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ORIGINAL ARTICLE

Coordinated transcriptional upregulation of oxidative metabolism proteins in long-lived endocrine mutant mice

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Abstract Caloric restriction (CR), which extends lifespan in rodents, leads to increased hepatic fatty acid β-oxidation and oxidative phosphorylation (OXPHOS), with parallel changes in proteins and their mRNAs. Genetic mutants that extend lifespan, including growth hormone receptor knockout (GHRKO) and Snell dwarf (SD) mice, have lower respiratory quotient, suggesting increased reliance on fatty acid oxidation, but the molecular mechanism(s) of this metabolic shift have not yet been worked out. Here we show that both GHRKO and SD mice have significantly higher mRNA and protein levels of enzymes involved in mitochondrial and peroxisomal fatty acid β-oxidation. In addition, multiple subunits of OXPHOS complexes I-IV are upregulated in GHRKO and SD livers, and Complex V subunit ATP5a is upregulated in liver of GHRKO mice. Expression of these genes is regulated by a group of nuclear receptors and transcription factors including peroxisome proliferator-activated receptors (PPARs) and estrogen-related receptors (ERRs). We found that levels of these nuclear receptors and their co-activator PGC-1α were unchanged or downregulated in liver of GHRKO and SD mice. In contrast, NCOR1, a co-repressor for the same receptors, was significantly downregulated in the two long-lived mouse models, suggesting a plausible mechanism for the changes in FAO and OXPHOS proteins. Hepatic levels of HDAC3, a co-factor for NCOR1 transcriptional repression, were also downregulated. The role of NCOR1 is well established in the contexts of cancer and metabolic disease, but may provide new mechanistic insights into metabolic control in long-lived mouse models.

Keywords Longevity · β-oxidation · OXPHOS · Mitochondria · NCOR1 · GHRKO

Introduction

Changes in cellular fuel utilization, including modifications in lipid synthesis, storage, and mobilization, have been linked to lifespan and age-dependent physiological status in invertebrates [1], mice [2, 3], and humans [4], and are likely to contribute to increased risks of metabolic syndrome, obesity, and diabetes in human populations [5, 6]. Many lifespan-extending interventions in mice have elevated fatty acid β-oxidation. Caloric restriction (CR) induces whole body fatty acid oxidation (FAO) and increases reliance

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on fatty acids for fuel [7]. CR also upregulates many enzymes involved in the β-oxidation pathway [8]. Transgenic overexpression of fibroblast growth factor 21 (FGF21) also extends murine lifespan and induces hepatic FAO [9, 10]. PTEN overexpression, another genetic intervention that extends murine lifespan, has been shown to induce FAO in calf hepatocyte cell cultures, and knockdown of PTEN inhibited FAO [11, 12]. Pharmacological interventions, like rapamycin, has also been shown to induce FAO in cell cultures of rat hepatocytes and skeletal muscle cells [13, 14]. Growth hormone receptor knockout (GHRKO) mice are more dependent on fat oxidation for fuel, more resistant to diet-induced obesity, and show higher levels of FAO [15–17].

Mitochondrial FAO is physically and functionally associated with oxidative phosphorylation (OXPHOS). For example, FAO provides reducing equivalents that enter the OXPHOS pathway at the level of complexes I and III. Additionally, FAO trifunctional protein (TFP) enzyme interacts with complex I of the OXPHOS pathway [18–20]. Using blue native polyacrylamide gel electrophoresis to separate OXPHOS complexes from liver mitochondrial extracts reveals co-migration of FAO enzymes with OXPHOS complexes [20]. Furthermore, many genetic disorders in OXPHOS proteins impair FAO, and conversely pharmacological inhibitors of FAO reduce OXPHOS capacity [21, 22]. In humans, fibroblasts from patients with loss of acyl-CoA dehydrogenase medium chain (ACADM) demonstrate defects in biogenesis, stability, and activity of OXPHOS complexes [23]. In terms of relationship to longevity, a recent study found that upregulation of OXPHOS is one of the major hepatic gene expression signatures associated with extended lifespan in multiple murine interventions including CR, growth hormone deficiency, and rapamycin treatment [24]. Another study found OXPHOS gene upregulation to be the top common transcriptional signature in cochlea, hippocampus, heart, liver, kidney, gastrocnemius, and white adipose tissues in CR mice. They also found this signature to be conserved in CR flies and in tissues from CR rats and rhesus monkeys [25].

Many OXPHOS and FAO genes are regulated by a group of nuclear receptors (NR) and transcription factors (TF) that have been implicated in longevity and metabolic reprogramming in many long-lived mouse models. Peroxisome proliferator-activated receptors (PPARs) α and δ, and estrogen-related receptors (ERRs), are the master transcriptional regulators for FAO and OXPHOS genes, respectively [26–31]. There is little information on ERRs in mouse longevity models, but PPARs have been studied in long-lived mice. Livers from CR mice had significantly higher protein levels of PPARα, significantly lower levels of PPARβ/δ, and no significant changes in levels of PPARγ. GHRKO mice had significantly higher levels of hepatic PPARα and γ, and significantly lower levels of PPARβ/δ. Skeletal muscle of both GHRKO and CR mice had significantly lower PPARα, β/δ, and γ levels [32, 33]. PPARs and ERRs, as well as other NRs/TFs, are co-activated by binding to peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α). Consistent with these findings, PGC-1α was reported in multiple studies to be upregulated in liver and adipose tissue of CR mice, and in liver of Snell Dwarf mice [34–38]. Many of the metabolic benefits of protein restriction (PR) diets, which also extend lifespan, have been linked to increased hepatic fibroblast growth factor 21 (FGF-21) and the subsequent increase in PGC-1α [10, 39–43]. PGC-1α in vitro overexpression in the 3T3-L1 preadipocyte cell line led to significant enrichment of PPAR signaling, fatty acid metabolism, and OXPHOS KEGG pathways [44].

Nuclear receptor corepressor 1 (NCOR1) is also able to regulate PPARs and ERRs [45, 46]. NCOR1 has recently been identified as a conserved metabolic switch that regulates oxidative metabolism signaling [46]. It forms a repressive complex that inhibits the activity of both PPARs and ERRs and downregulates their transcription targets [45, 47–50]. While the role of NCOR1 in metabolic regulation is well established, and resembles metabolic phenotypes seen in long-lived murine models, levels of NCOR1 in those models have not been evaluated.

Here we report levels of FAO enzymes and OXPHOS subunits in males and females of long-lived endocrine mutant mice, and evaluate transcriptional regulators known to influence these enzymes.

