transcriptomic analysis revealed that many murine anti-aging interventions produce lower levels of pro-inflammatory proteins. Here, we explore the hypothesis that different longevity interventions diminish NF-κB levels, potentially mediating some of the anti-inflammatory benefits of lifespan-extending interventions. We found that the NF-κB protein p65 is significantly downregulated in the liver of several kinds of slow-aging mice. These included both sexes of GHRKO and Snell Dwarf mutant mice, and in females only of PAPPA KO mice. P65 is also lower in both sexes of mice treated with rapamycin, canagliflozin, meclizine, or acarbose, and in mice undergoing caloric restriction. Two drugs that extend lifespan of male mice, i.e. 17α-estradiol and astaxanthin, however, did not produce lower levels of p65. We also measured other canonical NF-κB signaling regulators, including the activators IKKα and IKKβ and the inhibitor IκB-α. We found that those regulators do not consistently change in a direction that would lead to of NF-κB inhibition. In contrast, we found that NCoR1, an HDAC3 cofactor and a transcription co-repressor that regulates p65 activity, was also downregulated in many of these mouse models. Finally, we report downregulation of three p65 target proteins that regulate the metabolic and inflammatory states of the liver (HNF4α, IL-1β, and CRP) in multiple slow-aging mouse models. Together, these data suggest that NF-κB signaling, might be inhibited in liver of multiple varieties of slow aging mice. This establishes p65 as a potential target for novel longevity interventions.

Keywords Longevity · Inflammation · NF-kB · P65 · NCoR1

Introduction

Aging is accompanied by an increase in inflammation, reflected in upregulation of many pro-inflammatory signaling systems and a persistent activation of a dysregulated immune system [1]. Increased inflammation has been argued to be a driver of aging and aging-associated functional decline. It has also been suggested that increased activation of the immune system is a resilience mechanism induced by the aging-related disequilibrium in different tissues [2]. Moreover, an increase in pro-inflammatory signals

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has been reported to contribute to the progression of age-related diseases such as diabetes, Alzheimer's, atherosclerosis, and cardiovascular diseases [1, 3]. A recent paper by Widjaja et al., highlights the age-related increase in IL-11, and demonstrates that deletion or inhibition of IL-11 using specific antibodies leads to increased lifespan and improved metabolic health as shown by the higher respiratory exchange rate (RER), insulin sensitivity, lean mass, and lower fat mass [4].

Data from our group and others have also provided evidence that many anti-aging interventions reduce pro-inflammatory signals and associated phenotypes [5, 6]. For example, we found that in white adipose tissue (WAT) and brown adipose tissue (BAT) of slow-aging Snell Dwarf (SD) and growth hormone receptor knockout (GHRKO) mice, expression of pro-inflammatory M1 macrophage markers decreased, while anti-inflammatory M2 macrophage markers increased. This was accompanied by a corresponding decrease in mRNA for pro-inflammatory cytokines such as TNFα, IL-6, and MCP1 [7]. Canagliflozin, a drug that extends lifespan in male but not female mice, reduced age-associated microglial cytokine production in both sexes, and reduced microgliosis and astrogliosis only in male mice [8]. Caloric restriction (CR) has also been shown to reduce high-fat diet induced inflammation in mice, exhibited by lower production of pro-inflammatory cytokines IL-1β and TNF-α [9]. Rapamycin, which extends lifespan in male and female mice, acts as an immunosuppressant and diminishes inflammation by inhibiting production of inflammatory cytokines including IL-6 and TNF-α in macrophages and T helper cells tested in vitro and suppressing T-cell proliferation [10–12]. In vivo, rapamycin downregulated inflammatory markers TNF-α and COX-2 in the liver [13]. Previous data from our lab have shown a consistent decline in hepatic MAPK-p38 phosphorylation, an upstream activator of various pro-inflammatory pathways, including NF-κB, in long-lived mice treated with rapamycin, acarbose, and 17α-estradiol [14].

Understanding how major inflammatory pathways are modulated in different tissues in response to anti-aging interventions could help identify novel, more effective and/or targeted interventions. Here, we explore changes in the classical NF-κB transcription system in response to ten lifespan extending interventions ranging from genetic mutations (GHRKO, SD, and PAPPA KO), dietary (CR), and drug treatments (17α-estradiol, rapamycin, acarbose, meclizine, astaxanthin, canagliflozin). NF-κB is a pro-inflammatory protein complex composed primarily of five members: RelA (p65), RelB, c-Rel, NF-κB1 (p50), and NF-κB2 (p52) [15]. NF-κB transcription factors are found in the cytoplasm as homodimers and heterodimers containing combinations of the five components [15]. In classical NF-κB signaling, the dimer is initially bound to the inhibitor IκB-α, which prevents it from translocating to the nucleus and binding to its target DNA. NF-κB signaling is activated in response to pro-inflammatory and infectious stimuli via the IKK complex, which phosphorylates and degrades the inhibitor attached to the NF-κB dimer. The most common heterodimer in classical NF-κB signaling is p50:p65, while in alternative NF-κB signaling, the most common heterodimer is p52:RelB [16]. The only three members of NF-κB transcription factors that contain a transactivation domain are p65, RelB, and cRel [15, 16]. The other two members, p50 and p52, can form homodimers that binds to NF-κB DNA targets but do not activate transcription, thus serving as competitive inhibitors of NF-κB activity [17].

Activity of p65 is regulated at different levels, including nuclear translocation, DNA binding, and transcriptional activity. This regulation is achieved through multiple mechanisms including phosphorylation at different serine sites, modification of dimer composition, and binding to different transcription regulators [18–20]. Two regulators of p65 activity are nuclear receptor corepressor 1 (NCoR1) and a class of kinases called cyclin dependent kinases (CDKs) [21, 22]. NCoR1 is involved in transcriptional repression of many oxidative metabolism genes and is commonly associated with histone deacetylase 3 (HDAC3) [23–25]. It is commonly considered to be the functional opposite to PGC-1α, a transcription activator strongly associated with longevity phenotypes [26]. We have previously reported that NCoR1 is downregulated in livers of long-lived GHRKO and SD mice [23, 26, 27]. NCoR1, along with HDAC3, can also mediate cellular inflammation, and form a transcriptional complex with p65 [20, 22, 28, 29].

Hundreds of p65 targets have been reported [3, 18, 19]. Here, we focused on three targets whose downregulation can partially explain two of the main longevity-associated transcriptomic signatures shared by diverse slow-aging mice: decreased inflammation

and upregulated oxidative metabolism [5, 6]. First, we focused on Hepatocellular Nuclear Factor-4α (HNF4α). HNF4α is at the center of a complex transcriptional network that regulates glucose and lipid metabolism and mediates hepatocyte differentiation [30–32]. It is directly linked to NF-κB signaling as a target of p65 and a transcriptional regulator that shares many DNA binding motifs with it [33]. We also measured the level of IL-1β, a pro-inflammatory cytokine that is a main target of p65 and an activator of NF-kB [34–36]. Finally, since the complement cascade was one of the main pathways downregulated by different longevity interventions at the transcriptomic level, we measured the level of C-reactive protein (CRP) [5]. CRP is a protein produced by the liver and secreted into the systemic circulation, and is typically used as a marker for inflammation or infection [37].

In this study, we tested the hypothesis that downregulation of hepatic NF-κB signaling is a shared molecular pathway in most or all murine longevity models. We first measured the levels of the two main components of classical NF-κB dimer: p50 and p65 in the liver of slow-aging mice. Then, we measured the levels of NF-κB activators IKKα and IKKβ, and levels of total and phosphorylated inhibitor IκB-α. Finally, we also measured protein levels of p65 co-regulator NCoR1 and its downstream targets HNF4α, IL-1β, and CRP.

