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Supplementary Materials for

mTOR Inhibition Alleviates Mitochondrial Disease in a Mouse Model of Leigh Syndrome

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Materials and Methods

Animal Care

Ndufs4 +/- breeders were obtained from the Palmiter laboratory at the University of Washington. Following colony expansion by backcrossing to C57Bl/6 CR mice, obtained from the NIA, *Ndufs4* +/- mice were bred as harems with heterozygous parents bred to produce *Ndfus4 -/-* offspring. All mice were weaned at 20-21 days of age. *Ndufs4 -/-* animals were always housed with a minimum of one control littermate for warmth and stimulation. All mice were weighed daily and food and gel were provided on the bottom of each cage so that ability to find food or water did not become a limiting factor for survival. Mice were euthanized if they showed a 20% loss in maximum body weight, immobility, or were found prostrate or unconscious. Mice used for rotarod and QMR were separate from those used in the lifespan so that the any stress resulting from these assays would not be a complicating factor in determining survival. *Ndufs4* heterozygous mice and wild-type mice were identical in each assay described here and thus were pooled as controls for the experiments described.

All care of experimental animals was in accordance with the University of Washington institutional guidelines and experiments were performed as approved by the Institutional Animal Care and Use Committee.

Rapamycin Administration

Rapamycin was dissolved in DMSO to 100 mg/mL. This was diluted in 5% PEG-400/5% Tween-20 (vehicle) to a concentration of 1.2mg/mL, sterile filtered, aliquoted into 1mL portions, and stored at -80 for long-term storage. Rapamycin treated mice were injected with 66 microliters / 10g body weight for a final dosage of 8.0 mg/kg. Vehicle mice were injected with vehicle containing an equal volume of diluent and DMSO lacking rapamycin. Injections were performed intraperitoneally using 29 ½ gauge, 3/10 cc insulin syringes. The abdomen was briefly swabbed with an alcohol wipe prior to injection.

Rotarod

Rotarod parameters were set at beginning speed of 0 rpm with an acceleration rate of 0.1 rpm/s and a maximum speed of 40 rpm. Mice underwent practice sessions on two consecutive days, with one session per day. The animals were tested 24 hours after the second practice day; mice underwent three rotarod sessions on the testing day, and latency-to-fall times were recorded. Median and maximum times were then calculated. The number of mice used for each data point is as indicated in Figure S5.

Western Blotting

Whole-organs were rinsed of blood and flash-frozen in liquid nitrogen. Tissues were cryohomogenized on dry ice and the homogenized frozen powder was split for protein and RNA extractions. Protein was extracted by sonicating powdered tissue in RIPA buffer (10mM Tris-HCl pH 8.0, 1mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 140mM NaCl) with Roche cOmplete Ultra protease inhibitor and PhosSTOP phosphatase inhibitor tablets added prior to use. Protein lysates were BCA'd using standard techniques and 20 micrograms protein per sample were run using

Novex minigels. Antibodies used were ordered from the following companies: Hsp60: Cell Signaling Technology #4870, LC3: Cell Signaling, #4108, Akt: Cell Signaling, #2920, pAkt: Cell Signaling, #4060, IGFR: Cell Signaling, #3027, pIGFR: Cell Signaling, #4568, Beclin: Novus Biologicals, NBP1-00088, CytC: MitoSciences, #MSA06, CoxIV: Cell Signaling, #4844, GAPDH: Novus Biologicals, NB300-322, Ndufs3:Invitrogen, #439200, Ndufs9 MitoSciences (MS Catalog Number MS111), p62: Cell Signaling, #5114, S6: Cell Signaling, #5317, pS6: Cell Signaling, #4856. Blots were blocked in 3% milk-TBST and all primary antibodies were used at 1:1000 in 0.3%-TBST milk.

