PKC downregulation upon rapamycin treatment attenuates mitochondrial disease

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Leigh syndrome is a fatal neurometabolic disorder caused by defects in mitochondrial function. Mechanistic target of rapamycin (mTOR) inhibition with rapamycin attenuates disease progression in a mouse model of Leigh syndrome (Ndufs4 knock-out (KO) mouse); however, the mechanism of rescue is unknown. Here we identify protein kinase C (PKC) downregulation as a key event mediating the beneficial effects of rapamycin treatment of Ndufs4 KO mice. Assessing the impact of rapamycin on the brain proteome and phosphoproteome of Ndufs4 KO mice, we find that rapamycin restores mitochondrial protein levels, inhibits signalling through both mTOR complexes and reduces the abundance and activity of multiple PKC isoforms. Administration of PKC inhibitors increases survival, delays neurological deficits, prevents hair loss and decreases inflammation in Ndufs4 KO mice. Thus, PKC may be a viable therapeutic target for treating severe mitochondrial disease.

Results and discussion

Rapamycin similarly remodels the brain proteome in wild-type and NDUFS4-deficient mice. We analysed brain tissue of 30-day-old vehicle-treated wild-type (WT) mice and Ndufs4 KO mice treated with a daily intraperitoneal injection of 8 mg kg⁻¹ d⁻¹ rapamycin (KR) or vehicle (KO) for 20 d (Fig. 1a). Postnatal day 30 (P30) is approximately 1 week before the appearance of neurodegeneration and behavioural abnormalities in Ndufs4 KO mice. As expected, rapamycin-treated Ndufs4 KO mice had lower body weight and brain weight relative to vehicle-treated mice (Fig. 1b).

We quantified 6,231 proteins (Supplementary Table 1) with high reproducibility (Extended Data Fig. 1a), of which 16% (1,004 proteins) showed significant changes in abundance between groups (analysis of variance (ANOVA) test, false discovery rate (FDR) Q < 0.05). The mitochondrial proteome showed substantial changes in response to NDUFS4 loss, driven primarily by altered abundance of C-I proteins (Extended Data Fig. 1a). In agreement, principal component analysis (PCA) revealed changes to C-I proteins as the major discriminant among groups (Fig. 1c). No partitioning between sexes was observed either with or without rapamycin treatment (Fig. 1c). Most C-I proteins decreased substantially in Ndufs4 KO mouse brains (Fig. 1d–f and Extended Data Fig. 2), except for two C-I assembly proteins (ACAD9 and NDUFAP2) (Fig. 1d,f). Rapamycin treatment caused a modest increase of several C-I subunits (Fig. 1g–i and Extended Data Fig. 2), but is not sufficient to fully restore C-I function. In addition to the decrease in C-I abundance in Ndufs4 KO brain, we observed changes in other respiratory chain protein complexes (Fig. 1f) and Extended Data Fig. 2). In particular, Ndufs4 KO mice showed an overall increase in the

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Fig. 1 | Rapamycin remodels the brain proteome in NDufS4-deficient mice. a, Experimental design (N = 6 mice for the WT and Ndufs4 KO (KO) groups, N = 7 mice for the rapamycin-treated Ndufs4 KO (KR) group). b, Mouse body weight from the three experimental groups (mean ± s.d.). c, volcano plots show total brain weight at the end of the experimental trial (30 d) (t-test: *P < 0.01; **P < 0.001; N = 6–7 mice). d, Volcano plots comparing protein abundance in KO versus WT groups (genotype effect; N = 6 mice) at the level of individual proteins (d) or representative biological terms (e), transm., transmembrane; transport; vesic., vesicle. f, Mapping of protein abundance differences in individual subunits of the respiratory chain between the KO and WT groups. Assembly subunits of each mitochondrial respiratory complex are displayed at the bottom of each plot. LFQ-normalized abundance data were used, except for subunits with asterisks in which intensity IBAQ values were used. Subunits with significant changes are bolded (P < 0.05) and underlined if FDR Q < 0.05. In grey are subunits without abundance information. g, Volcano plots comparing protein abundance in rapamycin-treated KR versus KO groups (rapamycin effect; N = 6–7 mice) at the level of individual proteins (g) or representative biological terms (h), path., pathway; surv., surveillance; synt., synthesis; trans., transcription. In d and g, dotted lines indicate cut-off for significant changes: t-test FDR Q < 0.05 and artificial within-groups variance S0 = 0.1. e and h show significantly enriched annotation terms from one-dimensional enrichment analysis of log, differences between groups (Wilcoxon–Mann–Whitney test, FDR Q < 0.05); dot size is proportional to the number of proteins within that term. i, Same as in f but between KR and KO groups.

cytochrome c oxidase complex (C-I or COX), which was reverted by rapamycin treatment (Extended Data Fig. 2a). Rapamycin treatment of Ndufs4 KO mice caused additional changes in the brain proteome, including a significant decrease of the mTORC1 and mTORC2 core subunits mTOR and mLST8 (Fig. 1g). Although rapamycin is a specific inhibitor of mTORC1 (refs. 7,9), chronic rapamycin treatment has been reported to inhibit mTORC2 in mice10,11, which is also supported by our phosphoproteomic data where we observed a reduction in phosphorylation of the mTORC2 targets12,13, AKT and PKC (Extended Data Fig. 3a–f). Notably, all conventional PKC isoforms (PKC-α, PKC-β and PKC-γ) were significantly decreased (Fig. 1g and Extended Data Fig. 3d–f). Conventional PKC isoforms’ stability is compromised when the activation loop, turn motif or hydrophobic region is unphosphorylated, leading to its degradation via the ubiquitin–proteasome pathway14,15. Thus, decreased PKC phosphorylation upon rapamycin treatment may promote its instability and degradation. We observed similar results in brain extracts of rapamycin-treated mice.
Fig. 2 | Rapamycin restores the abundance level of several mitochondrial proteins in Ndufs4 KO mice. a, Heatmap and hierarchical clustering of proteins with significant changes in abundance (ANOVA test, FDR Q < 0.05; N = 6–7 mice). b, Significantly enriched GO terms from the main clusters of panel a (hypergeometric test, P < 0.001). dep., dependent; proc., process; prot., protein; reg., regulation. c,d, Scatter plot of individual protein abundances (c) and enriched GO terms (d) showing significant changes (t-test for proteins and Wilcoxon–Mann–Whitney test for GO terms, FDR Q < 0.05) between KO versus WT and KR versus KO groups, in the same direction (that is, rapamycin enhances Ndufs4 KO changes) or in opposite directions (that is, rapamycin rescues Ndufs4 KO changes).

at age 50 days (Extended Data Fig. 3g–j), an age at which untreated Ndufs4 KO mice already display severe neuropathic symptoms and are nearing their median lifespan. Our data suggest that inhibition of mTORC2 upon chronic rapamycin treatment reduces PKC activity, something that has been previously demonstrated by ablating mTORC2 function in vitro and also in vivo.