Methods

Mice

Growth hormone receptor knockout (GHRKO), Snell Dwarf (SD), and pregnancy-associated plasma protein A knockout mice (PAPP-A KO) mice were generated and maintained as previously described [38–42] and used at 5–6 months of age. Liver samples from mice fed with lifespan-extending drugs or placed on a calorically restricted diet were collected as previously described [43]. Briefly, genetically heterogeneous UM-HET3 mice were prepared for each sample group: control, acarbose (ACA), 17α-estradiol (17aE2), and rapamycin (Rapa), and caloric restriction (CR). The mice were treated with ACA (1000 mg kg^{-1}), 17aE2 (14.4 mg kg^{-1}), or Rapa (14 mg kg^{-1}) starting at 4 months. At 12 months, the mice fasted for 18 h and were euthanized for collection of liver samples. Finally, liver samples from Intervention Testing Program (ITP) mice were collected from three sites: University of Michigan, Jackson Labs, and The University of Texas, as previously described [44, 45]. In brief, genetically heterogeneous UM-HET3 mice were prepared for control, canagliflozin (CANA), meclizine (Mec), and astaxanthin (Asta). CANA was administered at 180 ppm starting at 7 months of age until 22 months of age. Mec (800 ppm) and Asta (4000 ppm) were both started at 12 months of age.

Experiments were conducted on 5–6 male and 5–6 female mice for control and anti-aging groups, unless otherwise specified. Mice used in this study were fed ad libitum, unless otherwise specified.

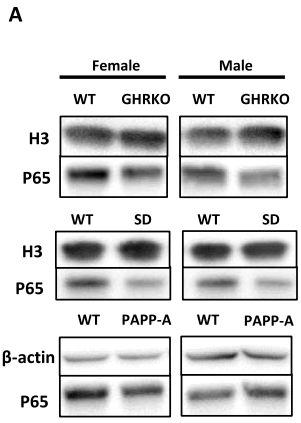
All experimental protocols were reviewed and approved by the University of Michigan Institutional Animal Care and Use Committee (IACUC).

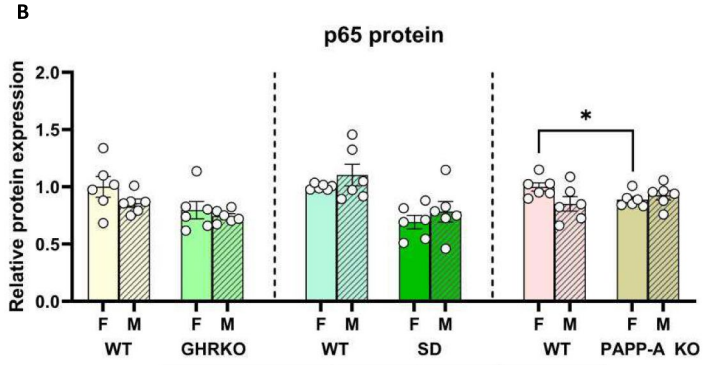
Western blotting

Proteins from liver tissue were homogenized and extracted in freshly prepared Laemmli lysis buffer supplemented with Protease and Phosphatase Inhibitor Cocktail (Thermo, PI78440). Protein concentration was measured using a BCA assay (Thermo, 23,227). The protein extracts were separated by SDS/PAGE on a 4–15% precast gel (BioRad, 4,561,096), and transferred to Immun-Blot PVDF Membrane (BioRad, 1,620,177). Membranes were then evaluated using EcoBright Femto HRP 100 (Innovative Solutions, EBFH100). GAPDH and Histone H3 were used as a protein loading control. A full list of primary antibodies used can be found in Table S1. Quantification was performed using ImageJ software.

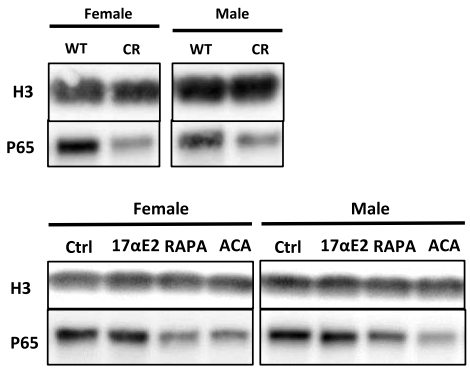
RNA isolation and cDNA synthesis

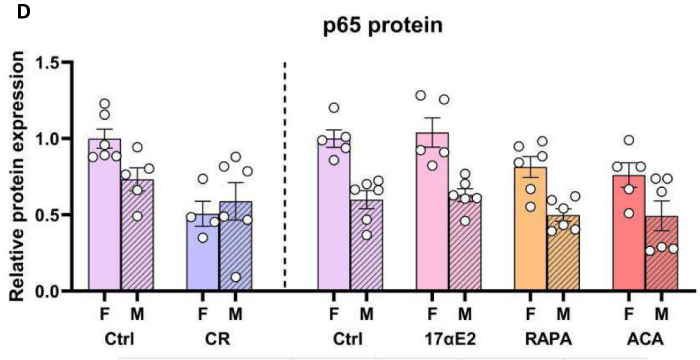
Murine liver tissue was homogenized, and RNA samples were extracted using the the Trizol Plus RNA purification kit (ThermoFisher, 1,218,355). The concentration of total RNA was measured using a Nanodrop One spectrophotometer (ThermoFisher). cDNA was reverse transcribed from 2 µg of total RNA using iScript Advanced cDNA Synthesis Kit (BioRad, 1,708,891).



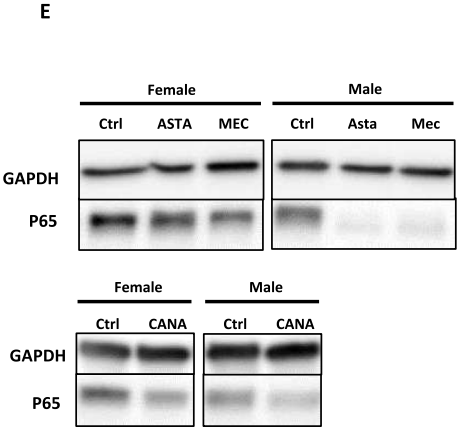


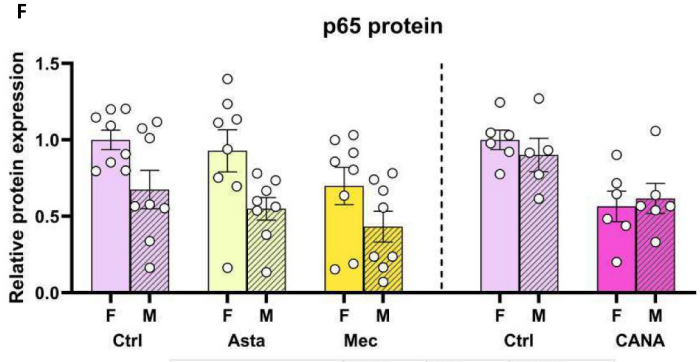
	GHRKO	SD	PAPP-A KO
Source of variation	P value	P value	P value
Interaction	0.485	0.931	0.045
Sex	0.134	0.195	0.225
Intervention	0.019	<0.001	0.677





	CR	17aE2	RAPA	ACA
Source of variation	P value	P value	P value	P value
Interaction	0.099	0.948	0.465	0.394
Sex	0.765	<0.001	<0.001	<0.001
Intervention	0.005	0.586	0.023	0.039





	Asta	Mec	CANA
Source of variation	P value	P value	P value
Interaction	0.796	0.784	0.279
Sex	0.002	0.009	0.643
Intervention	0.358	0.016	0.003

◂**Fig. 1** p65 protein is downregulated in the livers of diverse long-lived mouse models. (A,B) Western blot images **(A)** and levels **(B)** of p65 protein in livers of 6-months old GHRKO, Snell Dwarf, Snell Dwarf, and PAPP-A KO mice. (C,D) Western blot images **(C)** and levels **(D)** of p65 protein in livers of 12-months mice either calorically restricted or treated with 17αE2, RAPA, or ACA. (E,F) Western blot images **(E)** and levels **(F)** of p65 protein in livers of 22-months old mice treated with ASTA, MEC, and CANA. $n = 5–6$ for each group. Two-way ANOVA was used for analysis of intervention effect, sex effect, and their interaction. Unpaired t-test was used when the interaction term was significant. * $p < 0.05$

Quantitative real-time PCR

Quantitative PCR was performed using TaqMan Fast Advanced Master Mix (Applied Biosystems, 4,444,557). RT-PCR was performed using qPCR systems (Thermo) with corresponding TaqMan gene expression assay probes (Thermo, 4,331,182). 18S was simultaneously assayed as a housekeeping control gene. A full list of TaqMan assays used in this paper can be found in Table S2. Levels of 18S were not significantly altered and expression levels of different mRNAs were reported as CT values.