Methods

Mice

GHRKO and SD mice were generated and maintained as previously described [51–53], and used at 5–6 months of age. Experiments were conducted

on 5–6 male and 5–6 female mice for control and mutant 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

Liver samples were collected from mice at a specific time window (from 9:30 to 11:30 a.m.) to ensure the changes in protein levels were not influenced by circadian rhythms. 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, 23227). The protein extracts were separated by SDS/PAGE on a 4–15% precast gel (BioRad, 4561096), and transferred to Immun-Blot PVDF Membrane (BioRad, 1620177). Membranes were then evaluated using EcoBright Femto HRP 100 (Innovative Solutions, EBFH100). Histone H3 was used as a protein loading control, and no significant changes were found in GHRKO or SD Histone H3 levels (data not shown). 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 RNeasy Mini Kit (Qiagen, 74104). The concentration of total RNA was measured using Nanodrop One (Thermo). cDNA was reverse transcribed from 2 µg of total RNA using iScript cDNA reverse transcription kit (BioRad, 1708891).

Quantitative real-time PCR

qPCR was performed using TaqMan Fast Advanced Master Mix (Applied Biosystems, 4444557). RT-PCR was performed using quantitative PCR systems (Thermo) with corresponding TaqMan gene expression assay probes (Thermo, 4331182). 18S was simultaneously assayed as a loading control. A full list of TaqMan assays used in this paper can be found in Table S2. The expression levels of different mRNA were reported as CT values.

Statistical analysis

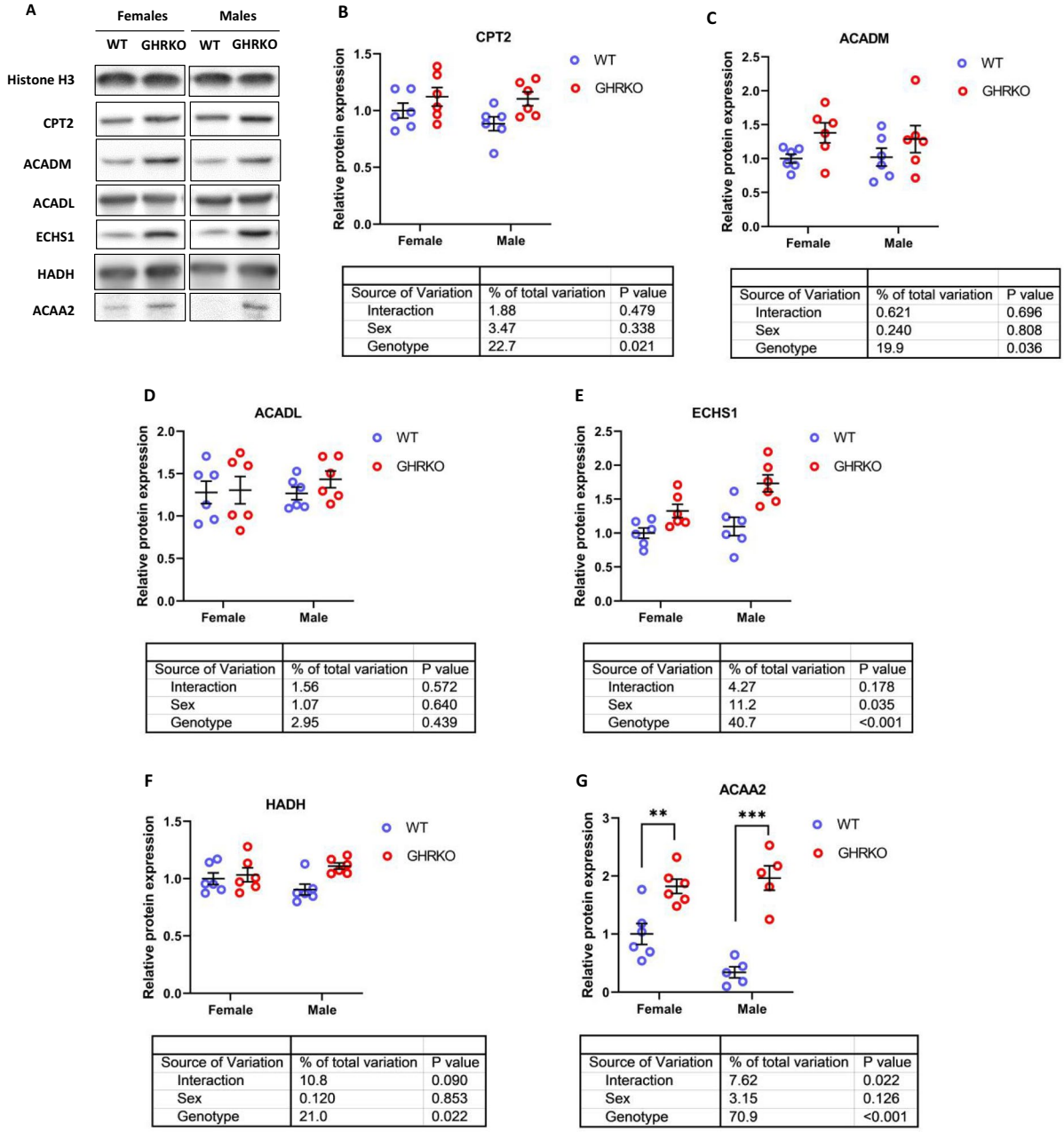
Statistical analyses and plotting were conducted in GraphPad Prism (version 9). All data are presented as mean ± SEM. Data from male and female mice were not pooled. 2-way ANOVAs were used for reporting sex effect, genotype effect, and their interaction. An unpaired Student t-test was performed when a significant interaction term was noted.

Results

Higher levels of mitochondrial fatty acid β-oxidation proteins in liver of SD and GHRKO mice

Multiple murine lifespan-extending interventions, including CR and FGF21 treatment, show higher levels of hepatic fatty acid β-oxidation [8–10]. CR mice were previously reported to have higher protein levels of multiple mitochondrial β-oxidation enzymes [8]. GHRKO mice were previously reported to have reduced respiratory quotient (RQ) indicating an increased reliance on fat oxidation rather than carbohydrate metabolism [17]. We therefore undertook a comprehensive survey of proteins involved in hepatic fatty acid oxidation in GHRKO and SD mice (Fig. S1).