To further explore the potential impact of rapamycin on mitochondrial function in the context of C-I deficiency, we performed hierarchical clustering of protein abundance changes which grouped proteins into four clusters (Fig. 2a). Clusters 1 and 4 contain protein changes associated with rapamycin treatment, cluster 3 includes protein changes associated with loss of NDUFS4 and cluster 2 is composed of proteins that increased in Ndufs4 KO mice and were restored to normal levels with rapamycin (Supplementary Table 2). Among proteins that decreased in Ndufs4 KO mice that increased levels of MPST, a neuroprotectant involved in mitochondrial fission (for example, MFF), and also proteins involved in other mitochondrial functions such as glutamate metabolism and cytochrome c oxidase activity (Fig. 2b,d) and Supplementary Table 2). Among proteins that decreased in Ndufs4 KO mice and returned to normal levels with rapamycin we found LPPR1 (Fig. 2c), a neuronal growth promoter. We also found in Ndufs4 KO mice that increased levels of MPST, a neuroprotectant mitochondrial enzyme involved in hydrogen sulfide production, were further enhanced by rapamycin (Fig. 2b).

To determine whether the rapamycin-associated changes observed in the brain proteome were specific to the Ndufs4 KO condition, we assessed the effect of the same rapamycin treatment in WT mice (Extended Data Fig. 4 and Supplementary Table 3). Rapamycin evoked a similar response in the brain of WT mice, with mTOR and PKC isoforms significantly reduced (Extended Data Fig. 3). Among proteins that increased in WT mice (Extended Data Fig. 4 and Supplementary Table 3). Rapamycin evoked a similar response in the brain of WT mice, with mTOR and PKC isoforms significantly reduced (Extended Data Fig. 3).
the abundance of the cold-inducible RNA-binding proteins CIRBP and RBM3 (Extended Data Fig. 4d), which have been reported to have neuroprotective effects. Rapamycin also increased histone abundance (that is, nucleosome) and upregulated processes associated with mTOR inhibition such as messenger RNA splicing and proteasomal degradation, while downregulating others such as myelination (Extended Data Fig. 4e). In contrast, C-I proteins increased slightly in KO but not in WT upon rapamycin treatment (Extended Data Fig. 4d,e). We do not know the cause or mechanisms for this selective control, but hypothesize that other complexes in the respiratory chain may impose an abundance threshold for C-I. These results demonstrate that chronic rapamycin treatment elicits a core response in the brain that is mostly independent of mitochondrial disease.

**Rapamycin reduces PKC activity in the mouse brain.** Given that mTOR functions as a kinase, we also sought to assess changes in global protein phosphorylation upon treatment with rapamycin (Fig. 3). We quantified 15,971 phosphorylation sites (Supplementary Table 4) in 3,091 proteins with high reproducibility (Extended Data Fig. 1c–f); to the best of our knowledge, 26% of these sites have not been previously reported. Globally, the phosphoproteome was altered to a lesser extent than the proteome (10% phosphosites with significant changes; ANOVA test, FDR Q < 0.05). Ndufs4 KO induced few changes in brain protein phosphorylation, consistent with the minimal changes in kinase abundance (Fig. 3a,b). Most of the changes were associated with rapamycin treatment (Fig. 3c,d) and correlated with changes in protein abundance (Extended Data Fig. 5), indicating that chronic rapamycin treatment largely rewires the signalling network in the mouse brain by changing protein levels. As in the proteome, these changes were mostly independent of the Ndufs4 KO background (Extended Data Fig. 4g and Supplementary Table 5). Of all the phosphorylation sites detected by our analysis, only 2% belong to mitochondrial proteins, and very few changed significantly after rapamycin treatment (Extended Data Fig. 1c). These involve proteins related to mitochondrial transport, metabolism and fission (Supplementary Table 6). The low occurrence of phosphorylation events on mitochondrial proteins is consistent with other studies and suggests that mitochondrial function may be largely regulated by changes in protein and substrate abundance or by other post-translational modifications. However, there is increasing evidence that protein phosphorylation may be important in some mitochondrial functions.

We detected several changes in protein phosphorylation with rapamycin treatment in Ndufs4 KO mice, including a decrease in phosphorylation of several mTOR complex members and known mTOR substrates (Extended Data Fig. 6a). To associate putative kinases targeting these phosphosites, we identified linear sequence motifs that were enriched in the set of rapamycin-regulated phosphosites (Fisher test, FDR Q < 0.05). We found an overrepresentation of basophilic motifs (for example, R>R>S* and R>S*; R, arginine; S, serine; S*, phosphoserine; x, any residue), which are typically targeted by kinases in the AGC (protein kinase A (PKA) and PKC) and CAMK (CAMK2) families (Fig. 3e). PKC and PKA themselves also exhibited significant changes upon rapamycin treatment, with PKC decreasing and PKA increasing in protein abundance (Fig. 3c) and phosphorylation levels (Fig. 3f). Global phosphorylation state has been recently correlated with kinase activity, suggesting that the observed changes in PKA and PKC phosphorylation may reflect differences in their activity.

To directly assess changes in kinase activity, we applied kinase–substrate enrichment analysis (KSEA), revealing that chronic treatment with rapamycin repressed PKC-β and induced PKA and CAMK2 activity (Fig. 3g). This result was further supported by direct inspection of phosphorylation changes of known substrates of these kinases (Extended Data Fig. 6b) and the kinase activation loop sites (Extended Data Fig. 6c). Given the decrease in phosphorylation of all conventional PKC isoforms (that is, PKC-α, PKC-β and PKC-γ), which require calcium for activation, we investigated whether rapamycin treatment also affected proteins involved in calcium homeostasis. We found that rapamycin reverted the hyperphosphorylation on the activation site of ITPR1 (Extended Data Fig. 6d), the main endoplasmic reticulum calcium release channel in the brain, and also decreased its levels and those of several calcium homeostasis-related proteins (Fig. 3h,i). These results suggest a tighter control of intracellular calcium homeostasis in Ndufs4 KO mice brains upon chronic rapamycin treatment which may preclude the activation of conventional PKCs and downstream effectors.