Statistical analysis

Statistical analyses and plotting were conducted in GraphPad Prism (version 9). All data are presented as mean ± SEM. 2-way ANOVAs were used for reporting sex effect, genotype effect, and their interaction. An unpaired Student t-test was performed when a significant interaction term was noted.

Results

p65 protein is downregulated in the liver of eight kinds of long-lived mice

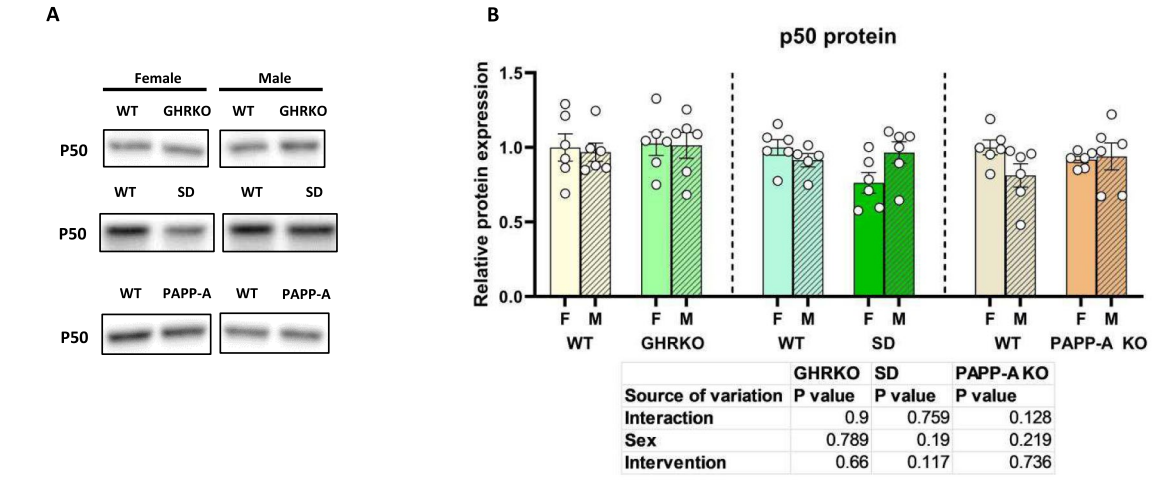
Classical NF-κB signaling is mainly mediated by a dimer of p65 and p50. We first measured the level of p65 in the livers of several kinds of slow-aging mice. We started by measuring p65 protein in young (5–6 months old) mice with the GHRKO mutation, which extends lifespan in both sexes [46]. Using western blotting, we found that p65 levels were significantly upregulated in livers of both sexes of GHRKO mice (Fig. 1A, B). We then measured p65 protein in SD mice, another long-lived mutant mouse model with growth hormone signaling disruption. We found similar diminution of p65 protein in both sexes of young (5–6 months old) SD mice (Fig. 1A, B), which have a loss-of-function mutation in the Pit1 gene that leads to lifespan extension in both sexes [38]. Finally, we tested the same hypothesis in PAPP-A KO mice, a third mutant model with lifespan extension in both sexes [47]. Here, the [Sex x Genotype] interaction term was significant at $p = 0.045$, implying that the effects of the PAPP-A KO mutation differed significantly between the two sexes. A subsequent unpaired t-test was performed to determine the genotype effect within each sex and revealed that p65 was significantly downregulated only in female PAPP-A KO mice (Fig. 1A, B).

Next, we applied this approach to interventions that extend lifespan of genetically normal mice by

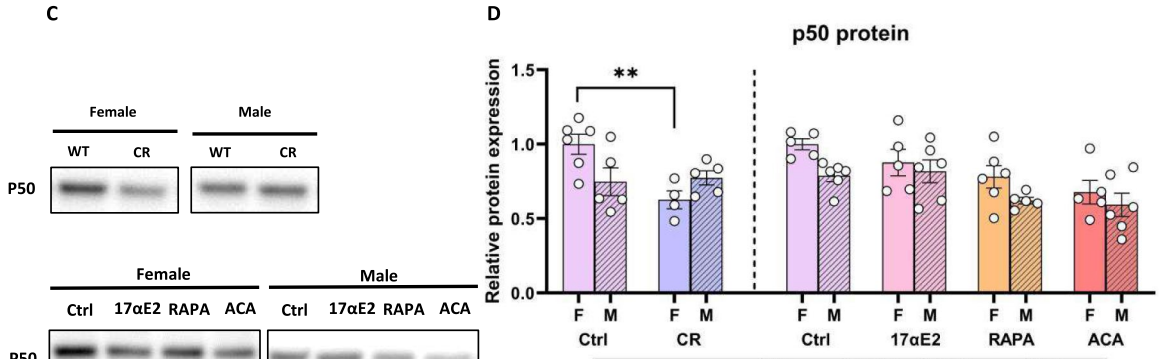
Table 1 Effects of different anti-aging interventions on levels of p65 and p50 protein in liver

Model			Age (Months)	p65	p50
Mutant mice		GHRKO	5–6	↓↓	-
		Snell Dwarf	5–6	↓↓	↓
		PAPP-A KO	5–6	↓↓ (F)	-
Drug treated and CR mice	LC	CR	12	↓↓	↓↓ (F)
		17α-estradiol	12	-	-
		Rapamycin	12	↓↓	↓↓
		Acarbose	12	↓↓	↓↓
	ITP	Astaxanthin	22	-	-
		Meclizine	22	↓↓	-
		Canagliflozin	22	↓↓	↓↓

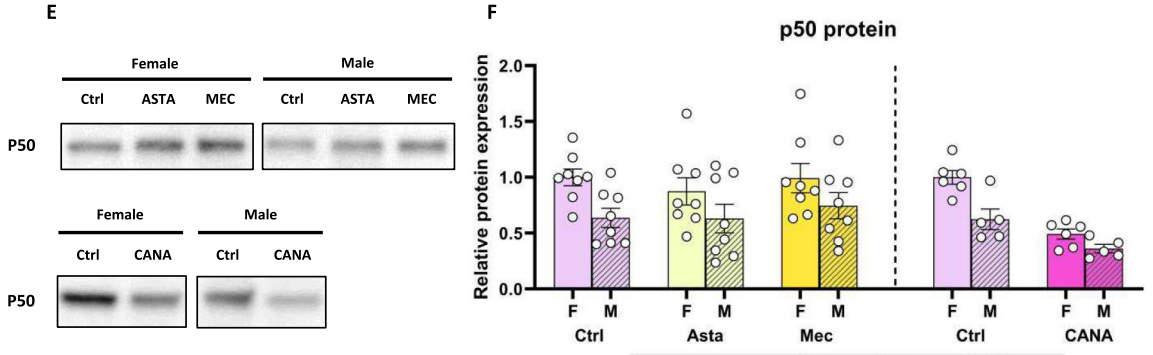
↓↓: significant downregulation (p-value < 0.05), ↓: trend downregulation (p-value < 0.2), F: change is seen only in female mice



	GHRKO	SD	PAPP-A KO
Source of variation	P value	P value	P value
Interaction	0.9	0.759	0.128
Sex	0.789	0.19	0.219
Intervention	0.66	0.117	0.736



	CR	17aE2	RAPA	ACA
Source of variation	P value	P value	P value	P value
Interaction	0.013	0.25	0.619	0.319
Sex	0.475	0.05	0.002	0.029
Intervention	0.027	0.48	0.001	<0.001



	Asta	Mec	CANA
Source of variation	P value	P value	P value
Interaction	0.0575	0.584	0.063
Sex	0.007	0.007	<0.001
Intervention	0.533	0.634	<0.001

diet (caloric restriction, CR), or by addition of drugs to food (17-α-estradiol (17αE2), rapamycin (RAPA), or acarbose (ACA)). CR extends lifespan in male and female mice [48, 49], including UM-HET3 mice [50]. CR reduced p65 in both sexes (Fig. 1C, D). The interaction term in the two-way ANOVA was