We found that levels of carnitine palmitoyltransferase 2 (CPT2), an enzyme that transports fatty acids across the mitochondrial membrane, were significantly upregulated in GHRKO and SD liver (Figs. 1A, B, S2A, and S2B). We measured protein levels of two acyl-CoA dehydrogenases, long chain (ACADL) and ACADM, which differ in specificity based on fatty acid chain length. We found that GHRKO and SD livers had significantly higher ACADM (Figs. 1C and S2C), but that ACADL levels were not significantly changed (Figs. 1D and S2D). This agrees with previously published data on ACADL in SD livers, and ACADM in CR livers [8, 54]. Enoyl-CoA Hydratase, Short Chain 1 (ECHS1), the enzyme responsible for the second step of mitochondrial fatty acid β-oxidation, was significantly upregulated in both



Source of Variation	% of total variation	P value
Interaction	1.88	0.479
Sex	3.47	0.338
Genotype	22.7	0.021

Source of Variation	% of total variation	P value
Interaction	0.621	0.696
Sex	0.240	0.808
Genotype	19.9	0.036

Source of Variation	% of total variation	P value
Interaction	1.56	0.572
Sex	1.07	0.640
Genotype	2.95	0.439

Source of Variation	% of total variation	P value
Interaction	4.27	0.178
Sex	11.2	0.035
Genotype	40.7	<0.001

Source of Variation	% of total variation	P value
Interaction	10.8	0.090
Sex	0.120	0.853
Genotype	21.0	0.022

Source of Variation	% of total variation	P value
Interaction	7.62	0.022
Sex	3.15	0.126
Genotype	70.9	<0.001

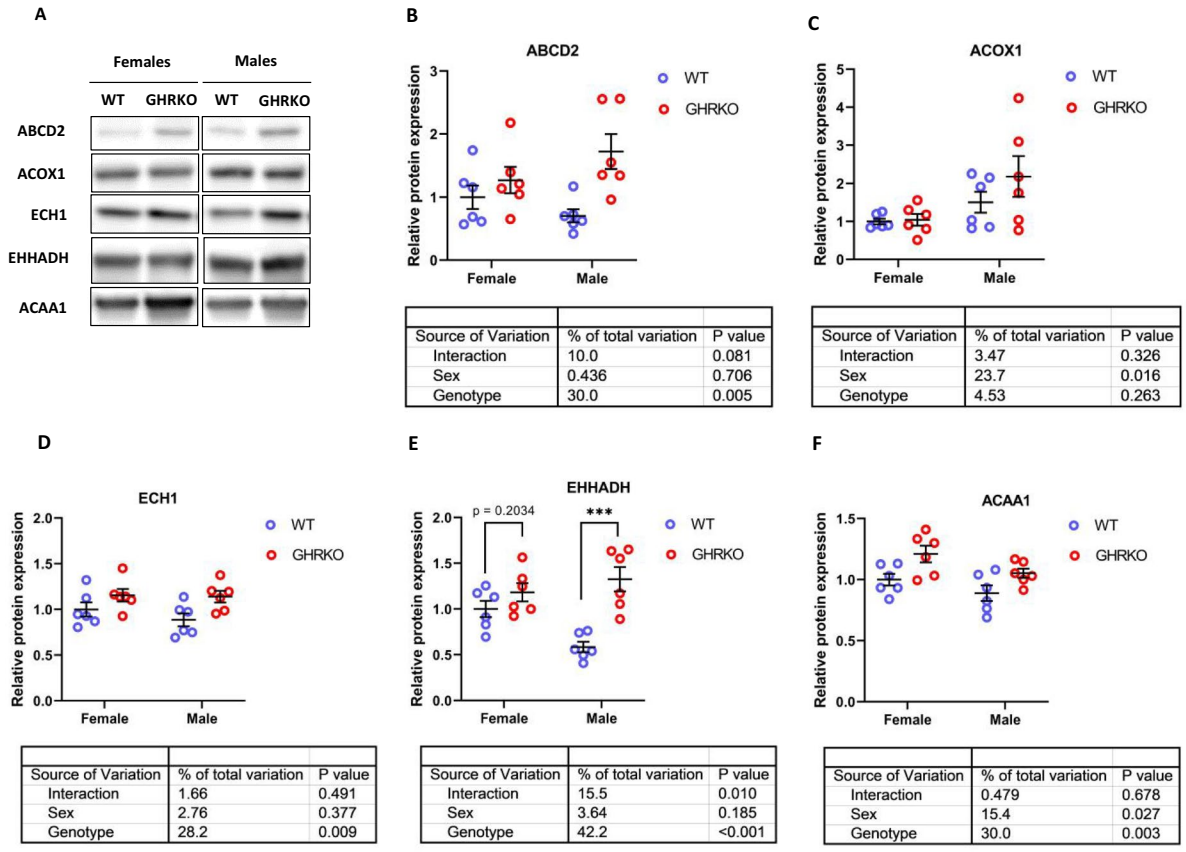
Fig. 1 Effect of growth hormone receptor knockout on mitochondrial fatty acid β-oxidation enzymes in liver. (**A**) Representative western blots for mitochondrial FAO enzymes in liver lysates from female and male wild type (WT) and GHRKO mice. (**B**-**G**) Scatter plots of CPT2 (**B**), ACADM (**C**), ACADL (**D**), ECHS1 (**E**), HADH (**F**), and ACAA2 (**G**). Data show mean ± S.E.M. Each symbol represents an individual mouse. n = 5–6 for each group. Two-way ANOVA was used for analysis of genotype effect, sex effect, and their interaction. Unpaired t-test was used for each sex separately when the interaction term was significant. ** $P < 0.01$, *** $p < 0.001$

GHRKO and SD livers (Figs. 1E and S2E). Hydroxyacyl-CoA Dehydrogenase (HADH) and acetyl-CoA acyltransferase 2 (ACAA2), the enzymes responsible for the final two steps of mitochondrial β-oxidation were both significantly upregulated in GHRKO and SD livers (Figs. 1F, G, S2F, and S2G).

GHRKO and SD mice have higher levels of peroxisomal fatty acid β-oxidation proteins

Peroxisomes are another cellular site for fatty acid β-oxidation (Fig. S1). Previous studies reported an increase in peroxisomal FAO proteins in liver of acarbose-treated and SD mice [55, 56]. In CR mice, acyl-CoA Oxidase 1 (ACOX1), which is responsible for the first of four peroxisomal β-oxidation reactions, was downregulated compared to wild type mice [8].

ATP Binding Cassette Subfamily D Member 2 (ABCD2), the protein responsible for transporting fatty acids into the peroxisome, was significantly upregulated in GHRKO and SD livers (Figs. 2A, B, S3A, and S3B). ACOX1 was elevated in SD liver (Fig. S3C), but did not show a significant effect in GHRKO liver (Fig. 2C). In both models, ACOX1 was significantly higher in males than in females. Enoyl-CoA Hydratase (ECH1), the peroxisomal counterpart of mitochondrial ECHS1, was significantly upregulated in both GHRKO and SD livers (Figs. 2D and S3D). Enoyl-CoA Hydratase And 3-Hydroxyacyl CoA Dehydrogenase (EHHADH), which catalyzes the third reaction in the peroxisomal fatty acid β-oxidation pathway, was significantly upregulated in male GHRKO mice and in both sexes of SD mice, with a non-significant effect ($p=0.2$) in female GHRKO mice (Figs. 2E and S3E). Peroxisomal acetyl-CoA acyltransferase 1 (ACAA1) was significantly upregulated in GHRKO mice, but not in SD mice (p-value = 0.09 in SD) (Figs. 2F and S3F).