Tissue Staining

Tissues were fixed overnight in 4% formalin in PBS and cryoprotected by soaking in 30% sucrose in PBS until floating (24-48 hours). Oil-red-o staining was performed by the University of Washington Histology and Specialized Pathology Services core facilities by standard methods. Staining for Iba1 and GFAP were performed using 30-µm free-floating sections were used. Paraffin sections were rehydrated through a series of graded ethanol solutions and boiled for 20 min in sodium citrate buffer, pH 6.0, for antigen unmasking. Sections were blocked with 10% normal donkey serum (NDS) in PBS-0.2% Triton X (PBST) for 1 h at room temperature and then incubated overnight at 4 °C in a wet chamber with primary antibodies diluted in 1%NDS-PBST (1:1,000 anti-GFAP, Sigma #G3893 , or 1:1,000 anti–Iba-1, Cell Signaling #019–19741). Slides were washed in PBST and incubated for 1 h at RT with Cy2- or Cy3-conjugated secondary antibodies (1:200 in 1% NDS-PBST; Jackson Immunoresearch). Sections were washed in PBS and counterstained with DAPI (Sigma) and coverslipped with aqueous mounting media (Fluo romount G; Electron Microscopy Science).

Microscopy

All images were gathered with a Nikon Eclipse E600 bright field and fluorescence microscope at the University of Washington Keck Microscopy center. All image analysis was performed using the free analysis program ImageJ (http://rsbweb.nih.gov/ij/).

Respiration assays

Respiration of freshly isolated mitochondria was measured with a Seahorse XF24 flux analyzer (Seahorse BioScience, Billerica, MA) following the manufacurers guidelines (http://www.seahorsebio.com/resources/tech-writing/iso-mito-xf24.pdf). Briefly: 5ug of mitochondria were adsorbed to the bottom of each sample well by spinning 50ul of mitochondrial suspension at 2000g for 20min at 4°C. The medium was MAS (70mM sucrose, 220mM mannitol, 10mM KH_2PO_4, 5mM $MgCl_2$, 2mM HEPES, 1mM EGTA, 0.02% fatty acid-free BSA, pHed to 7.2 with KOH at 37°C) supplemented with electron donor substrate. Electron donor combinations for complex I dependent respiration were 5mM malate plus either 10mM pyruvate, 10mM glutamate or 10mM α-ketoglutarate. For measuring complex I-independent respiration the complex II substrate succinate (13mM) in combination with the complex I inhibitor rotenone (2µM) was utilized. Before loading the plate into the Seahorse the assay volume of all wells, samples, and blanks was adjusted to 500ul by adding more of the same medium (MAS plus electron donors).

The instrument was programmed to execute the following protocol at 37°C: wait 10 min, mix 1min, wait 3 min, mix 1 min, wait 3 min, mix 1 min, measure 3 min, mix 1 min, measure 3 min state 2 respiration, mix 1min, inject ATP synthetase substrate ADP (4mM, pH'd to 7.2), mix 1min, measure 3min state3 respiration, mix 1min, inject ATP synthetase inhibitor oligomycin (2.5ug/ml), mix 1min, measure 3min state 4 respiration, mix 1min, inject uncoupler carbonyl cyanide 4-(trifluoromethoxy)-phenylhydrazone (= FCCP) (5uM), mix 1min, measure 3min uncoupled respiration, mix 1min, inject complex III inhibitor antimycin A (4uM), mix 1min, measure 3min respiratory chain-independent respiration. Concentrations given signify concentrations after injection in assay. Respiration rates for each well were determined using the Akos algorithm for OCR (oxygen consumption rate) (2009 Gerencser et al. AnalChem81 6868 - Seahorse - quantitative microplate-based respirometry with correction for oxygen diffusion), background corrected and normalized to ug of loaded protein. State3 and uncoupled respiration rates declined during the 3min measurement intervall and were therfore determined as the initial (maximum) respiration rate measured. State2 and state4 rates were stable and therefore reported as the average over the respective measurement interval. For each mitochondrial preparation these parameters were reported as the mean of 5 technical replicates.