◂**Fig. 2** P50 protein is downregulated in the livers of some long-lived mouse models. (A,B) Western blot images **(A)** and levels **(B)** of p50 protein in livers of 6-months old GHRKO, Snell Dwarf, Snell Dwarf, and PAPP-A KO mice. (C,D) Western blot images **(C)** and levels **(D)** of p50 protein in livers of 12-months mice either calorically restricted or treated with 17αE2, RAPA, or ACA. (E,F) Western blot images **(E)** and levels **(F)** of p50 protein in livers of 22-months old mice treated with ASTA, MEC, and CANA. n = 5–6 for each group. Two-way ANOVA was used for analysis of intervention effect, sex effect, and their interaction. Unpaired t-test was used when the interaction term was significant. ** $p < 0.01$

not significant (p = 0.1), but the data suggest that the effect of CR on p65 may be stronger in female mice. RAPA and ACA extend lifespan in both male and female mice [44, 45], and each led to lower levels of p65 (Fig. 1C, D) with no evidence for a sex-specific effect. In contrast, 17aE2, which leads to male-specific lifespan extension [45], did not have a significant effect on p65 in either sex (Fig. 1C, D).

We also evaluated three drugs shown by the ITP to extend lifespan in male mice only: Canagliflozin (CANA), meclizine (MEC), and astaxanthin (ASTA) [51, 52]. The available samples were from 22-month-old mice, rather than the 12-month-old mice used for the tests of CR, RAPA, 17aE2, and ACA. Consequently, we used 22-month-old untreated control animals for this set of experiments. MEC and Cana, but not ASTA, led to significant declines in p65 protein (Fig. 1E, F), with no evidence for sex-specificity despite the male-specific lifespan increases shown by each of these drugs.

In summary, of the ten interventions tested, 7 led to diminished p65 levels in both sexes, one led to a sex-specific decline in p65, and two (ASTA, 17aE2) did not lower p65 to a significant degree in liver tissue (Table 1).

Hepatic p65 is downregulated at the mRNA level in long-lived mice

We then tested whether p65 protein declines were accompanied by parallel changes in mRNA. P65 mRNA was significantly lower in GHRKO, Snell, CR, and CANA mice, and no sex-specific effects were found (Supp. Figure 1A). In contrast, we found no effects of ACA on p65 mRNA. mRNA samples from RAPA mice were not available for testing. This demonstrates that p65 is transcriptionally downregulated in long-lived mice, but it does not rule out the involvement of other regulatory pathways such as translational, post-translational, or proteolytic mechanisms. Levels of 18S RNA were used as an internal control and were not significantly altered in any of the models (Supp. Figure 1B).

Hepatic p50 protein level is downregulated in a smaller subset of long-lived mice

Next, we measured the level of p50, the second component of NF-κB dimer, in the same mouse samples used for tests of p65. Using western blotting, we found that hepatic p50 level is unchanged in young (i.e. 5–6 months old) GHRKO livers compared to WT littermate mice (Fig. 2A, B). Female SD mice demonstrated a non-significant downregulation in p50 levels (≈ 20%) (Fig. 2A, B). Finally, hepatic p50 was also unchanged in the third mutant model, PAPP-A KO mice.

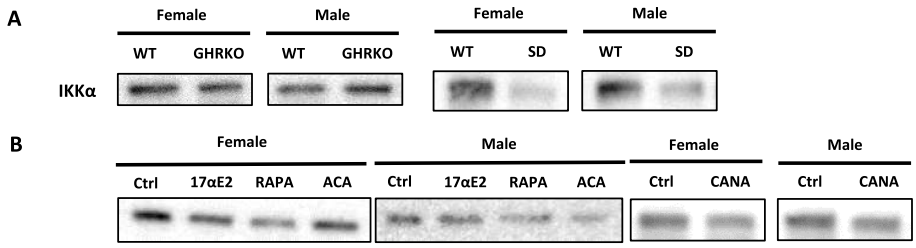
In 12-month-old UM-HET3 mice, CR reduced p50 in females only (Fig. 2C, D). RAPA and ACA induced a significant downregulation in p50 levels in both sexes, while 17αE2 did not affect its level (Fig. 2C, D). Interestingly, in all UM-HET3 mice, p65 and p50 levels appeared to be significantly lower in male mice compared to female mice, regardless of the varying drug-induced changes. This led to a significant sex effect in the two way ANOVA for the three treatments (17αE2, RAPA, ACA) (Fig. 1C, D, Fig. 2C, D).

In 22-month-old UM-HET3 mice, ASTA and MEC did not affect p50 levels, while CANA significantly downregulated p50 levels in both sexes. Similar to 12-month-old mice, male UM-HET3 mice had significantly lower p50 -and p65- when compared to female UM-HET3 mice, as shown by the significant sex factor p-value for all three drugs for p50, and in MEC and CANA for p65 (Fig. 1E, F, Fig. 2E, F).

In summary, p50 protein level was measured in 10 different slow-aging mouse models. It was significantly downregulated in both sexes for four models (RAPA, ACA, Mec, and CANA), downregulated in females only of CR mice, and unchanged in 5 models (GHRKO, SD, PAPP-A KO, 17αE2, and Asta). (Table 1).

Activators and inhibitors of classical NF-κB signaling do not consistently change in response to anti-aging interventions

The activity of NF-κB dimer can be modulated via a group of activator kinases (IKKα and IKKβ) that



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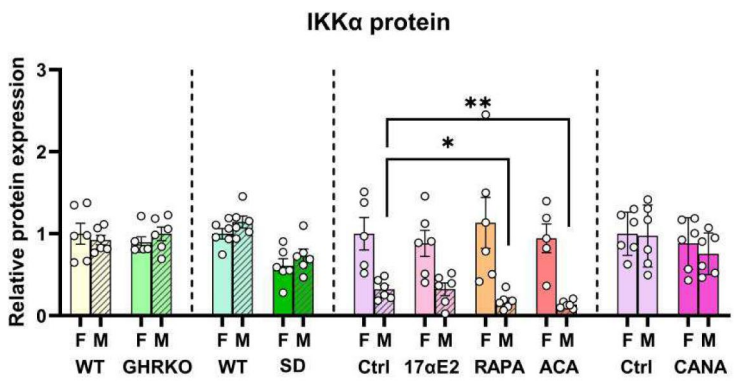
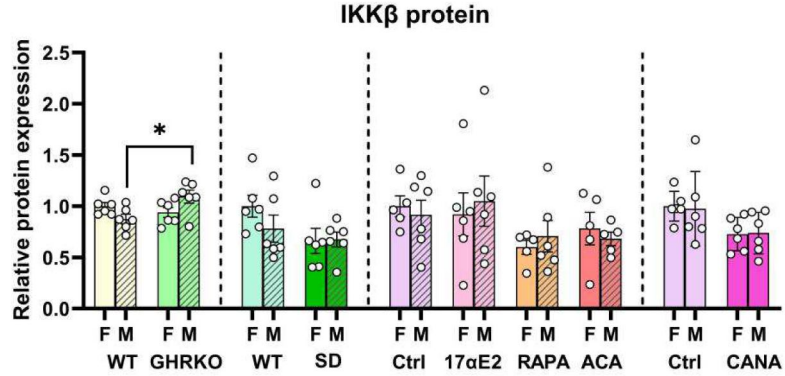


	GHRKO	SD	17αE2	RAPA	ACA	CANA
Source of variation	P value	P value	P value	P value	P value	P value
Interaction	0.322	0.885	0.651	0.469	0.606	0.691
Sex	0.888	0.109	<0.001	<0.001	<0.001	0.543
Intervention	0.877	<0.001	0.659	0.978	0.34	0.193

F



	GHRKO	SD	17αE2	RAPA	ACA	CANA
Source of variation	P value	P value	P value	P value	P value	P value
Interaction	0.011	0.308	0.583	0.467	0.945	0.855
Sex	0.761	0.365	0.905	0.926	0.475	0.945
Intervention	0.127	0.059	0.891	0.029	0.092	0.015

◂**Fig. 3** IKKs are differentially regulated in livers of slow-aging mice. (A, B) Western blot images of IKKα in livers of mutant slow aging GHRKO and SD mice **(A)** and livers of mice treated with 17αE2, RAPA, ACA, or CANA **(B)**. **(C)** Levels of IKKα protein in livers of different slow aging mouse models shown in panels A and B. (D, E) Western blot images of IKKα in livers of mutant slow aging GHRKO and SD mice **(D)** and livers of mice treated with 17αE2, RAPA, ACA, or CANA **(E)**. **(F)** Levels of IKKα protein in livers of different slow aging mouse models shown in panels D and E. $n = 5–6$ for each group. Two-way ANOVA was used for analysis of intervention effect, sex effect, and their interaction. * $p < 0.05$. ** $p < 0.01$

phosphorylate the IκB-α inhibitor, which frees the dimer to translocate to the nucleus [15, 53]. We also measured total and phosphorylated levels of the NF-κB inhibitor IκB-α and IκB-β; increased phosphorylation of IκB-α leads to NF-κB activation [16].