Source of Variation	% of total variation	P value
Interaction	10.0	0.081
Sex	0.436	0.706
Genotype	30.0	0.005

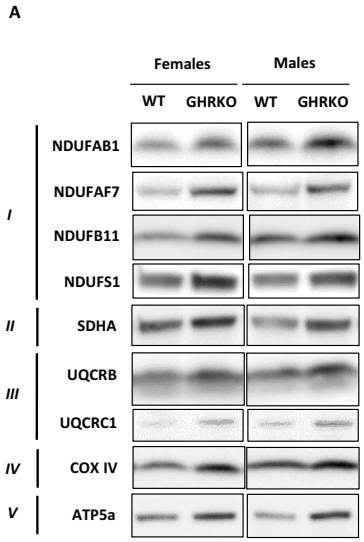
Source of Variation	% of total variation	P value
Interaction	3.47	0.326
Sex	23.7	0.016
Genotype	4.53	0.263

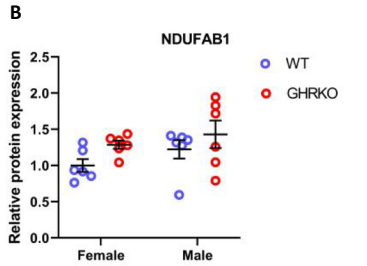
Source of Variation	% of total variation	P value
Interaction	1.66	0.491
Sex	2.76	0.377
Genotype	28.2	0.009

Source of Variation	% of total variation	P value
Interaction	15.5	0.010
Sex	3.64	0.185
Genotype	42.2	<0.001

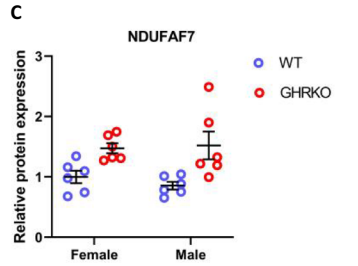
Source of Variation	% of total variation	P value
Interaction	0.479	0.678
Sex	15.4	0.027
Genotype	30.0	0.003

Fig. 2 Hepatic levels of peroxisomal fatty acid β-oxidation enzymes in WT and GHRKO livers. (**A**) Representative images of western blot data for peroxisomal FAO enzymes in liver lysates from female and male WT and GHRKO mice. (**B**-**F**) Scatter plots of ABCD2 (**B**), ACOX1 (**C**), ECH1 (**D**), EHHADH (**E**), and ACAA1 (**F**). Data show mean ± S.E.M. Each symbol represents an individual mouse. n = 5–6 for each group. Two-way ANOVA was used for analysis of genotype effect, sex effect, and their interaction. Unpaired t-test was used when the interaction term was significant. *** $p<0.001$

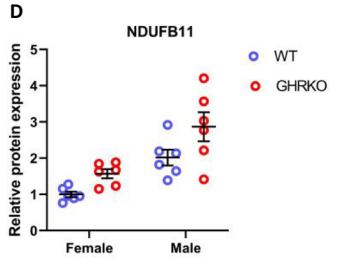




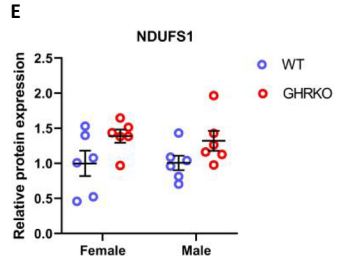
Source of Variation	% of total variation	P value
Interaction	0.383	0.755
Sex	8.18	0.160
Genotype	14.7	0.064



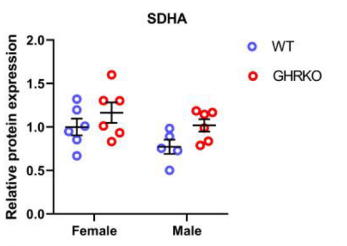
Source of Variation	% of total variation	P value
Interaction	1.28	0.493
Sex	0.342	0.722
Genotype	45.7	<0.001



Source of Variation	% of total variation	P value
Interaction	0.653	0.565
Sex	44.4	<0.001
Genotype	16.7	0.008



Source of Variation	% of total variation	P value
Interaction	0.287	0.784
Sex	0.191	0.823
Genotype	25.5	0.016



Source of Variation	% of total variation	P value
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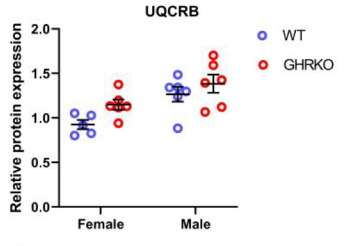
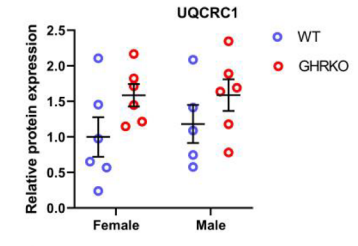
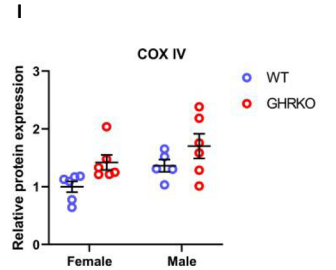
Sex	14.2	0.064
Genotype	17.3	0.043



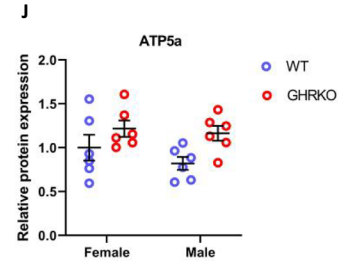
Source of Variation	% of total variation	P value
Interaction	1.23	0.510
Sex	36.6	0.002
Genotype	12.9	0.043



Source of Variation	% of total variation	P value
Interaction	0.621	0.704
Sex	0.648	0.698
Genotype	18.6	0.048



Source of Variation	% of total variation	P value
Interaction	0.262	0.778
Sex	15.4	0.041
Genotype	21.4	0.018



Source of Variation	% of total variation	P value
Interaction	1.28	0.550
Sex	4.35	0.275
Genotype	25.3	0.013

◂**Fig. 3** Hepatic levels of OXPHOS subunit proteins in WT and GHRKO livers. (**A**) Representative western blot images for OXPHOS subunits in liver lysates from female and male WT and GHRKO mice. (**B**-**J**) Scatter plots of NDUFAB1 (**B**), NDUFAF7 (**C**), NDUFB11 (**D**), NDUFS1 (**E**), SDHA (**F**), UQCRB (**G**), UQCRC1 (**H**), COX IV (**I**), and ATP5a (**J**). Data show mean ± S.E.M. Each symbol represents an individual mouse. n = 5–6 for each group. Two-way ANOVA was used for analysis of genotype effect, sex effect, and their interaction

Decreased growth hormone signaling also elevates expression of oxidative phosphorylation proteins

Mitochondrial fatty acid oxidation is physically and functionally linked to the oxidative phosphorylation (OXPHOS) pathway, with proteins in both pathways co-regulated on the transcriptional level by common nuclear receptors and shared transcription factors [18, 20, 35, 44, 45, 50]. Hence, our next aim was to measure the levels of different protein subunits that make up the five OXPHOS complexes.