Blue Native Gels (BNG)

BNG-PAGE was performed as described by Wittig et al. (2006 Nature Protocols 1(1) 418) with the modification that precast minigels (Novex, NativePAGE 3-12% Bis-Tris, BN2011BX10, 1mm thick) were used in an XCell SureLock Mini-Cell (Invitrogen). Key features of the sample treatment: 150ug of previously frozen mitochondria samples were extracted with either digitonin (6:1 detergent:protein w:w) or Triton X-100 (5:1 detergent:protein w:w) and protein complexes in the 15,000g supernatant were negatively charged with Coomassie Blue G-250 (1:8 detergent:dye w:w). Key features of the run: After 1h electrophoresis at 100V blue cathode buffer was replaced by cathode buffer without Coomassie blue and 300V were applied for an additional 1.5h. At the end of the run the overall protein distribution in the gel could be imaged without any additional Coomassie staining. Complex I in-gel activity (IGA) staining: Gels were incubated at room temperature with 2.5mM NADH and 0.5mM nitroblue tetrazolium (mod. of Sabar et al. PlantJ44 893). The purpelish hue of the formazan reaction product identifies bands with NADH dehydrogenase activity were. (A prominent cross reactivity at about 200 kD is probably caused by dehydrolipoamide dehydrogenase (2009 EH Meyer et al. PlantPhysiol151 603)).

Metabolomics

Approximately 150 micrograms of cryohomogenized frozen tissue powder from each sample were sonicated, with 50 micron glass beads in 500 microliters of 80:20 methanol:H_20 on dry ice, incubated for 30 minutes, then centrifuged for 10 min at 14,000 rpm in a microcentrifuge at 4 degrees C. Supernatant was moved to a new tube, on dry ice, and the extraction was repeated two more times. The total volume of extract was combined and lyophilized using a microcentrifuge rotovac at 14,000 rpm, 30 degrees C, with carbon dioxide filling the rotovac chamber prior to applying the vacuum and starting

centrifugation. Samples were submitted to the University of Washington Nutrition Obesity Research Center Analytic Core facility for analysis.

Dried samples were reconstituted in 5 mM ammonium acetate in 95% water/5% acetonitrile + 0.5% acetic acid and filtered prior to LC-MS analysis. The filtered samples were injected to the LC system which was composed of two Agilent 1260 binary pumps, an Agilent 1260 auto-sampler and Agilent 1290 column compartment containing a column-switching valve (Agilent Technologies, Santa Clara, CA). The chromatography was performed using Solvents A (5 mM ammonium acetate in H2O + 0.5% acetic acid + 0.5% acetonitrile) and B (acetonitrile + 0.5% acetic acid + 0.5% water), with 5% B for 2 min, 5% B to 80% B in 3 min, 80% B for 3 min, 80% B to 5% B in 3 min, and 5% B for 7 min. After the chromatographic separation, MS ionization and data acquisition was performed using an AB Sciex QTrap 5500 mass spectrometer (AB Sciex, Toronto, ON, Canada) equipped with electrospray ionization (ESI) source. Multiple-reaction-monitoring (MRM) mode was used for targeted data acquisition of 158 metabolites. The extracted MRM peaks were integrated using MultiQuant 2.1 software (AB Sciex, Toronto, ON, Canada). Reproducibility of the quality control samples indicated excellent technical reproducibility with an average CV of 7% for positive ion mode and 10% for negative ion mode detected metabolites.

Statistical Analysis

All data were presented as means +/- SEM. Comparisons between groups were performed using student t-tests, 2-tails. $P<0.05$ was considered significant and $p<0.005$ considered highly significant (designated * and ** on bar graphs, respectively). Statistical comparisons of lifespan curves were performed using the log-rank test as indicated.

Metabolomic data q-value multiple testing correction was performed using the R plugin qvalue available at http://genomics.princeton.edu/storeylab/qvalue/.

Rapamycin Blood Level Analysis

Whole-blood samples were collected through cardiac puncture immediately following euthanasia into tubes containing EDTA. Samples were frozen and shipped on dry ice to the Javors lab in the Department of Psychiatry at the University of Texas Health Science Center San Antonio for analysis by HPLC.