**Rapamycin attenuates inflammation in Ndufs4 KO brain.** Among the multiple PKC isoforms with reduced abundance upon rapamycin treatment, PKC-β showed the most significant decrease in activity (Fig. 3g). PKC-β mediates activation of the NF-κB pathway via IKK-α and IκB phosphorylation and plays a key role in activation of the immune system and inflammatory response. In addition to inhibition of PKC-β upon rapamycin treatment, we observed a global decrease in phosphorylation of proteins from pro-inflammatory signalling pathways (for example, adipocytokine signalling system, T-cell receptor signalling pathway, BMP signalling pathway) (Fig. 4a) and a decrease in abundance of the inflammatory regulator GSK3B (ref. 9) (Fig. 3c), together with increased phosphorylation of its inhibitory site (Extended Data Fig. 6c). Rapamycin treatment also reduced brain cytokine levels (Fig. 4b). Western blot analysis of brain extracts confirmed downregulation of PKC-β phosphorylation (Extended Data Fig. 3d–f) as well as phosphorylation of IκK-α (Fig. 4c,d). Consistent with the role of IKK-α in activating NF-κB by phosphorylating the NF-κB suppressor IκB and promoting its degradation, we observed that rapamycin treatment decreased IκB phosphorylation while increasing total IκB levels compared with vehicle-treated mice (Fig. 4c,d).

This model was further supported by analysis of 50-day-old Ndufs4 KO mice, which exhibit pathological features such as neuroinflammation, weight loss and moribundity. Western blot analysis of 50-day-old Ndufs4 KO brain extracts indicated that rapamycin treatment deactivates mTORC1 and mTORC2 (Extended Data Fig. 3g,h), decreases abundance of phosphorylated and total PKC levels (Extended Data Fig. 3i,j) and downregulates phosphorylation of the inflammation-activating kinase IκK-α, IκB and NF-κB (Fig. 4e,f). Consistent with the rapamycin-mediated delay of neurodegeneration, levels of the neuroinflammatory marker GFAP decreased upon rapamycin treatment (Fig. 4e,f). These results collectively indicate that rapamycin reduces neuroinflammation and glial activation concomitant with inhibition of PKC.

**Inhibition of PKC reduces skin inflammation in Ndufs4 KO mice.** To test the hypothesis that chronic rapamycin treatment is improving health in Ndufs4 KO mice through downregulation of PKC signalling, we asked whether pharmacological inhibitors of PKC would be sufficient to reduce inflammation. We treated Ndufs4 KO mice with three different PKC inhibitors: the pan-PKC inhibitors Go6983 and GF109203X, and the PKC-β-specific inhibitor roxubistaurin.

We first examined the effect of PKC inhibition on skin inflammation because previous studies have indicated that Ndufs4 KO mice experience a profound inflammatory burst in skin, which results in hair loss near the time of weaning. We observed that all three PKC inhibitors largely suppressed this hair loss phenotype, even more potently than rapamycin treatment alone (Fig. 5a and Extended Data Fig. 7). Histological analysis indicated that Ndufs4 KO mice exhibit substantial skin abnormalities at weaning (P21), including dilated hair follicles; aberrant keratin features; fractured, bent or twisted hair shafts; and chronic-active...
Fig. 3 | Rapamycin reduces PKC activity in Ndufs4 KO mouse brain. a, Volcano plot comparing the abundance of protein kinases between KO and WT groups (genotype effect). Kinases with significant changes are coloured and labelled (t-test FDR Q < 0.05; N = 6–7 mice). b, Volcano plot comparing phosphosite abundance differences between KO and WT groups (genotype effect; N = 12 samples; 6 mice and duplicated IMAC enrichment and LC–MS/MS analysis). Dotted lines indicate cut-off for significant changes: t-test FDR Q < 0.05 and artificial within-groups variance S0 = 0.1. c, d, Same as in a and b, respectively, but comparing differences between KR and KO groups (rapamycin effect; N = 12–14 samples; 6–7 mice and duplicated IMAC enrichment and LC–MS/MS analysis). e, Enriched kinase motifs in significantly changing phosphosites (Fisher test, FDR Q < 0.05). Inner graph shows overrepresented linear sequence motifs. f, Average rapamycin-induced changes in phosphorylation of kinases. Dot size is proportional to the number of phosphosites considered for each kinase. Only kinases with n ≥ 3 sites are shown. g, KSEA showing kinases with significant changes in activity upon rapamycin treatment (Kolmogorov–Smirnov test, P < 0.05). h, Mapping of protein abundance changes in proteins involved in intracellular calcium homoeostasis between the Ndufs4 KO mice treated (KR) and untreated (KO) with rapamycin. Only significant changes are coloured (t-test P > 0.05; N = 12–14 samples; 6–7 mice and duplicated IMAC enrichment and LC–MS/MS analysis); nonquantified proteins are shown in grey.
inhibition throughout the dermis characterized by increased infiltration of macrophages, lymphocytes and neutrophils (Fig. 5b and Extended Data Fig. 8a–c). Ruboxistaurin treatment largely prevented this pathological state and reduced skin inflammation, having a more pronounced effect than rapamycin (Fig. 5b and Extended Data Fig. 8a–c). We made similar observations in skin sections of mice at age 30 d, and also noticed the presence of atrophied subcutaneous fat in the untreated Ndufs4 KO mice (Extended Data Fig. 8d–f). In addition, we found a general decrease in skin cytokines upon ruboxistaurin and rapamycin treatments (Extended Data Fig. 8g), further supporting an attenuation of the skin inflammatory response by PKC inhibition.

PKC inhibition reduces neuroinflammation and improves survival in Ndufs4 KO mice. We next asked whether PKC inhibition would be sufficient to increase health and survival in Ndufs4 KO mice. All three PKC inhibitors were able to significantly increase survival (Fig. 5c) and delay the onset of neurological symptoms (that is, clasping; Fig. 5d). Interestingly, inhibition of PKC did not result in the reduced weight and delayed growth characteristics of rapamycin treatment (Figs. 1b and 5e).