First, we measured the levels of four subunits in complex I. NDUFAF7, NDUFB11, and NDUFS1 were all significantly upregulated in GHRKO livers, and NDUFAB1 showed a similar effect that approached conventional significance thresholds ($p = 0.06$) (Fig. 3A–E). In SD livers, NDUFAF7, NDUFB11, and NDUFAB1 were all significantly upregulated, while NDUFS1 showed a similar trend (p-value = 0.10) (Figures S4A-S4E). We also measured levels of SDHA (complex II), UQCRB and UQCRC1 (complex III), COX IV (complex IV), and ATP5a (complex V). The subunits from complexes II to V were all significantly upregulated in GHRKO livers (Fig. 3F–K). This is consistent with previous studies showing that upregulation of OXPHOS is a common transcriptional signature of long-lived models including CR and GHRKO [24, 25]. In Snell Dwarf livers, subunits from complexes II to IV (SDHA, UQCRB, UQCRC1, and COX IV) were significantly upregulated, while ATP5a showed a similar trend (p-value = 0.06) (Figure S4F-S4K).

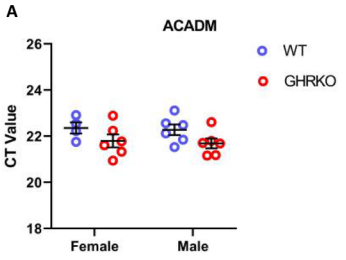
Upregulation of mRNA encoding proteins of fatty acid oxidation and oxidative phosphorylation

We then sought to determine if these changes in fatty acid oxidation enzymes and OXPHOS proteins reflected transcriptional changes. We found that mRNA levels of mitochondrial fatty acid β-oxidation enzymes ACADM, ECHS1, and ACAA2 were all significantly upregulated in GHRKO and SD livers, except for mRNA for ACAA2 in GHRKO livers, which approached our significance threshold ($p = 0.06$) (Figs. 4A–C and S5A-S5C). mRNA for peroxisomal enzymes ECH1 and EHHADH was also significantly upregulated in GHRKO mice. mRNA for ABCD2 showed a [Sex x Genotype] interaction in GHRKO mice, with significant elevation in males, and a similar trend ($p = 0.09$) in female mice (Fig. 4D–F). In SD mice, only ECH1 mRNA was significantly upregulated in both males and females, while ABCD2 and EHHADH were significantly upregulated in male mice, with female mice showing similar trends ($p = 0.08$ and $p = 0.1$, respectively) **(S5D-S5F)**. Finally, OXPHOS genes NDUFB11, NDUFS1, SDHA, and UQCRB were all significantly increased at the mRNA level in both mouse models (Figs. 4G–J and S5G-S5J). The OXPHOS data are consistent with previous reports showing that OXPHOS genes are upregulated in CR, GHRKO, and SD livers [24]. The levels of 18S mRNA were used as a control and did not show any statistically significant changes in GHRKO or SD liver samples (Figs. 4K and S5K).

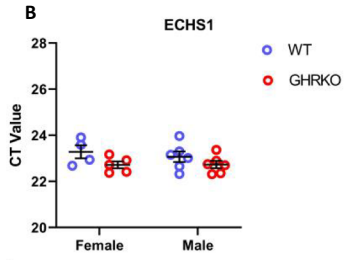
Transcriptional upregulation of oxidative metabolism proteins in GHRKO and SD livers is not explained by levels of corresponding transcription factors

The proteins involved in fatty acid β-oxidation and OXPHOS are transcriptionally controlled via a group of transcription factors called nuclear receptors, which includes peroxisome proliferator-activated receptors (PPARs) and estrogen-related receptors (ERRs) [44, 45]. Protein levels and activity of different PPARs have long been hypothesized to play an important role in metabolic regulation in long-lived murine models [28, 31, 37, 44, 55, 57, 58]. A common co-activator for those receptors, peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α), has also been positively implicated in multiple murine lifespan-extending pathways [21, 31, 33, 44].

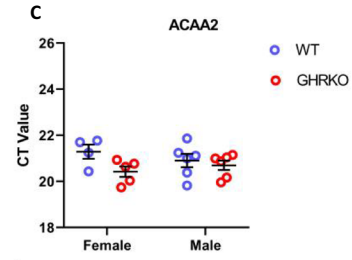
Previous studies have reported an increase in PPARα protein in GHRKO and SD liver tissue [33, 55].While we saw an upward trend in GHRKO livers, the effect did not reach statistical significance (Fig. 5A and B).



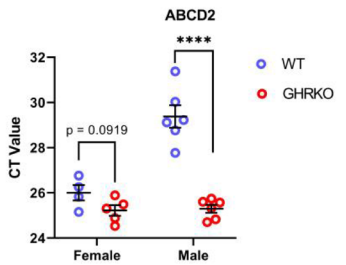
Source of Variation	% of total variation	P value
Interaction	0.0104	0.961
Sex	0.592	0.714
Genotype	22.4	0.035



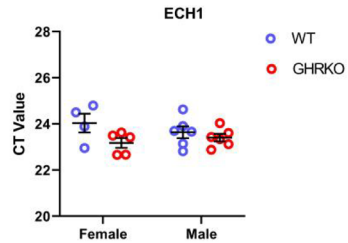
Source of Variation	% of total variation	P value
Interaction	1.33	0.596
Sex	1.05	0.638
Genotype	21.5	0.045



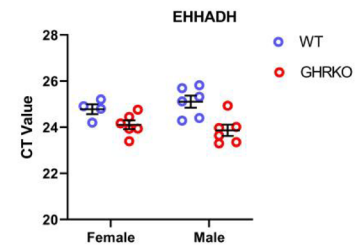
Source of Variation	% of total variation	P value
Interaction	7.20	0.224
Sex	0.201	0.836
Genotype	18.9	0.057



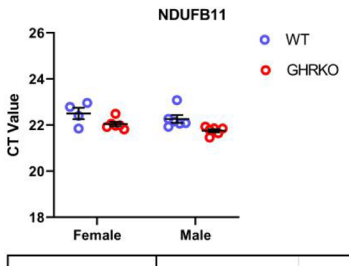
Source of Variation	% of total variation	P value
Interaction	17.8	<0.001
Sex	19.4	<0.001
Genotype	38.5	<0.001



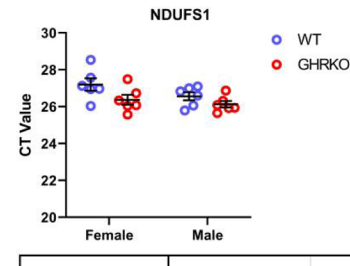
Source of Variation	% of total variation	P value
Interaction	6.61	0.239
Sex	0.478	0.747
Genotype	20.6	0.046