Levels of IKKα were unchanged in GHRKO, 17αE2, RAPA, ACA, or CANA mice, but was significantly downregulated in SD mice. (Fig. 3A-C). This suggests that, unlike p65 downregulation, IKKα downregulation in SD mice could be a model-specific mechanism of NF-kB inhibition. IKKα also showed a similar sex-specific pattern to p50 and p65in 12-month-old UM-HET3 mice, where male mice had significantly lower amounts of IKKα compared to female mice. IKKβ was significantly downregulated in RAPA and CANA mice, non-significantly downregulated in SD and ACA mice (p-value = 0.059 and 0.092, respectively), and was unchanged in GHRKO or 17αE2, (Fig. 3D-E) (Table 2). Since the two-way ANOVA interaction term for IKKβ was significant in GHRKO mice, a subsequent t-test was performed and revealed that IKKβ level goes up only in male GHRKO mice.

Total levels of the inhibitor IκB-α were unchanged in GHRKO, 17αE2-, RAPA-, or ACA-treated mice livers, but were downregulated in CANA-treated mice (Supp. Figure 2A, B). The inactivated phosphorylated form of IκB-α (p-IκB-α) was significantly downregulated in GHRKO mice, downregulated to a suggestive extent in CANA mice (p-value = 0.065), and unchanged in 17αE2-, RAPA-, or ACA-treated mice (Supp. Figure 2C, D). Again, there was a significant sex effect where male GHRKO had lower levels of the inactivated NF-κB inhibitor p-IκB-α compared to their female counterparts. Interestingly 22-month-old UM-HET3 mice appeared to have an opposite sex effect, where control and CANA-treated male mice had significantly higher levels of p-IκB-α. Finally, we also calculated the ratio of p-IκB-α to total IκB-α. None of the tested interventions (GHRKO, 17αE2, RAPA, ACA, and CANA) induced any changes to the p-IκB-α ratio, but 22-month-old UM-HET3 male mice demonstrated significantly higher ratio of p-IκB-α:IκB-α when compared to their female counterparts (Supp. Figure 3E) (Table 2). Overall, the inter-animal variability of the IKKs, IκB, and p- IκB data was higher than other markers, as evident by relatively high SEM.

Regulators of p65 activity are downregulated in multiple slow-aging mice

Except for p65, NF-κB signaling regulators including p50, IKKα, IKKβ, IκB-α, and IκB-β did not appear to be consistently modulated by different anti-aging interventions. Next, we wanted to evaluate regulators that are not integral parts of the NF-κB system but have been reported to modulate p65 activity. We were particularly interested in NCoR1, because we previously found it to be downregulated in liver of GHRKO and SD mice

Table 2 Protein level changes in activators and inhibitors of NF-κB signaling in different slow-aging mouse models

Model		IKKα	IKKβ	IkB-α	p-IkB-α
Mutant mice	GHRKO	-	↑↑ (M)	-	↓↓
	SD	↓↓	↓	NA	NA
Drug treated and CR mice	17α-estradiol	-	-	↑	-
	Rapamycin	↓↓ (M)	↓↓	-	-
	Acarbose	↓↓ (M)	↓	-	↓
	Canagliflozin	↓	↓↓	↓↓	↓

↓↓: significant downregulation (p-value < 0.05), ↑↑: significant upregulation (p-value < 0.05), ↓: trend downregulation (p-value < 0.2), ↑: trend upregulation (p-value < 0.2), M: change is seen only in male mice, NA: data not available

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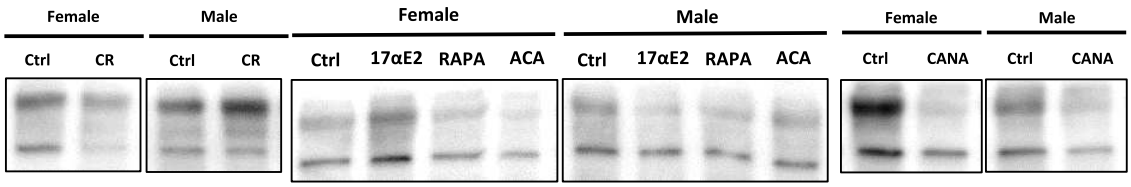

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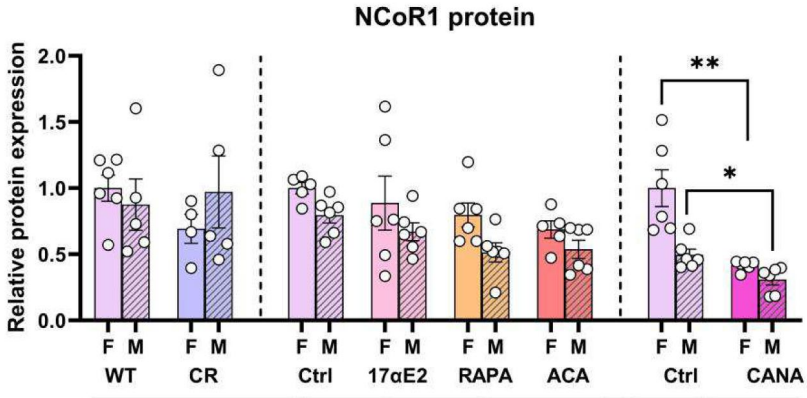

	CR	17aE2	RAPA	ACA	CANA
Source of variation	P value	P value	P value	P value	P value
Interaction	0.291	0.962	0.59	0.659	0.017
Sex	0.682	0.091	0.003	0.01	<0.001
Intervention	0.575	0.332	0.003	<0.001	<0.001

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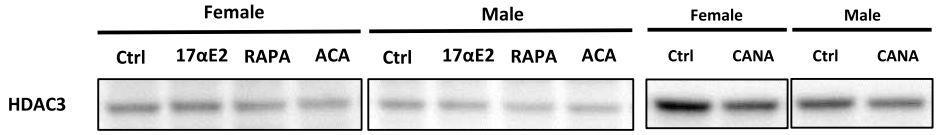

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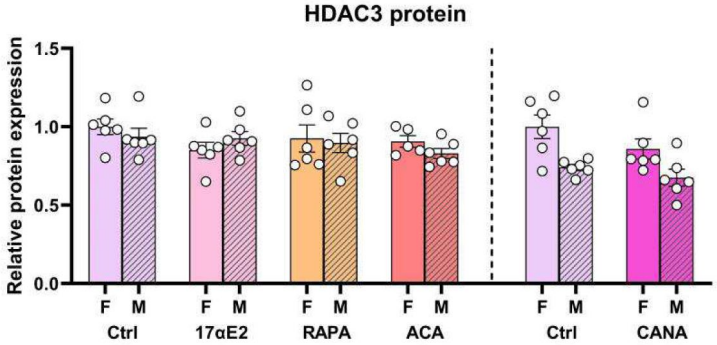


	17aE2	RAPA	ACA	CANA
Source of variation	P value	P value	P value	P value
Interaction	0.176	0.786	0.895	0.509
Sex	0.913	0.481	0.139	0.001
Intervention	0.126	0.394	0.041	0.086