Neurological symptoms in Ndufs4 KO mice have been found to correlate with neuroinflammation. As expected, vehicle-treated Ndufs4 KO mice showed a large increase in GFAP in whole-brain extracts at age 50 d (Fig. 5f,g). Treatment with the PKC-β-specific inhibitor ruboxistaurin significantly decreased GFAP levels in both WT and Ndufs4 KO mice (Fig. 5f,g), consistent with the delay of clasping (Fig. 5d). Treatment with ruboxistaurin also downregulated the NF-κB inflammatory response in the brain, as indicated by decreased phosphorylation of IKK-α, IκB and NF-κB, as well as by increases in abundance of total IκB levels (Fig. 5f,g). This was largely independent of genotype as similar effects were observed in ruboxistaurin-treated WT mice (Fig. 5f,g).

Conclusion

Taken together, these results suggest that inhibition of PKC-β and downstream NF-κB-mediated inflammation contributes to suppression of mitochondrial disease and increased survival from chronic rapamycin treatment in Ndufs4 KO mice. These effects may be mediated through reduced abundance and activity of mTORC2, a known regulator of PKC. It has been suggested that inhibition of mTORC2 upon chronic rapamycin treatment leads to metabolic dysregulation and detrimental side effects in the context of normative aging in mice35,36; however, our data raise the possibility that inhibition of mTORC2 may also have beneficial anti-inflammatory and metabolic consequences, at least in the context of severe mitochondrial disease. Recently, it was reported that rapamycin and rapamycin derivatives can improve outcomes in patients suffering from paediatric Leigh syndrome37 and adult-onset mitochondrial encephalopathy, lactic acidosis and stroke-like episodes syndrome37. The findings presented here suggest that PKC inhibitors may also have therapeutic value for
treated mitochondrial disease and may be particularly useful in patients where side effects associated with mTOR inhibition represent a notable concern.

**Methods**

**Animals and animal care.** All studies with mice were performed in strict accordance with the Guide for the Care and Use of Laboratory Animals of the...
heterozygous mice were generously provided by the Palmiter Laboratory at the University of Washington and backcrossed with C57Bl/6 cr mice. Breeders of littermate (ruboxistaurin), 8 mg kg⁻¹ was used in proteomic experiments were housed with one control littermate for warmth and companionship. Ndufs4⁻/− mice used in proteomic experiments were housed with one Ndufs4⁻/− control littermate for warmth and companionship. Both male and female mice were used on the bottom of the cage. Mice used in lifespan experiments were checked daily for signs of clamping and were euthanized if they showed a 30% loss in maximum body weight or loss of righting reflex, were found prostrate or were generally unresponsive. The minimum sample size for mouse lifespan experiments was determined to be 7 using continuous endpoint, two-independent-sample study calculations to determine a 30% change in lifespan considering an average lifespan of 50 ± 10 d for untreated mice with a confidence interval of 0.05 and 80% power. Drug treatment. Rapamycin (LC Laboratories) and GO6983 (LC Laboratories) were dissolved in dimethylsulfoxide and diluted 100-fold in 5% or 30% PEG/400/500/water (v/v/v). Sterile filtered solutions were stored at −80°C until needed for use. GF10920X (Cayman Chemical) and ruboxistaurin hydrochloride (Synnovator) were directly dissolved in vehicle containing 1% dimethylsulfoxide. Starting at P10, mice were treated daily between 14:00 and 18:00 (via intraperitoneal injection with 29 gauge, 1/2” insulin syringes) using 6.66 µg per gram of body weight of these solutions for final doses of 10 mg kg⁻¹ (rapamycin), 2 mg kg⁻¹ (ruboxistaurin). Rapamycin (GO6983). Control mice were generally treated with vehicle using 6.66 µg per gram of body weight. For the lifespan experiments control mice were untreated, as it has been reported that untreated Ndufs4−/− mice exhibit identical phenotypes as vehicle-treated Ndufs4−/− mice, and we also show this here for lifespan, onset of clamping and weight gain (Extended Data Fig. 9).

Sample preparation for proteomic analysis. WT and Ndufs4−/− mice were treated daily with rapamycin or vehicle as described above from P10 until P29. At that age, mice were fasted overnight (for 12 h), treated with rapamycin and re-fed in the morning for ~4 h. Unanaesthetized mice were then euthanized by cervical dislocation. Brains were immediately isolated, flash-frozen in liquid N₂, and stored at −80°C. Frozen brains were then ground in liquid nitrogen. About 15 mg of ground tissue was resuspended in 600 µl of lysis buffer composed of 8 M urea, 75 mM NaCl, 50 mM Tris pH 8.2 and a mix of protease inhibitors (Rocke Complete EDTA-free) and phosphatase inhibitors (50 mM β-glycerophosphate, 10 mM sodium pyrophosphate, 1 mM sodium orthovanadate and 50 mM sodium fluoride). Samples were then subjected to two cycles of bead beating (1 min of beating, 1.5 min of rest) with 0.5-mm-diameter zirconia beads and sonicated for 5 min in ice. Samples were centrifuged at 4°C to remove debris and lystate protein concentration was measured by BCA assay (Pierce). Protein was reduced with 50 mM DTT (in 20 mM Tris pH 8.2) and alkylated with 15 mM iodoacetamide in the dark for 30 min at room temperature. The alkylation reaction was quenched by incubating with additional 5 mM dithiothreitol for 15 min at room temperature. Samples were diluted fivefold with 50 mM Tris pH 8.2. Proteolytic digestion was performed by adding trypsin at 1:200 enzyme/protein ratio and incubating at 37°C overnight. The digestion was quenched by addition of trifluoroacetic acid to pH 2. Samples were centrifuged to remove insoluble material and peptides were desalted over a 50-µg C18 SepPak cartridge (Waters). Briefly, cartridges were conditioned with 1 ml of methanol, 3 ml of 100% acetonitrile, 1 ml of 70% acetonitrile, 0.25% acetic acid and 1 ml of 40% acetonitrile, 0.5% acetic acid; and equilibrated with 3 ml of 0.1% trifluoroacetic acid. Next, peptide samples were loaded into the cartridges, washed twice with 1 ml of 0.1% trifluoroacetic acid and eluted with 0.5% acetic acid and then sequentially eluted first with 0.5 ml of 40% acetonitrile, 0.5% acetic acid and then with 0.5 ml of 70% acetonitrile, 0.25% acetic acid. Then, 20-µg and 200-µg aliquots of eluted peptides were dried by vacuum centrifugation and stored at −80°C for proteomic and phosphoproteomic analysis, respectively.