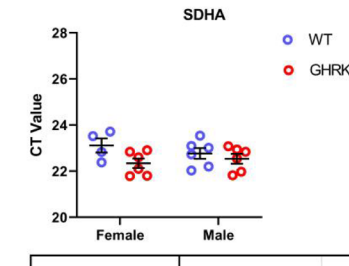
Source of Variation	% of total variation	P value
Interaction	3.79	0.253
Sex	0.0896	0.858
Genotype	43.2	<0.001



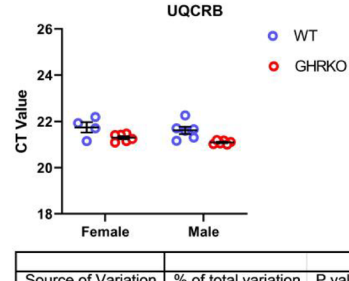
Source of Variation	% of total variation	P value
Interaction	0.0951	0.864
Sex	10.3	0.088
Genotype	35.1	0.004



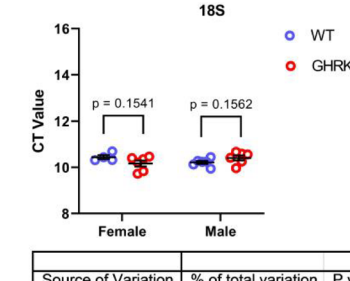
Source of Variation	% of total variation	P value
Interaction	1.96	0.456
Sex	9.90	0.103
Genotype	20.4	0.023



Source of Variation	% of total variation	P value
Interaction	5.55	0.271
Sex	0.440	0.753
Genotype	19.1	0.049



Source of Variation	% of total variation	P value
Interaction	0.266	0.765
Sex	5.25	0.194
Genotype	43.7	0.001



Source of Variation	% of total variation	P value
Interaction	21.4	0.039
Sex	0.0221	0.944
Genotype	0.618	0.710

◂**Fig. 4** Hepatic mRNA levels of FAO and OXPHOS genes in WT and GHRKO livers. (**A**-**K**) Scatter plots of ACADM (**A**), ECHS1 (**B**), ACAA2 (**C**), ABCD2 (**D**), ECH1 (**E**), EHHADH (**F**), NDUFB11 (**G**), NDUFS1 (**H**), SDHA (**I**), UQCRB (**J**), and 18S (**K**). Data shows mean ± S.E.M. Each symbol represents an individual mouse. n = 4–6 for each group. Two-way ANOVA was used for analysis of genotype effect, sex effect, and their interaction. Unpaired t-test was used when interaction was significant. **** $p < 0.0001$

In SD livers, PPARα protein was significantly downregulated (Figures S6A and S6B), contradicting previous reports. The discrepancy could stem from the difference in genetic backgrounds or diets of SD mice [55]. PPARδ, another PPAR receptor that acts as a transcription factor for mitochondrial and peroxisomal β-oxidation enzymes, showed no significant change in GHRKO livers (Fig. 5C) and was not evaluated in SD livers. ERRα, a nuclear receptor and transcription factor responsible for OXPHOS proteins transcription, was also unchanged in GHRKO livers (Fig. 5D); both proteins did show sexual dimorphism, with PPARδ higher in males and ERRα higher in females. In SD livers, EERα showed a decline in female mice that approached statistical significance ($p = 0.051$) (Fig. S6C). Surprisingly, PGC-1α, a co-activator for PPARs and ERRs, was not significantly changed in GHRKO or SD livers (Figs. 5E and S6D). This disagrees with a previously published study that found PGC-1α to be significantly upregulated in SD livers [38]. This difference could be due to changes in feeding conditions of the mice or in time of the day when the samples were collected, since PGC-1α influences and is in turn influenced by circadian rhythms [59].

Overall, the changes in transcription factors and co-activator levels did not explain the significant transcriptional upregulation observed in most of the downstream targets.

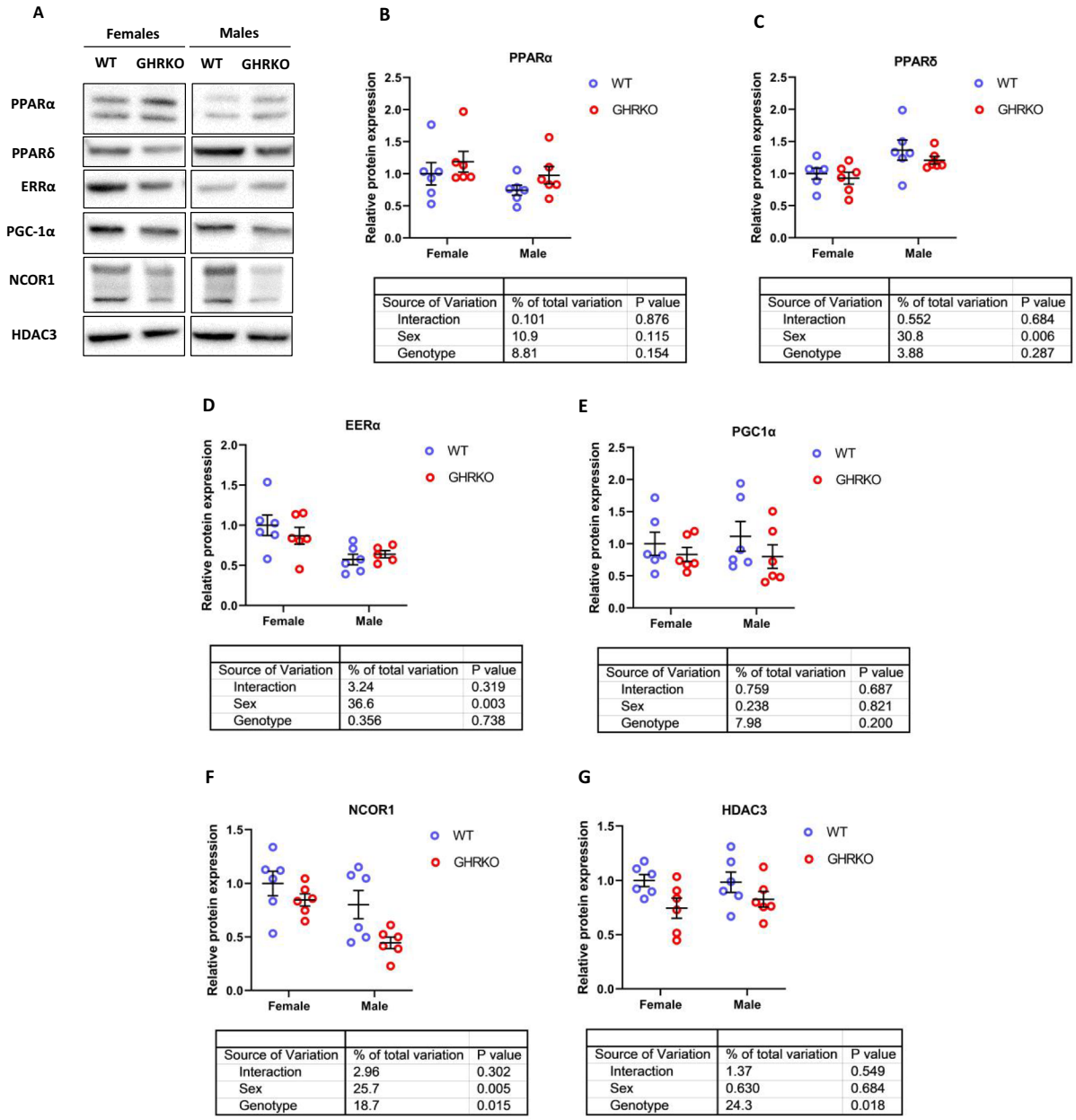
Diminished levels of nuclear receptor co-repressor NCOR1 in GHRKO and Snell dwarf mice