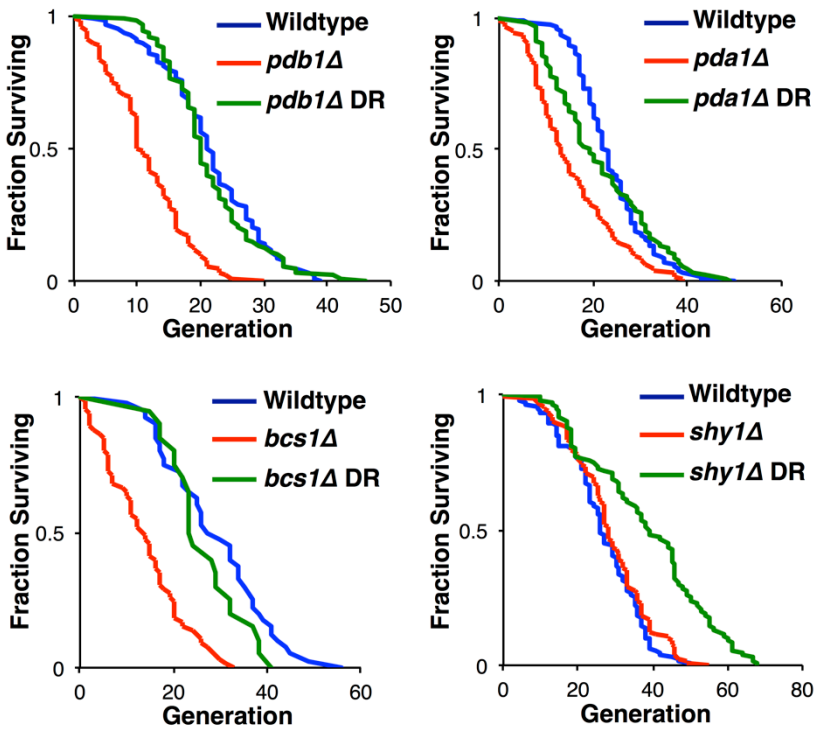


Fig. S1.

Glucose Restriction Extends the Replicative Lifespan of Leigh Syndrome Homolog Mutants in Yeast. Glucose restriction at 0.05% extends the replicative lifespan of *pdb1Δ*, *pda1Δ, bcs1Δ*, and *shy1Δ* cells. *pdb1Δ*, *pda1Δ, bcs1Δ* are short-lived compared to WT cells on regular media (2% glucose) while *shy1Δ* cells have a WT lifespan on regular media. Each plot shows experiment-matched data.