Phosphopeptide enrichment. Phosphopptide enrichment was done by immobilized metal affinity chromatography (IMAC). First, 200 µg of peptides were resuspended in 150 µl of 80% acetonitrile, 0.1% trifluoroacetic acid. To prepare IMAC slurry, Ni-NTA magnetic agarose (Qagen) was first stripped with 40 mM EDTA for 30 min, reloaded with 10 mM FeCl₃ for 30 min, washed three times and resuspended in 80% acetonitrile, 0.1% trifluoroacetic acid. Phosphoprotein enrichment was performed using a KingFisher Flex robot (Thermo Scientific) programmed to incubate peptides with 150 µl of 50% bead slurry for 30 min; wash three times with 150 µl of 80% acetonitrile, 0.1% trifluoroacetic acid; and elute with 60 µl of 1:1 acetonitrile/1% ammonium hydroxide. The eluates were aciddified with 30 µl of 10% formic acid, 75% acetonitrile; dried by vacuum centrifugation; and stored at −80°C until MS analysis. Both sample processing and phosphopeptide enrichment were performed in technical duplicate.

Liquid chromatography–tandem mass spectrometry analysis. Peptide and phosphopeptide samples were dissolved in 4% formic acid, 3% acetonitrile; loaded onto a 100-µm-inner-diameter x 3-cm precolumn packed with Reprosil C18 3-µm beads (Dr. Maisch); and separated by reverse-phase liquid chromatography on a 100-µm-inner-diameter x 30-cm analytical column packed with 1.9-µm beads of the lifespan experiment and housed into a column heater set at 50°C. As peptides eluted off the column, they were online-analysed by MS.

Peptides for proteome analysis were eluted into a Q-Exactive (Thermo Fisher) mass spectrometer by gradient elution delivered by an EasyII nanoLC system (Thermo Fisher). The gradient was 9–30% acetonitrile in 0.125% formic acid over the course of 90 min. The total duration of the method, including columns wash and re-equilibration, was 120 min. MS and MS/MS spectra were acquired in the Orbitrap mass analyser and stored in centroid mode. Full MS scans were acquired from 300 to 1,500 m/z at 70,000-full-width-at-half-maximum (FWHM) resolution with a fill target of 3 x 10⁶ ions and maximum injection time of 100 ms. The 20 most abundant ions on the full MS scan were selected for fragmentation using a 2-m/z precursor isolation window and beam-temperature higher-energy collision-activated dissociation (HCD) with 26% normalized collision energy. Tandem mass spectrometry (MS/MS) fragmentation spectra were collected at 17,500-FWHM resolution with a fill target of 5 x 10⁶ ions and maximum injection time of 50 ms. Fragmented precursors were dynamically excluded from selection for 30 s. Two tandem replicate filtered phosphoproteome and phosphoprotein spectrum database searches were conducted using MaxQuant V1.6.0.1 (ref. 38) against the Uniprot mouse canonical plus isoform protein sequence database (downloaded August 2017, 51,434 entries) with common contaminants added. The precursor mass tolerance was set to 6 ppm, and the fragment ion tolerance was set to 20 ppm. A static modification on cysteine residues and N-terminal acetylation were used for all searches except for phosphopeptide-enriched samples, where phosphorylation on serine, threonine and tyrosine residues was also included as a variable modification. Trypsin/P was the specified enzyme allowing for up to two missed cleavages. The minimum required peptide length was 8 amino acids. The target-decoy strategy was used to guide filtering and estimate FDRs. All data were filtered to 1% FDR at both peptide and protein levels. The ‘match between runs’ option was enabled with a time window of 0.7 min to match identifications between replicates. Proteins with at least two peptides (one of them unique to the protein) were considered identified. Label-free quantification (LFQ) and intensity-based absolute quantification (iBAQ) algorithms for protein quantification were selected. LFQ intensity data were used for individual protein comparisons between experimental groups, except for specific analysis including low-abundance proteins that had no LFQ intensity values and so iBAQ-normalized intensities were used instead.

For protein and phosphorylation site analysis we used the generated ‘proteinGroups.txt’ and ‘PhosphoGroups.txt’ datasets and ‘MYSTySites.txt’ tables, respectively, after filtering off contaminants and reverse hits. Protein intensity measurements from technical replicates were aggregated, whereas phosphoryte intensities were treated individually due to the stochastic inherent to data-dependent mass spectrometry sampling of individual phosphopeptides. Perseus v1.6.0.7 software(42) was used for statistical (for example, PCA) and bioinformatic (for example, one-dimensional and two-dimensional enrichment analysis) analyses using log-transformed data from LFQ-normalized protein intensities and median-normalized phosphopeptide intensities. Phosphoproteins were considered localized with a localization probability >0.75.

Annotations were extracted from Gene Ontology (GO), the Kyoto Encyclopedia of Genes and Genomes (KEGG) and mouse MetaCarta 2.0 (ref. 43) databases. GOrilla application was used for GO terms enrichment analysis(44), using the whole list of identified proteins as background. Kinase–substrate sequence information were obtained from the PhosphositePlus database(45). Motif extractor tool (motif-x) was used to extract overrepresented motif patterns in sequences surrounding phosphosites (+7 amino acids)46. Kinase...
activity was determined using kinase-substrate enrichment analysis (KSEA), which uses Kolmogorov–Smirnov test to assess whether a predefined set of kinase substrates is statistically enriched in phospho- and non-phospho-well-timed tissue sections. KSEA activity denotes the log$_2$-transformed $P$ values from the enrichment analysis of kinase substrates and is signed based on the average sign of all substrates (that is, if the majority of substrates present increased or reduced phosphorylation, the kinase is predicted as activated (+) or inactivated (−), respectively).

Sample preparation for western blot. Vehicle-treated Ndufs4−/− and Ndufs4+/+ mice, or ruboxistaurin- or rapamycin-treated Ndufs4−/− and Ndufs4+/+ mice, were treated daily from P10 until P27–31 or P47–51. Mice were then fasted overnight (12h), treated with vehicle or small molecule, and re-fed. Unanesthetized mice were then euthanized by cervical dislocation 3–4.5h later. Brains were immediately isolated and flash-frozen in liquid N$_2$ for western blot. Brains were ground using a cryogenic homogenizer. Approximately 50 mg of homogenized brain tissue was lysed with 1 ml of RIPA buffer containing Pierce protease and phosphatase inhibitor tablets (Thermo Fisher) for 30 min. Samples were then centrifuged to remove insoluble cell debris, quantified by BCA, diluted to 2 mg ml$^{-1}$ and used for western blot.