Nuclear receptor co-repressor 1 (NCOR1) is an inhibitor of PPARs and ERRs [45, 47, 48]. Tissue-specific knockdown of NCOR1 has been shown to increase oxidative phosphorylation and fatty acid oxidation in murine liver and muscle tissues [50, 60]. We tested the hypothesis that NCOR1 decline in GHRKO and SD livers could be responsible for increased transcription of genes regulated by PPARs and ERRs in mouse liver. We found that NCOR1 was indeed significantly downregulated in GHRKO and SD livers by at least 15% and 25%, respectively (Figs. 5F and S6E). NCOR1 forms a complex with Histone deacetylase 3 (HDAC3) that is necessary for metabolic functions of NCOR1 [61, 62]. HDAC3 levels were also significantly reduced in GHRKO and SD livers (Figs. 5G and S6F). These results suggest that reduced NCOR1 and HDAC3 may contribute to the coordinated increase of transcription of OXPHOS subunits and β-oxidation enzymes in GHRKO and Snell mice.

Discussion

Murine lifespan extension can be achieved via dietary changes (e.g., CR and methionine restriction), genetic mutations (e.g., GHRKO and SD), or pharmacologically (e.g., rapamycin and acarbose) [2, 56, 63–65]. Finding shared signatures and mechanisms for longevity among these models would provide important clues for developing new lifespan extending interventions and for a more detailed understanding of pathways that control aging rate and risks of late-life diseases. One such shared signature is the upregulation of mRNA for fatty acid β-oxidation and OXPHOS in long-lived mice [24, 25]. RNA-seq data show OXPHOS and FAO pathways to be upregulated in CR and GHRKO livers [7, 25]. Furthermore, male mice treated with rapamycin for the first 45 days of life were long-lived, had lower levels of enrichment of inflammatory pathway genes including interferon α and IL6-JAK-STAT3 signaling, and higher levels of enrichment of metabolic pathway genes including OXPHOS, FAO, gluconeogenesis, and adipogenesis [66]. Overexpression of phosphatase and tensin homolog (PTEN), which also extends lifespan in mice, increases FAO in calf hepatocytes [11]. Consistently, knocking down PTEN in calf hepatocytes inhibits FAO [12].

One weakness of inference based on mRNA data alone (i.e. on "genetic signatures") is that there can be substantial discrepancies between mRNA levels and levels of the corresponding proteins [54, 63, 67–69]. Many studies of long-lived mice have highlighted the importance of differential translation of specific sets



Source of Variation	% of total variation	P value
Interaction	0.101	0.876
Sex	10.9	0.115
Genotype	8.81	0.154

Source of Variation	% of total variation	P value
Interaction	0.552	0.684
Sex	30.8	0.006
Genotype	3.88	0.287

Source of Variation	% of total variation	P value
Interaction	3.24	0.319
Sex	36.6	0.003
Genotype	0.356	0.738

Source of Variation	% of total variation	P value
Interaction	0.759	0.687
Sex	0.238	0.821
Genotype	7.98	0.200

Source of Variation	% of total variation	P value
Interaction	2.96	0.302
Sex	25.7	0.005
Genotype	18.7	0.015

Source of Variation	% of total variation	P value
Interaction	1.37	0.549
Sex	0.630	0.684
Genotype	24.3	0.018

Fig. 5 Hepatic levels of PPAR signaling network proteins in WT and GHRKO livers. (**A**) Representative images of western blot data for different nuclear receptors and their co-regulators in liver lysates from female and male WT and GHRKO mice. (**B**-**G**) Scatter plots of PPARα (**B**), PPARδ (**C**), ERRα (**D**), PGC-1α (**E**), NCOR1 (**F**), and HDAC3 (**G**). Data show mean ± S.E.M. Each symbol represents an individual mouse. n = 5–6 for each group. Two-way ANOVA was used for analysis of genotype effect, sex effect, and their interaction

of mRNAs. For example, cap-independent translation of many mitochondrial proteins is upregulated in GHRKO, SD, rapamycin-treated, and acarbose-treated mice without increases in the mRNA levels of the corresponding genes [38, 63]. Additionally, proteolysis via ubiquitination, autophagy, or chaperone-mediated

autophagy can also alter the levels of specific sets of proteins in long-lived mouse models [54, 67, 70–72]. Differences in genetic signatures based on mRNA levels are not sufficient to show that a specific pathway is causative for, linked to, or even present in models of lifespan extension. Our new data help to clarify these ambiguities by evaluation of proteins related to OXPHOS, FAO, and their transcriptional regulators. We found that FAO and OXPHOS proteins, as well as mRNA, are significantly upregulated in GHRKO and SD livers. This finding adds to the body of evidence that OXPHOS and FAO pathways are upregulated in long-lived mutant mice, as they are in CR animals [8]. Interestingly, we found that ACADM, one of the enzymes responsible for the first β-oxidation step in the mitochondria, was significantly upregulated in both GHRKO and SD livers. ACADL, which catalyzes the same reaction for longer fatty acids, was not significantly changed in either model. This could suggest that in both GHRKO and SD livers, different acetyl-CoA dehydrogenases could be differentially regulated based on hepatic use of fatty acids in specific length classes.

Our results are consistent with previous studies on metabolic phenotypes in Ames dwarf and GHRKO mice, which rely more on fatty acids than on carbohydrates. The previous data included measures of energy expenditure, respiratory quotient, oxygen consumption, and body temperature [15, 17].

Most of the changes we report were seen in both sexes, but sex-specific peroxisomal FAO enzymes were an exception to this generalization. ABCD2 mRNA levels in GHRKO and SD, EHHADH protein level in GHRKO livers, and its EHHADH mRNA level in SD livers all showed a significant interaction term, and in all these cases, male mice showed the more extreme changes.

Increased oxidative metabolism and β-oxidation could reflect increased abundance of these proteins, or perhaps increased enzymatic activity with minimal change in protein levels. Previous research provided some evidence to support each of these hypotheses. For example, in liver of CR mice, protein levels of CPT1, ACADM, ACADL, and HADHB were found to be significantly upregulated [8]. There are also multiple reports that post-translational modifications of those enzymes (e.g., SIRT3-mediated hyperacetylation of OXPHOS and FAO enzymes) contribute to their modulated activity and stability [8, 73–75]. In this study, we confirmed increased abundance of many proteins involved in those two pathways. More work will be needed to see if activity of these enzymes is also higher in the long-lived mice.