◂**Fig. 4** Levels of NCoR1/HDAC3 co-repressor components in the liver of multiple long-lived mouse models. (A,B) Western blot images **(A)** and levels **(B)** of NCoR1 protein in livers of CR mice or mice treated with 17αE2, RAPA, ACA, or CANA. (C,D) Western blot images **(C)** and levels **(D)** of HHDAC3 protein in livers of mice treated with 17αE2, RAPA, ACA, or CANA. $n = 5–6$ for each group. Two-way ANOVA was used for analysis of intervention effect, sex effect, and their interaction. Unpaired t-test was used when the interaction term was significant. * $p < 0.05$, ** $p < 0.01$

[23]. NCoR1 is a histone deacetylase 3 (HDAC3) co-factor that was linked to p65 through multiple lines of evidence. For example, macrophage-specific deletion of NCoR1 inhibited p65 activation via phosphorylation [29]. ChIP-Seq analysis further revealed that NCoR1 deletion in macrophages led to reduced p65 transcriptional output, without changes in its DNA binding, leading to uncoupling NF-κB binding and subsequent steps required for pro-inflammatory gene activation [20]. Finally, it was found that signaling by the receptor activator of nuclear factor κB (RANK) converts the NCoR/HDAC3 complex from a co-repressor role to a co-activator of NF-κB target genes [22]. In our present study, we found that NCoR1 was unchanged in response to 17αE2 or CR but was significantly reduced by RAPA and ACA. NCoR1 was significantly reduced by CANA as well, with greater effects in female mice (Fig. 4A, B). Those effects, along with our previously reported downregulation of NCoR1 in GHRKO and SD mice, mirror to a high degree the effects of different interventions on p65 protein level, except for CR.

We previously reported downregulation of HDAC3 in GHRKO and SD mice. Here, we measured HDAC3 levels in 12-month-old drug-treated slow-aging mice. We found that HDAC3 level was unchanged in 17αE2 or RAPA mice but was significantly downregulated in ACA mice. In 22-month-old CANA mice, there was a decline in HDAC3 level with a p-value that approached statistical significance levels ($p = 0.086$) (Fig. 4C, D).

Finally, we were also interested in measuring the levels of CDKs, which regulate p65 activity by phosphorylation [18, 21, 54]. CDK5 levels were significantly lower in liver of Snell, GHRKO, RAPA, and ACA mice (Supp. Figure 4A, C), with parallel but non-significant declines in 17aE2, CANA, and CR mice (p-value = 0.108, 0.086, and 0.089, respectively) (Supp. Figure 3) (Table 3).

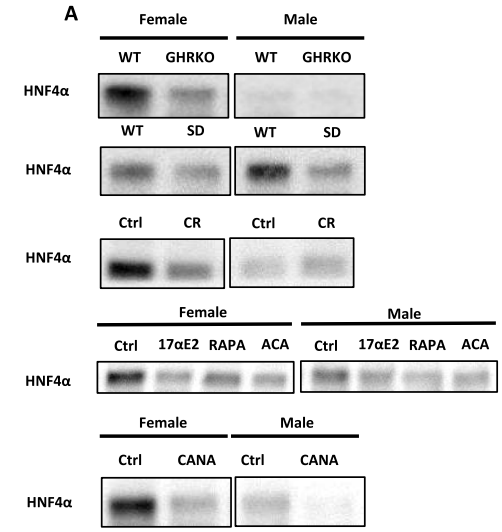
Downstream p65 target proteins decline in a similar, but not identical, pattern to p65 protein

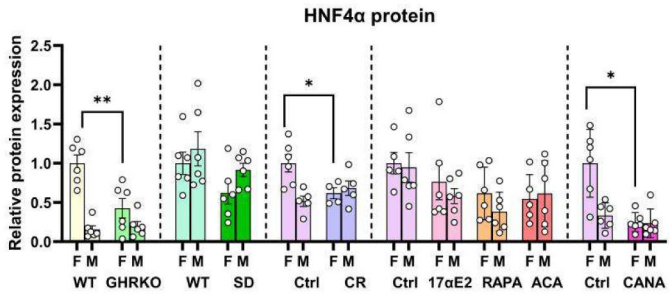
There are hundreds of genes downstream to NF-κB and under p65 control. In the liver, those genes mediate diverse functions including pro-inflammatory and metabolic processes [16, 18, 19, 36, 55, 56]. Here, we were first interested in the hepatocyte nuclear factor 4 alpha (HNF4α) due to its multiple links to the NF-κB pathway. For example, transcription of HNF4α is p65-dependent, and it further amplifies NF-κB inflammatory response by stimulating the expression of interleukin-1 receptor 1 (IL-1R1) [33]. Furthermore, HNF4α recruits the NCoR1/HDAC3 complex in mouse liver to control a gene program responsible for regulating lipid

Table 3 Changes in regulators and downstream targets of p65 in livers of long-lived mouse models

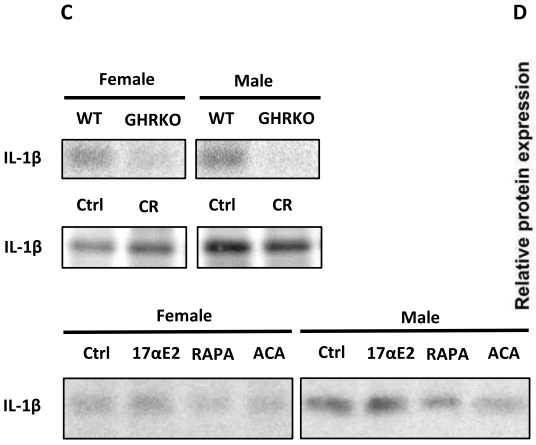
Model		Regulators of p65			Downstream targets of p65		
		NCoR1	**HDAC3**	**CDK5**	**HNF4α**	**IL-1β**	**CRP**
Mutant mice	GHRKO	↓↓[23]	↓↓[23]	↓↓	↓↓ (F)	↓↓	↓↓ (M)
	Snell Dwarf	↓↓[23]	↓↓[23]	↓↓	↓↓	NA	NA
Drug treated and CR mice	CR	-	NA	↓	↓↓ (F)	-	↓↓
	17α-estradiol	-	↓	↓	↓	-	-
	Rapamycin	↓↓	-	↓↓ (M)	↓↓	↓↓	-
	Acarbose	↓↓	↓↓	↓↓ (M)	↓↓	↓↓	↓↓
	Canagliflozin	↓↓	↓	↓	↓↓ (F)	NA	NA

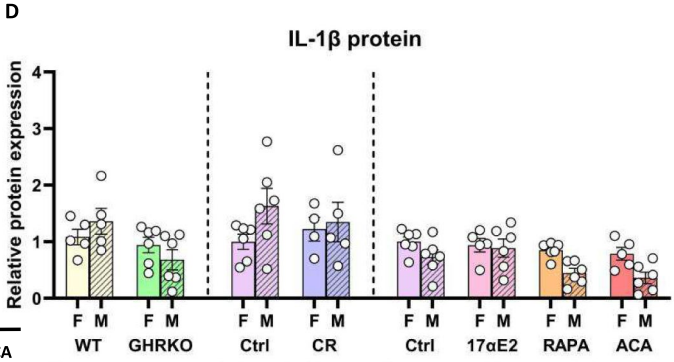
↓↓: significant downregulation (p-value < 0.05), ↓: trend downregulation (p-value < 0.2), F: change is seen only in female mice, M: change is seen only in male mice, NA: data not available. Data from previous research is indicated by a citation number