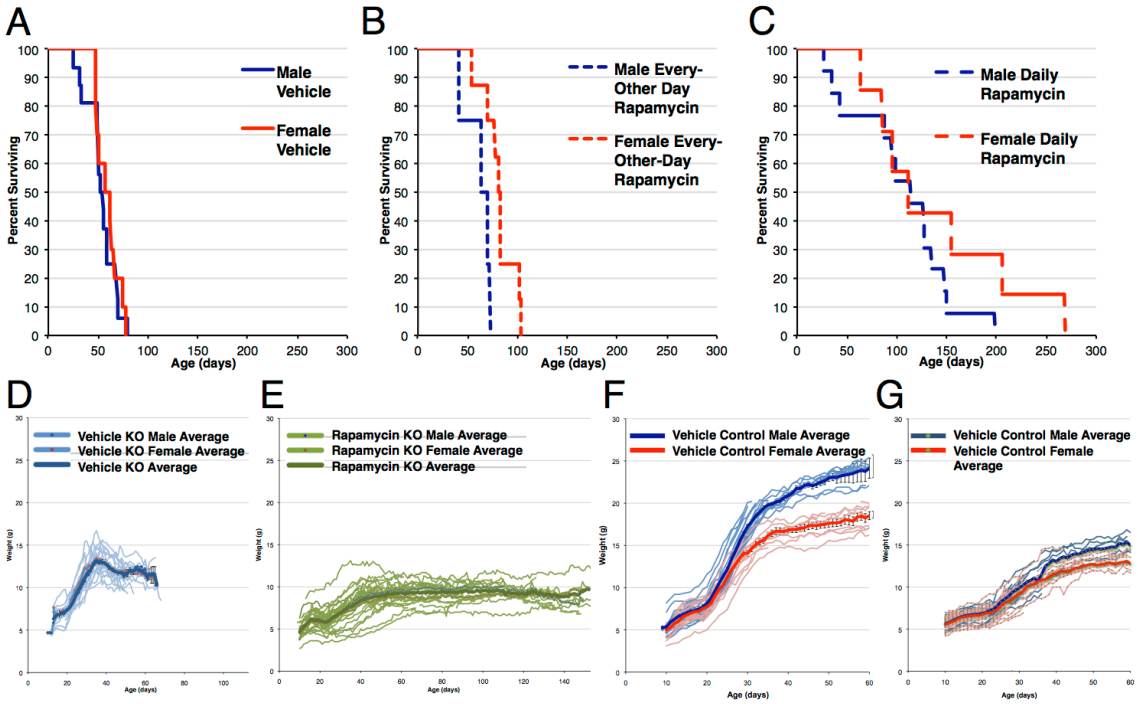


Fig. S2

Lifespan and weight tracking data split by gender. (A) Vehicle treated Ndufs4 -/- mouse lifespan split by gender (n=16 and 10 for males and females, respectively). (B) Every-other-day rapamycin injected Ndufs4 -/- mouse lifespan split by gender (n=4 and 8 for males and females, respectively). (C) Daily rapamycin injected Ndufs4 -/- mouse lifespan split by gender (n= 13 an 7 for males and females, respectively). (D-G) Weight tracking of vehicle treated Ndufs4 -/- (E), daily rapamycin treated Ndufs4 -/- (F), vehicle treated control (G), and daily rapamycin treated control mice.

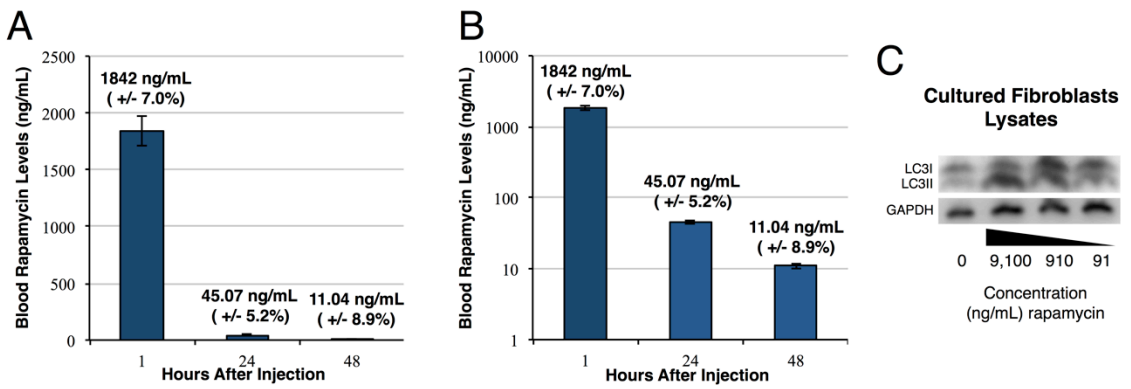


Fig. S3

Blood levels of rapamycin following injection of 8mg/kg. (A-B) Analysis of blood levels of rapamycin show a rapid decrease in blood levels by 24 hours post-injection (shown in linear (A) and log (B) scale). (C) Western blotting for LC3, a marker of mTOR inhibition, treated with rapamycin for 6 hours suggests that levels of rapamycin in mice at 24 hours post-injection are below levels necessary to induce pathways downstream of mTOR. Error bars represent +/- standard error of the mean (SEM), n=3 animals per data point.