Western blotting. Relative protein levels were determined through western blotting of ~20 µg of protein lysate diluted in 4x Laemmli sample buffer, 10x reducing agent and RIPA buffer. The samples were heated at 95°C for 5 min, at 70°C for 10 min or at 4°C for 1 h, then were loaded onto a NuPAGE 8% or 10% Bis-Tris MDEI or MINI gel and run at 120V in MOPS running buffer. Protein was transferred to a PVDF membrane using a Trans-Blot Turbo Transfer System according to manufacturer instructions (an additional 0.2% SDS was added to the transfer buffer). Unless otherwise noted, the blots were blocked with 5% BSA or 5% milk in TBST at room temperature for ~1 h and incubated with primary antibody in body in TBST overnight at 4°C, followed by ~2 h at room temperature. Primary antibodies used were: p235/236-S6 ribosomal protein (1:1,000, Cell Signaling Technology 4858), S6 ribosomal protein (1:1,000, Cell Signaling Technology 2217), p5473-AKT (1:1,000, Cell Signaling Technology 4060), pT308-AKT (1:1,000, Cell Signaling Technology 9275), pT450-AKT (1:1,000, Cell Signaling Technology 4691), pS222-PKC-α (1:2,000, Cell Signaling Technology 1056), pT638/641-PKC-α (1:1,000, Cell Signaling Technology 2056), pT638/641-PKC-β/δ (1:1,000, Cell Signaling Technology 9375), pS192/193-ERK (1:500, Cell Signaling Technology 9102), pS223/226-SAPK (1:1,000, Cell Signaling Technology 4511), pS556/NF-κB p65 (1:100, Abcam ab13685), pS76/S80-NF-κB p65 (1:200, 1:200 in 1% BSA, Santa Cruz Biotechnology sc-8404), IκBα (1:100, 500 ml, Abcam ab3225), pS536-NF-κB p65 (1:100 in 1% BSA, Santa Cruz Biotechnology sc-136548), NF-κB p65 (1:200 in 1% BSA, Cell Signaling Technology cs-8242), GRAP (1:5,000 in 5% milk, Cell Signaling Technology 12389) or β-actin HRP conjugate (1:25,000 at room temperature for 2 h, Cell Signaling Technology 5125). The blots were then rinsed three times at room temperature with TBST for 10 min each. The blots were incubated with the secondary antibody in 5% BSA or 5% milk for 1–2 h. Secondary antibodies used were donkey anti-rabbit IgG HRP conjugate (1:25,000 at room temperature for 2 h, Cell Signaling Technology 7110), swine anti-rabbit HRP (1:14,000, Cell Signaling Technology 7150), swine anti-mouse HRP (1:25,000, Cell Signaling Technology 7140), swine anti-rabbit HRP (1:15,000, Cell Signaling Technology 7140), and goat anti-rabbit HRP (1:4000, Cell Signaling Technology 7140). The primary antibody was incubated with TBST and then revealed after incubation with Amersham ECL or Thermo Fisher SuperSignal West Pico PLUS, Femto or Atto Western Blotting Detection Reagent for 5 min. Western blots were imaged using an iBright CL1500 system (Thermo Fisher) and analysed using Thermo Fisher Connect iBright Analysis Software.

Skin cytokine analysis. Skin protein was extracted from skin tissue of treated mice at age ~30 d. Skin was excised and stored in PBS, and the carcass was stored in 70% ethanol. The carcass was submitted to the Histology and Imaging Core at the University of Washington or Zyagen (San Diego, CA) for haematolysis and eosin staining of skin using established protocols. Three sections of skin from the top, middle and bottom sections of the back torso were used. The hair follicle and skin pathology was established protocols. Three sections of skin from the top, middle and bottom sections of the back torso were used. The hair follicle and skin pathology was estimated using a unique couple with activities far beyond the cold.
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Author contributions
M.M.-P. designed, conducted, analysed and interpreted all the proteomic experiments; prepared associated figures; wrote the initial draft; and revised and edited the manuscript. A.S.G. designed, conducted and interpreted phenotypic and lifespan experiments with the PKC-β inhibitor; conducted and interpreted all the western blot, cytokine and histological data; prepared associated figures; and wrote, revised and edited the manuscript. T.K.I. conceptualized the study; designed, conducted and interpreted phenotypic and lifespan experiments with rapamycin and broad-spectrum PKC inhibitors; and wrote, revised and edited the manuscript. A.S.V. conducted KSEA analysis and revised and edited the manuscript. J.H. conceptualized the study and obtained the brain tissue samples for proteomic analysis. S.W.E. assisted with the proteomic analysis and revised and edited the manuscript. H.Z.H. and D.K. assisted with mouse experiments. M.Y. assisted with statistical analysis and revised and edited the manuscript. M.K. conceived and coordinated the project; supervised the mouse work; provided instrumentation resources and funding for M.M.-P. A.S.V. and S.W.E.; and revised and edited the manuscript.

Competing interests
The authors declare no competing interests.