The transcription of FAO and OXPHOS genes is regulated by PPARs and ERRs, respectively [26, 27, 29, 30, 57]. There are some previous data suggesting PPARα upregulation in livers of CR, GHRKO, and SD mice [33, 55], and data on muscle showing that PPARα is unchanged in GHRKO mice and downregulated in CR mice [32]. Indeed, many studies have reported PGC-1α to be upregulated in liver, muscle, and adipose tissue of different longevity models [38, 44]. Since PGC-1α acts as a co-activator for many NRs and TFs, including PPARs and ERRs, it has attracted interest as a potential target for anti-aging interventions.

In this study, we did not find any significant upregulation in PPARs, ERRs, or PGC-1α that could explain FAO and OXPHOS transcriptional upregulation in GHRKO and SD mice. It is possible that this discrepancy may reflect circadian control of PPARs and PGC-1α [59, 76]. Indeed, the relationship between circadian clock and energy metabolism is strong and well-established. For example, it was previously shown that caloric restriction induces better rhythmicity in hepatic ketogenesis as well as higher levels of PPARα, FGF21, ACADL and ECHS1 [77]. Additionally, previous work has demonstrated that PGC-1α influences circadian clock genes, and liver PGC-1α is crucial for integrating mammalian clock with energy metabolism [59]. Similarly, and of relevance to the current study, disruption of HDAC3-NCOR1 interaction led to changes in circadian behavior, and generated mice that are leaner and have higher energy expenditure and insulin sensitivity. Levels of different FAO enzymes and PPARs in mice with disrupted HDAC3-NCOR1 appeared to have a circadian phase-shift that resulted in them being higher than or similar to WT levels depending on time of day. For example, PPARα level in that model was almost the same as WT mice at ZT5 (five hours from lights on), and was significantly higher than WT mice at ZT10, before falling back to lower than WT levels at ZT15 [76]. Other studies have also demonstrated that activity of PPARs can be modulated without alteration in PPAR protein levels [32, 50].

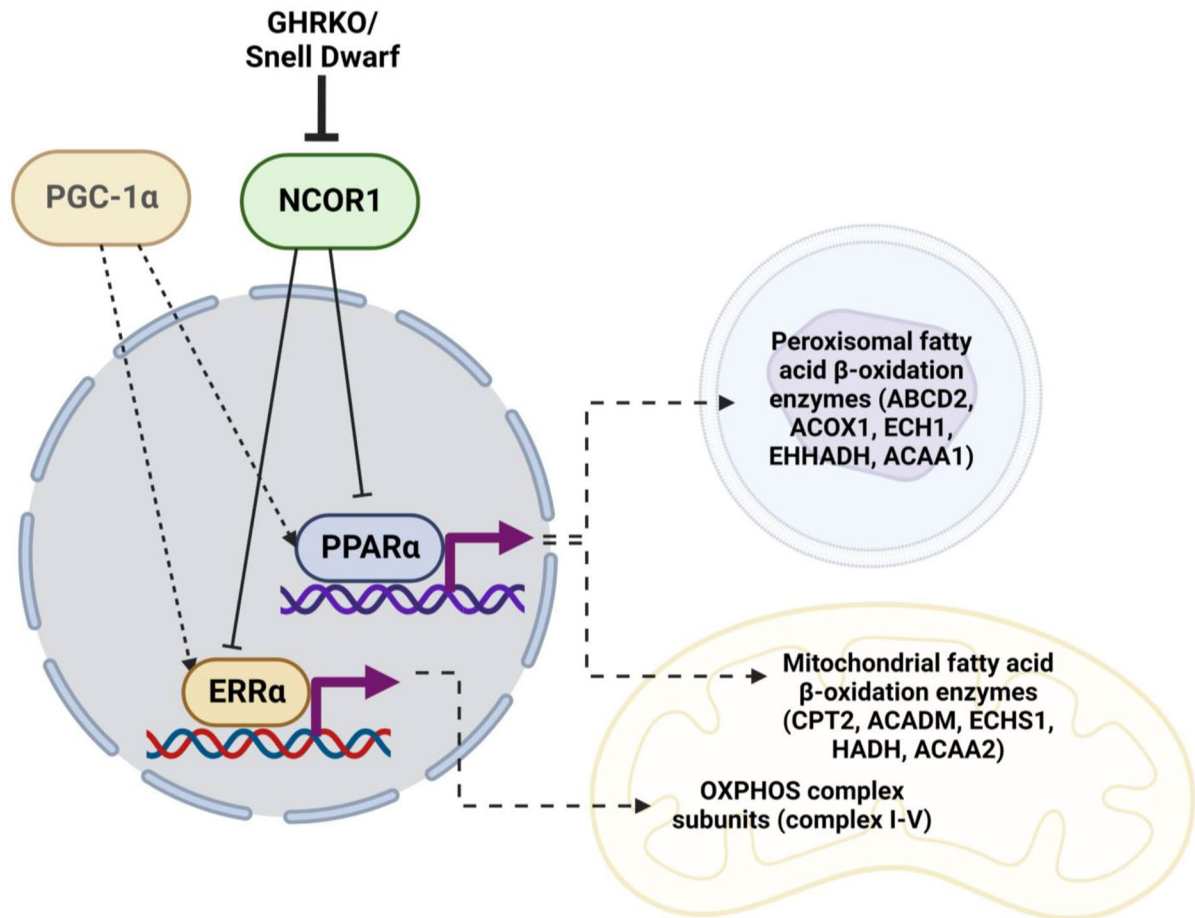


Fig. 6 Postulated mechanism of NCOR1 regulation of PPARα- and ERRα-mediated transcription in GHRKO and SD. Created with BioRender.com

Transcription of OXPHOS and FAO mRNA is also influenced by other co-regulators, including receptor-interacting protein 140 (RIP140), silencing mediator for retinoid or thyroid-hormone receptors (SMRT), and NCOR1 [47, 48, 78]. Those regulators have not previously been evaluated in slow-aging mice.. NCOR1 antagonizes PGC-1α in skeletal muscle oxidative metabolism [45]. Deletion of NCOR1 in skeletal muscles or adipose tissue leads to increased insulin sensitivity [50, 79]. Additionally, mTORC1 controls fasting-induced ketogenesis through phosphorylation of S6K and subsequent activation of NCOR1 [80]. NCOR1 mediates this inhibition of NRs by forming a repressive complex with HDAC3 [61, 62]. Our results show downregulation of hepatic NCOR1 and HDAC3 in both GHRKO and SD mice, and suggest that the NCOR1-HDAC3 repressive complex may play an important role as a regulator of metabolic phenotypes linked to increased longevity (Fig. 6).

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Data availability All data supporting the findings of this study are included within this paper and its Supplementary Information.

Declarations

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

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