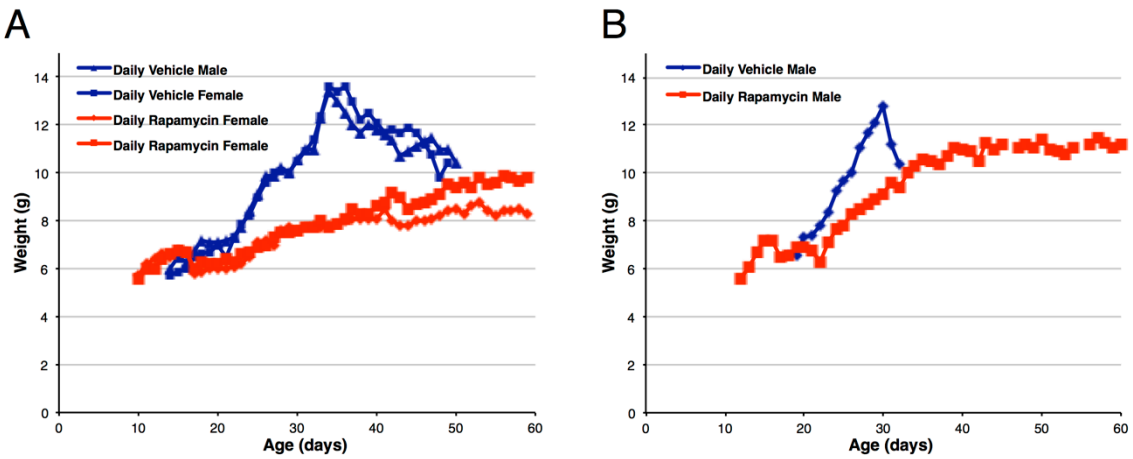
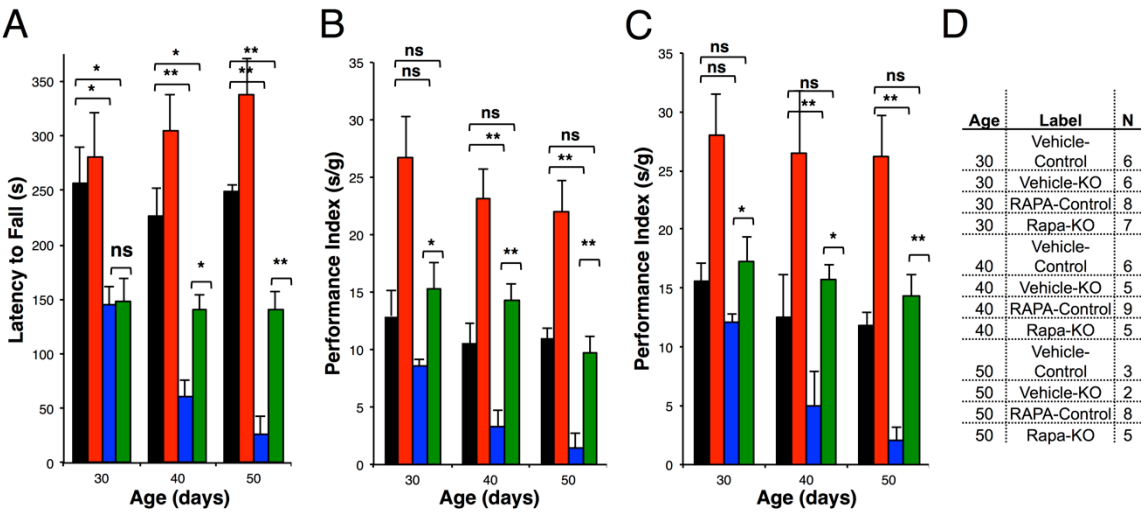


Fig. S4

Daily rapamycin injection effect highly reproducible even among mice within the same litter. (A-B) Two representative litters where pups were randomly assigned to vehicle or rapamycin treatment. In both litters the rapamycin response was robust. Response was also gender independent at this dose (see also Figure S1).



Age	Label	N
30	Vehicle-Control	6
30	Vehicle-KO	6
30	RAPA-Control	8
30	Rapa-KO	7
40	Vehicle-Control	6
40	Vehicle-KO	5
40	RAPA-Control	9
40	Rapa-KO	5
50	Vehicle-Control	3
50	Vehicle-KO	2
50	RAPA-Control	8
50	Rapa-KO	5

Fig. S5

Additional Rotarod Data. (A) Maximum latency to fall. (B) Weight-normalized median latency to fall (see Figure 1 for raw median latency to fall data). (C) Weight-normalized maximum latency to fall. (D) Number of mice assayed for each condition.