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Extended Data Fig. 1 | See next page for caption.
Extended Data Fig. 1 | Proteome and phosphoproteome analysis statistics. **a**, Number of total (all), mitochondrial (mitoc) and Complex I (C-I) proteins quantified (gray) and those with significant changes among WT, KO, and KR experimental groups (ANOVA test, FDR q-value < 0.05) (red). Mitochondrial proteins annotations were extracted from mouse MitoCarta 2.0 database. **b**, Correlations of log$_2$ transformed LFQ-normalized protein abundance measurements between samples (N = 6-7 mice). **c**, Same as in **a** but for phosphorylation sites. **d**, Correlations of log$_2$ transformed median-normalized phosphorylation site intensity values between samples and replicates. Two technical replicates of IMAC phosphopeptide enrichment were performed for each brain sample to increase phosphoproteome coverage (N = 12-14 samples; 6-7 mice and duplicated IMAC enrichment and LC-MS/MS analysis). **e**, Distribution of Pearson’s r correlation values among all samples and only for technical replicates. Box plots include the median line, the box denotes the interquartile range (IQR), whiskers denote ±1.5×IQR. **f**, PCA analysis of log$_2$ transformed median-normalized phosphorylation site intensities data (hollow/solid symbols indicates female/male samples respectively).
Extended Data Fig. 2 | Global changes in respiratory chain related proteins. a, b. Aggregated protein abundance changes in respiratory chain complexes (a) and respiratory chain assembly proteins (b). Box plots include the median line, the box denotes the interquartile range (IQR), whiskers denote ±1.5 × IQR. Sum of relative iBAQ intensities for all members of each complex or protein group were used (N = 6-7 mice). T-test significance p-values are indicated (* p < 0.05; ** p < 0.01; *** p < 0.001).
Extended Data Fig. 3 | See next page for caption.
Extended Data Fig. 3 | Western blot analysis of brain extracts from P30 and P50 mice treated with vehicle or rapamycin from P10 to P30 or P50. **a,b,** Western blot analysis of mTORC1 and mTORC2 markers of brain lysates from P30 wild-type (WT) and Ndufs4 KO mice treated daily with vehicle (KO) or rapamycin (KR) from P10 to P30. **c,** Densitometry (relative to actin) of western blot data from (a) and (b) normalized to wild-type levels (N = 6 mice). **d,e,** Western blot analysis of PKC isoforms of brain lysates from P30 wild-type (WT) and Ndufs4 KO mice treated daily with vehicle (KO) or rapamycin (KR) from P10 to P30. **f,** Densitometry (relative to actin) of western blot data from (d) and (e) normalized to wild-type levels (N = 6 mice). **g,h,** Representative WB images and densitometry (relative to actin) normalized to wild-type (WT) levels showing relative phosphorylated and total levels of proteins involved in mTORC1 and mTORC2 (N = 4 mice). **i,j,** Representative WB images and densitometry (relative to actin) normalized to WT levels showing relative phosphorylated and total levels of PKC proteins (N = 4 mice). Each lane corresponds to a brain lysate from a single mouse. T-test significance p-values are indicated (* p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001).
Extended Data Fig. 4 | See next page for caption.
Extended Data Fig. 4 | Rapamycin exerts similar effects in the brains of wild-type mice to Ndufs4 KO mice. a, Experimental design to evaluate rapamycin-mediated effects in the brain of wild-type mice. b, Body weight gain in mice from the two experimental groups (mean ± s.d.; N = 4–6 mice). c, Total brain weight at the end of the experimental trial (N = 4–6 mice). T-test significance p-values are indicated (* p < 0.05). Box plots include the median line, the box denotes the interquartile range (IQR), whiskers denote ±1.5 × IQR. d, Comparison of rapamycin mediated changes between wild-type and knock-out mice in individual protein levels. Pearson’s correlation values are indicated. e, 2D-enrichment analysis of GO and KEGG terms comparing the effect of rapamycin between wild-type and knock-out mice in the proteome (Wilcoxon-Mann-Whitney test, FDR q-value < 0.05). f, Comparison of rapamycin mediated changes between wild-type and knock-out mice in phosphorylation sites. Pearson’s correlation values are indicated. g, 2D-enrichment analysis of GO and KEGG terms comparing the effect of rapamycin between wild-type and knock-out mice in the phosphoproteome (Wilcoxon-Mann-Whitney test, p-value < 0.01). In 2D enrichment analysis (panels (e) and (f)) most data points are close to the diagonal dashed line (that is identity function), indicating no differences in the effect of rapamycin on wild-type and Ndufs4 KO mice.
Extended Data Fig. 5 | Correlation between rapamycin effects in Ndufs4 KO mice at the proteomic (x axis) and phosphoproteomic (y axis) levels. Pearson’s r coefficient and goodness-of-fit test p-value of linear curve fitted line (dashed line) are indicated.
Extended Data Fig. 6 | See next page for caption.
Extended Data Fig. 6 | Phosphorylation changes in brain proteins of Ndufs4 KO mice upon rapamycin treatment. a, Phosphorylation sites on proteins of the mTOR complexes and associated substrates that show significant changes among experimental groups (WT: wild-type; KD: Ndufs4 KO; KR: rapamycin-treated Ndufs4 KO). b, Average (mean ± s.e.m.) changes in phosphorylation of kinase substrates upon rapamycin treatment in Ndufs4 KO mice brain. Each dot represents an individual phosphorylation site substrate. Only kinases with more than 9 substrates found are shown. c, Significant changes in phosphorylation on activity regulatory sites of specific kinases (*activation loop sites, #inhibitory sites). d, Significant changes in activating phosphorylation sites of the main two calcium-release channels from the endoplasmic reticulum. All box plots include the median line, the box denotes the interquartile range (IQR), whiskers denote ±1.5×IQR. T-test significance p-values are indicated (* p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001; N=12-14 samples; 6-7 mice and duplicated IMAC enrichment and LC-MS/MS analysis, if no missing values are found).
Extended Data Fig. 7 | See next page for caption.
Extended Data Fig. 7 | Treatment of Ndufs4 KO mice with PKC inhibitors largely prevents the alopecia phenotype at weaning (~P21). a, Wild-type mice at weaning show no hair loss. b, Untreated Ndufs4 KO mice normally exhibit alopecia (that is hair loss) at 21-days old due to a TLR2/4 innate immune response. In contrast, minimal hair loss was observed in 21-day old Ndufs4 KO mice treated with c, d, GF109203X and ruboxistaurin from P10 to P21. e, Some hair loss was observed in 21-day old Ndufs4 KO mice treated with rapamycin from P10 to P21.
Extended Data Fig. 8 | See next page for caption.
Extended Data Fig. 8 | Histological analysis of skin pathology at P21 or P30. a, Representative images (20X zoom) of haematoxylin and eosin staining of skin sections of P21 WT and Ndufs4 KO mice treated with vehicle, rapamycin, or ruboxistaurin from P10 to P21. Each picture corresponds to an image from an individual mouse. b, Blinded skin inflammation pathology scores from haematoxylin and eosin staining of skin sections of P21 WT and Ndufs4 KO mice treated with vehicle, rapamycin, or ruboxistaurin from P10 to P21. Increasing scores represent increasing severity of pathology. c, Blinded hair follicle pathology scores from haematoxylin and eosin staining of skin sections of P21 WT and Ndufs4 KO mice treated with vehicle, rapamycin, or ruboxistaurin from P10 to P21. d, Representative images (20X zoom) of haematoxylin and eosin staining of skin sections of P30 WT and Ndufs4 KO mice treated with vehicle, rapamycin, or ruboxistaurin from P10 to P30. Each picture corresponds to an image from an individual mouse. e, Blinded skin inflammation pathology scores from haematoxylin and eosin staining of skin sections of P30 WT and Ndufs4 KO mice treated with vehicle, rapamycin, or ruboxistaurin from P10 to P30. Increasing scores represent increasing severity of pathology. f, Blinded hair follicle pathology scores from haematoxylin and eosin staining of skin sections of P30 WT and Ndufs4 KO mice treated with vehicle, rapamycin, or ruboxistaurin from P10 to P30. Increasing scores represent increasing severity of pathology. Each point represents the score for an individual mouse. T-test significance p-values are indicated (* p < 0.05; ** p < 0.01; *** p < 0.001; **** p < 0.0001; N = 6 mice). g, Cytokine levels in mouse skin (N = 3–6 mice) of P30 WT and Ndufs4 KO mice treated with vehicle, rapamycin, or ruboxistaurin from P10 to P30. Cytokine levels were measured using a cytokine array and z-score normalized. Left, violin plot of combined z-score normalized values of median abundance of individual cytokines (each point represents an individual cytokine and the dashed line indicates median z-score value for each group; N = 14). Zero mean score is indicated by a dotted line. Right, individualized information in a heatmap. T-test significance p-values are indicated for the different treatments compared to their respective wild-type or knock-out mice values (^ p < 0.1; * p < 0.05; ** p < 0.01; N = 3–6 mice).
Extended Data Fig. 9 | Untreated and vehicle-treated Ndufs4 KO mice exhibit similar symptoms of disease. 