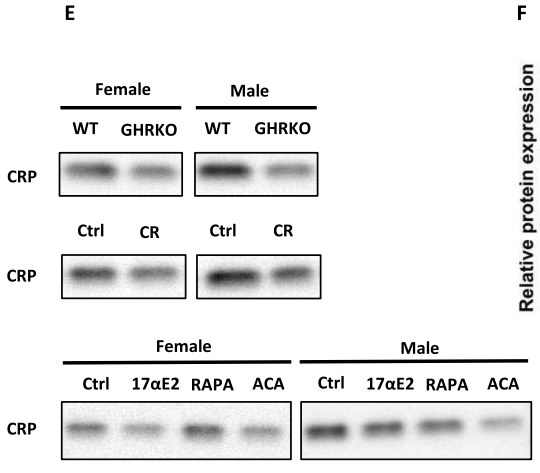


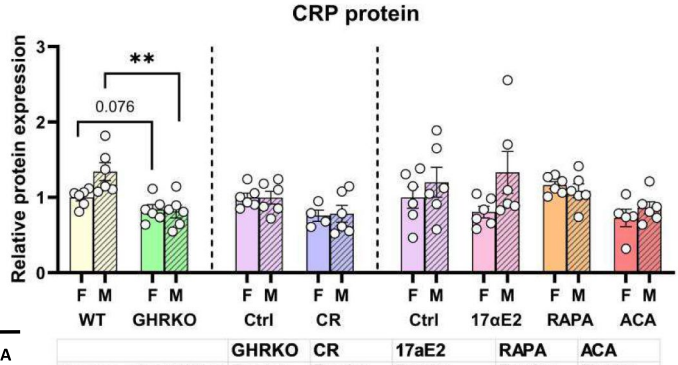
	GHRKO	SD	CR	17aE2	RAPA	ACA	CANA
Source of variation	P value	P value	P value	P value	P value	P value	P value
Interaction	0.003	0.72	0.01	0.716	0.543	0.719	0.005
Sex	<0.001	0.131	0.04	0.507	0.337	0.965	0.004
Intervention	0.007	0.045	0.267	0.097	0.004	0.029	<0.001





	GHRKO	CR	17aE2	RAPA	ACA
Source of variation	P value	P value	P value	P value	P value
Interaction	0.137	0.369	0.369	0.481	0.494
Sex	0.961	0.185	0.222	0.002	0.005
Intervention	0.029	0.913	0.7	0.031	0.016





	GHRKO	CR	17aE2	RAPA	ACA
Source of variation	P value	P value	P value	P value	P value
Interaction	0.041	0.875	0.399	0.308	0.823
Sex	0.072	0.897	0.068	0.643	0.257
Intervention	<0.001	0.019	0.868	0.85	0.048

◂**Fig. 5** NF-κB downstream target proteins are differentially regulated in the livers of different long-lived mouse models. (A,B) Western blot images **(A)** and levels **(B)** of HNF4α protein in livers of GHRKO, SD, CR, 17αE2, RAPA, ACA, and CANA mice. (C,D) Western blot images **(C)** and levels **(D)** of IL-1β protein in livers of GHRKO, CR 17αE2, RAPA, and ACA mice. (E,F) Western blot images **(E)** and levels **(F)** of CRP protein in livers of GHRKO, CR 17αE2, RAPA, or ACA mice. $n = 5–6$ for each group. Two-way ANOVA was used for analysis of intervention effect, sex effect, and their interaction. Unpaired t-test was used when the interaction term was significant. * $p < 0.05$, ** $p < 0.01$

homeostasis in the liver through inhibition of hepatic triglyceride content [24, 30]. Six of the seven interventions tested led to significant decline in HNF4α, though with variation in sex-specificity. HNF4α was significantly downregulated in both sexes in SD, RAPA-, and ACA-treated mice, and significantly downregulated in females only in GHRKO, CANA, and CR mice (Fig. 5A, B). 17aE2 showed some evidence for a decline, though it did not reach statistical significance (p = 0.097). These data correlate with the downregulation of p65 in all of those models except in 17αE2-treated mice.

The next p65 target we evaluated was the pro-inflammatory cytokine IL-1β. Similar to HNF4α, IL-1β is both a target and an activator of NF-κB signaling [34–36]. We found that IL-1β was significantly downregulated in both sexes of GHRKO and in mice treated with RAPA or ACA but was unchanged in CR and 17αE2-treated mice (Fig. 5C, D).

Finally, we also measured CRP protein. CRP is a complement protein, and a major inflammatory marker in the liver [37]. Here, we found that CRP was significantly downregulated in male GHRKO mice and non-significantly downregulated in females (p-value = 0.076). It was also significantly downregulated with CR and ACA-treated mice, and unchanged in 17αE2- or RAPA-treated mice (Fig. 5E, F).

To summarize, we measured the protein levels of three p65 targets involved in various inflammatory and metabolic pathways in the liver. HNF4α was downregulated in 6 out of 7 models, with female specific downregulation in CANA and CR mice. IL-1β and CRP were each downregulated in 3 out of 5 models. 17αE2, one of the two models that did not demonstrate p65 downregulation in our initial assays, consistently showed no downregulation in all three targets (Table 3).

Discussion

NF-κB is a major pro-inflammatory transcription factor that regulates a wide range of cytokines, chemokines, and enzymes [3, 16, 57]. Changes in NF-κB could potentially contribute to anti-inflammatory effects of anti-aging interventions. We hypothesized that NF-κB signaling would be downregulated in slow-aging mice based on different lines of data. First, NF-κB is consistently upregulated with age in multiple tissues including liver, white adipose tissue, skeletal muscle, and brain [1, 3, 16, 57]. Analysis of data from 294 microarrays spanning six human tissue types including kidney, brain, muscle, and fibroblasts, and three murine tissues including heart, kidney, and hematopoietic stem cells, suggested that NF-κB was the most strongly enriched motif in aging, and suggested NF-κB signaling was a major mediator of age-dependent changes in gene expression in human and mouse [58]. The second reason NF-κB presented an attractive pathway to study in various longevity models was that inhibition of NF-κB in different tissues can mediate health and lifespan benefits. For example, downregulation of NF-κB signaling in the liver via tissue-specific p65 knockdown leads to enhanced insulin sensitivity [19]. Inhibition of NF-κB signaling in the hypothalamic microglia by introduction of a lentiviral vector expressing a dominant negative form of IKKβ extended mouse lifespan [53]. Finally, hepatic NF-κB signaling can mediate systemic level changes in inflammation by upregulation of the acute phase response (APR) proteins [55, 56]. Recently, another group reported that rapamycin reduces NF-κB activity in the liver by increasing the interaction between the NF-κB protein p65 and its inhibitor IκB-α [13].

In this study, we found that p65, the main active protein unit in classical NF-κB signaling, is consistently downregulated in response to diverse anti-aging interventions. We tested a total of ten interventions: three mutant models using mice 5–6 months old (GHRKO, SD, and PAPP-A KO), four longevity consortium models using 12 months old mice (17αE2, RAPA, ACA, and CR), and three ITP models using 22 months old mice (CANA, MEC, astaxanthin). We found p65 to be significantly downregulated in both sexes of seven of those models (GHRKO, SD, RAPA, ACA, CR, CANA, and MEC), in females