**a**, Vehicle treatment does not alter the lifespan of Ndufs4 KO mice compared to untreated controls. Untreated vs. vehicle p-value = 0.7898, log-rank. 

**b**, Vehicle treatment does not alter the onset of clasping of Ndufs4 KO mice compared to untreated controls. 

**c**, Vehicle treatment does not alter weight gain of Ndufs4 KO mice compared to untreated controls (mean ± s.d). N = 8 mice for vehicle and N = 11 mice for untreated controls in all plots.
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Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection
Mass spectrometry data for proteome samples was acquired in a QExactive using XCalibur instrument software v.2.2.1.1646. Mass spectrometry data for phosphoproteome samples was acquired in an Orbitrap Velos using XCalibur instrument software v2.6.0 SP3.

Data analysis
Acquired mass spectra raw files were searched with MaxQuant v1.6.0.1 (https://www.maxquant.org/).
Perseus v1.6.0.7 software was used for bioinformatic and statistical analysis (https://maxquant.net/perseus/).
GORilla application was used for GO terms enrichment analysis [http://cbl-gorilla.cs.technion.ac.il/].
Motif extractor tool (motif-x) was used to extract overrepresented motif patterns in sequences surrounding phosphosites (http://motif-x.med.harvard.edu.sire.ub.edu/).
Kinase activity was determined using kinase set enrichment analysis (KSEA) R package (https://github.com/evocellnet/ksea).
Western blot data was analyzed using Thermo Fisher Connect iBright Analysis Software.

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All mass spectrometry raw files and searches have been deposited in the MassIVE repository with dataset identifier PXD012158. Protein and phosphorylation quantification results are provided as Supplementary Tables 1-6.

List of figures and tables with associated proteomic and phosphoproteomic raw data:
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- **Sample size**: Sample size for proteomic experiments was decided based on prior quantiative proteomic studies using mouse tissue. The minimum sample size for mouse lifespan experiments was determined to be 7 using Cochrans's Formula to determine a 30% change in lifespan considering an average lifespan of 50 +/- 10 days for untreated mice with a confidence interval of 0.05 and 80% power.

- **Data exclusions**: No data was excluded from the manuscript.

- **Replication**: All data was obtained using multiple biological replicates as indicated throughout the text and when possible multiple technical replicates were used minimize error from data acquisition.

- **Randomization**: Mice were randomly assigned between treatment groups after ensuring similar sex distributions between groups.

- **Blinding**: Genotypes of mice were blinded from mouse technicians. Histology data was blinded before pathology scoring. All other data was blinded prior to analysis whenever possible.

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### Materials & experimental systems

| n/a | Involved in the study |
|-----|-----------------------|
| ☐   | Antibodies            |
| ☐   | Eukaryotic cell lines |
| ☐   | Palaeontology         |
| ☐   | Animals and other organisms |
| ☐   | Human research participants |
| ☑   | Clinical data         |

### Methods

| n/a | Involved in the study |
|-----|-----------------------|
| ☐   | ChIP-seq              |
| ☐   | Flow cytometry        |
| ☑   | MRI-based neuroimaging |

### Antibodies

**Antibodies used**

All antibodies used were commercially available.

Primary antibodies used were: p5235/236-56 ribosomal protein (1:1,000, Cell Signaling Technology 4858), S6 ribosomal protein (1:1,000, Cell Signaling Technology 2217), p5473-Akt (1:1,000, Cell Signaling Technology 4060), pT308-Akt (1:500, Cell Signaling Technology 9275), pT450-Akt (1:1,000, Cell Signaling Technology 9267), Akt (1:1,000, Cell Signaling Technology 4691), p6222-PKC-α (1:2,000, AbClonal AP0599), PKC-α (1:2,000, Cell Signaling Technology 2056), pT638/641-PKC-α/β (1:5,000, Cell Signaling Technology 9375), PKC-β (1:500, Cell Signaling Technology 46809), pT674-PKC-γ (1:1,000, Abcam ab5797), PKC-γ (1:2,000, Santa Cruz Biotechnology sc-166385), pS176/180-ERK1/2 Antibody (1:1,000, Cell Signaling Technology 2694), IKB-κ (1:200 in 1% milk, Novus Biologicals NB-100-56704), pS32-Kβ (1:200 in 1% BSA, Santa Cruz Biotechnology sc-8404), Kβ (1:50,000, Abcam ab-32518), pS36-NF-κB p65 (1:100 in 1% BSA, Santa Cruz Biotechnology sc-136548), NF-κB p65 (1:2,000 in 1% BSA, Cell Signaling Technology cs 8242), GAP41 (1:5,000 in 5% milk, Cell Signaling Technology 12389), or β-actin HRP conjugate (1:25,000 at room temperature for 2 hours, Cell Signaling Technology 5125).
Secondary antibodies used were donkey anti-rabbit IgG HRP conjugate (1:10,000 or 1:25,000, Thermo Scientific 31458) or mouse-<i>IgG</i>κ BP HRP conjugate (1:2,000, Santa Cruz Biotechnology sc-516102).

Validation
Antibodies were validated by the commercial source and by the observed molecular weight.

Animals and other organisms
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Laboratory animals
Animal studies throughout manuscript used the Ndufs4 KO mice and its WT counterpart in a C57Bl/6 background. Both male and female mice were used and ages ranged from postnatal day 10 to ~100.

Wild animals
N/A

Field-collected samples
N/A

Ethics oversight
All animal experiments were approved by the IACUC at the University of Washington.

Note that full information on the approval of the study protocol must also be provided in the manuscript.