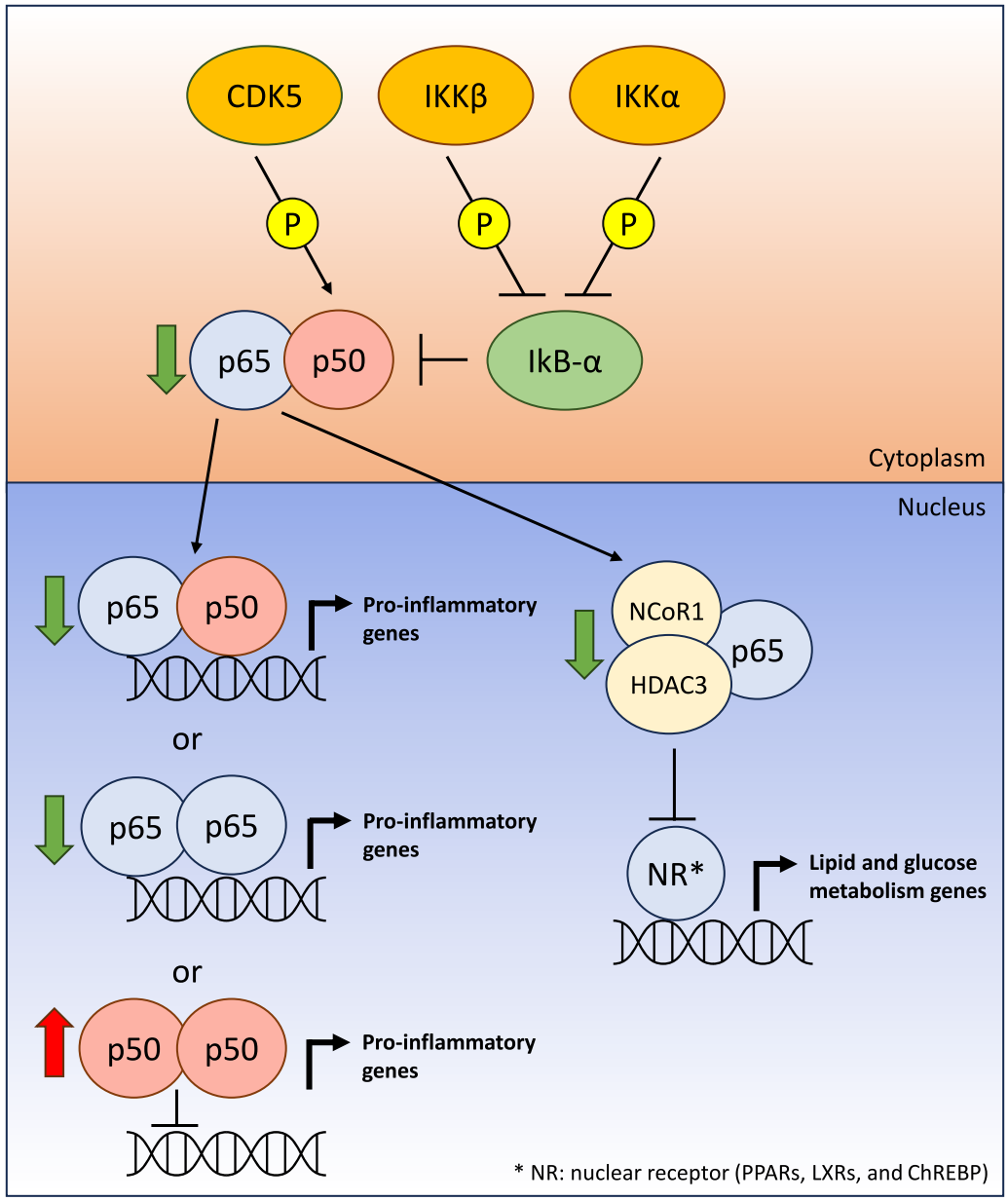


Fig. 6 Potential mechanisms through which p65 downregulation can regulate pro-inflammatory and metabolic genes in livers of slow aging mice. Black arrows show the established pathways. Green and red arrows indicate changes (or potential changes) in slow aging mice livers

only of PAPP-A KO mice, and to be unchanged in two models (17αE2 and astaxanthin). Overall, those results demonstrate that hepatic p65 downregulation is seen in many, but not all, anti-aging interventions. The difference in age between different groups of models represent both a strength and a limitation to the study. The strength was that p65 appeared to be downregulated in most of those models regardless of the age of the mice. However, since we have not tested the same model at two or more different ages, we are not able to conclude that p65 downregulation is equivalent, for all interventions tested, throughout the lifespan of slow aging models. Additionally, the two models that did not demonstrate p65

downregulation (i.e., 17αE2 and astaxanthin) could possibly have led to changes in p65 at ages we did not evaluate.

The downregulation of p65 in the livers of long-lived mice is a novel finding suggesting at least a partial downregulation of NF-κB target genes, which agrees with previous research [5]. On the other hand, we report either a lack of changes or inconsistent downregulation of p50 protein in different anti-aging models. It is important to note that unlike p65, p50 lacks the transactivating domain (TAD) that initiates transcription [17]. Indeed, it was reported that p50 dimers can bind to DNA targets of NF-κB, but cannot activate their transcription, acting as a steric inhibitor of NF-κB signaling in the process [17, 59, 60]. Downregulation of p65 with no significant change in p50 could indicate an inhibition of p65-p50 heterodimer formation, p65-p65 homodimer formation, and/or upregulation of the NF-κB inhibitor p50-p50 homodimer formation. The models where p50 was significantly downregulated (CR females, RAPA, ACA, or CANA) suggest downregulation in the p50-p65 heterodimer. Importantly, rapamycin does not share many of the metabolic phenotypes reported in other anti-aging interventions (e.g., GHRKO, CR, and CANA) including increased insulin sensitivity and oxidative metabolism [23, 44, 61–69]. However, it shows a similar hepatic p65 downregulation to the other models suggesting that rapamycin may uncouple metabolic and anti-inflammatory pro-longevity phenotypes.

Other methods of inhibition of classical NF-kB signaling could potentially include downregulation of activators IKKα and IKKβ, upregulation of the inhibitor IkB-α, or inhibition of its phosphorylation. We assayed the levels of those changes but found no consistent phenotype across different slow-aging mice. IKKα was significantly downregulated in both sexes of SD mice, and in male ACA and RAPA mice (Fig. 3A-C), while IKKβ was significantly downregulated in RAPA and CANA mice, and non-significantly downregulated in SD livers (p-value = 0.059) (Fig. 3D-F). IkB-α was non-significantly upregulated in 17αE2 (p-value = 0.124), while its phosphorylation level was significantly downregulated in GHRKO mice and almost significantly downregulated in CANA mice (p-value = 0.065) (Supp. Figure 2A-D). These results indicate that other intervention-specific ways to inhibit NF-kB signaling may exist, but they are not as commonly shared by different interventions as p65 downregulation. For example, while GHRKO and SD mice share many phenotypes that are related to or caused by disruption of growth hormone signaling, there were few NF-kB features that were only downregulated in SD, but not in GHRKO livers, including downregulation of p50 (p-value = 0.117), IKKα (p-value < 0.001) and IKKβ (p-value = 0.059). Those differences between the two slow aging mutant mouse models could be due to different background strains or the hypothyroidism phenotype previously reported in SD mice [70, 71].

We also measured levels of p65 regulators [20, 21, 29, 54]. The rationale behind this was two-fold: first, to seek evidence that the other members of the p65 transcription complex were downregulated, and second, to if downregulation of p65 regulators could be another consistent phenotype between anti-aging models, potentially even in models like 17αE2 where p65 levels were unchanged. Since we previously reported the transcription repressor NCoR1 to be downregulated in mutant models like GHRKO and SD mice [23], we were interested to see if NCoR1 was also modified in other varieties of slow-aging mice. NCoR1 is a co-factor for HDAC3, and they can function together as a transcriptional repressor for many transcription factors that mediate oxidative metabolism, including PPARs and LXRs [23, 26, 27, 72, 73]. The transcriptional repressor complex of HDAC3/NCoR1 was previously reported to activate NF-kB signaling through inhibition of IkBα transcription [74]. The complex of NCoR1 and HDAC3 has been reported to bind to p65 and modulate its transcriptional activity [20, 29, 75]. Interestingly, NCoR1 can also bind to p65 and acts as an activator instead of a suppressor for a specific transcription program that was reported in osteoclast differentiation (Fig. 6) [22]. The NCoR1/HDAC3/p65 co-activator complex have also been reported to activate transcription on inflammatory genes in macrophages [76]. Future studies will be needed to learn whether p65 and NCoR1 downregulation in multiple slow aging mouse models reduces repression of oxidative metabolism genes, decreased transcription of pro-inflammatory genes, or both. Unphosphorylated p65 has also been reported to act as a transcription repressor by recruiting histone deacetylases to gene sites in proximity to its own targets [77]. Additionally, certain roles of p65, including its role in activating apoptosis, were unrelated to its

role as a transcription factor [78]. This suggests that shared downstream effects of p65 downregulation in long-lived mice could include phenotypes that are not directly related to its transcription factor activity.

This study was limited by multiple factors. First, the results are mainly descriptive, with some limited causal inferences, intended to set a foundation for future studies. Second, the data are limited to liver tissue; studies of NF-kB in other cells and tissues may well also be informative.

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Declarations

Competing interests The authors have no relevant financial or non-financial interests to disclose.

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