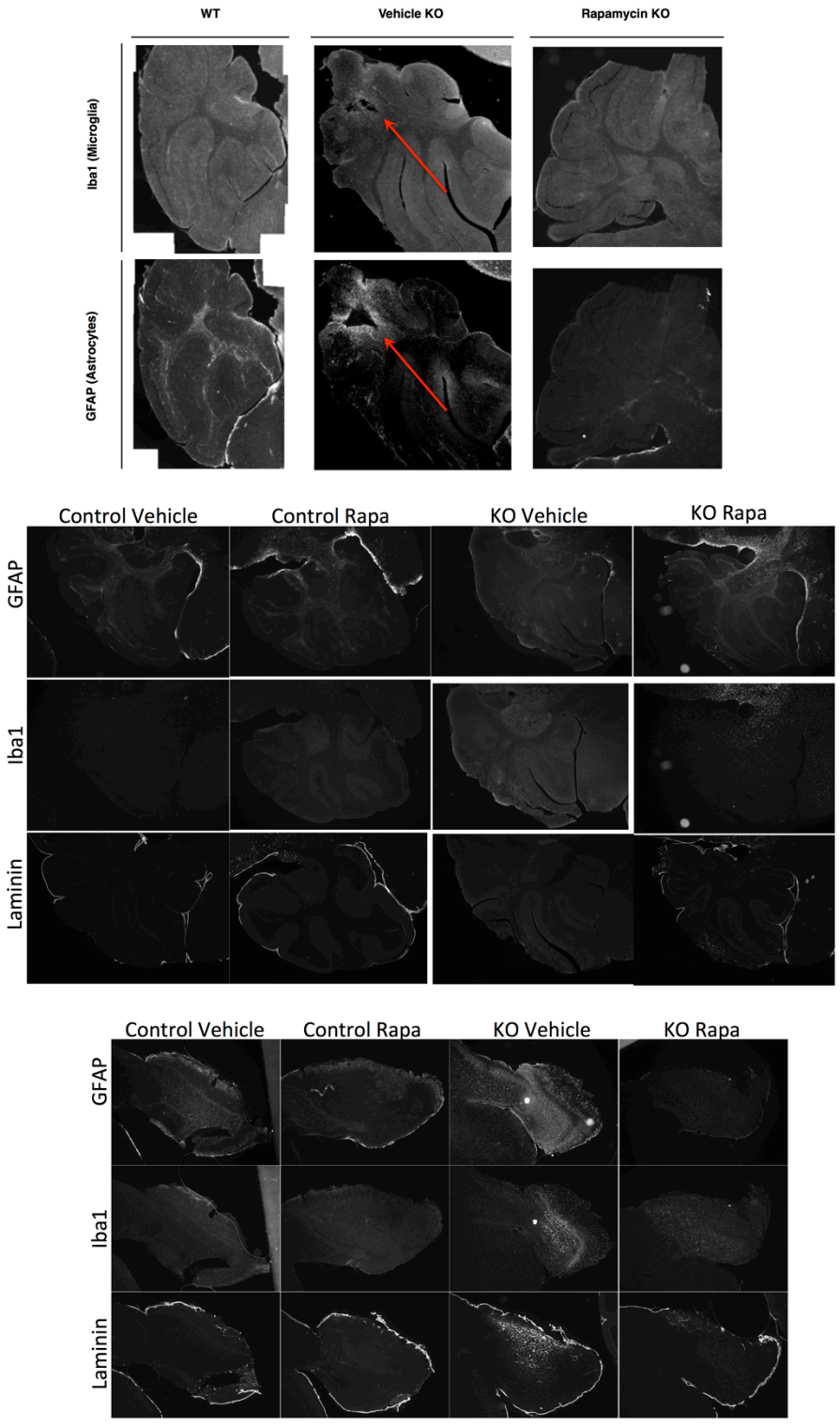


Fig. S6

Additional Histology Images. (Top two panels) Additional age-matched samples showing rescue of lesions in the cerebellum by rapamycin. (Bottom panels) Rapamycin has no overt effects on staining pattern or intensity in control animals.

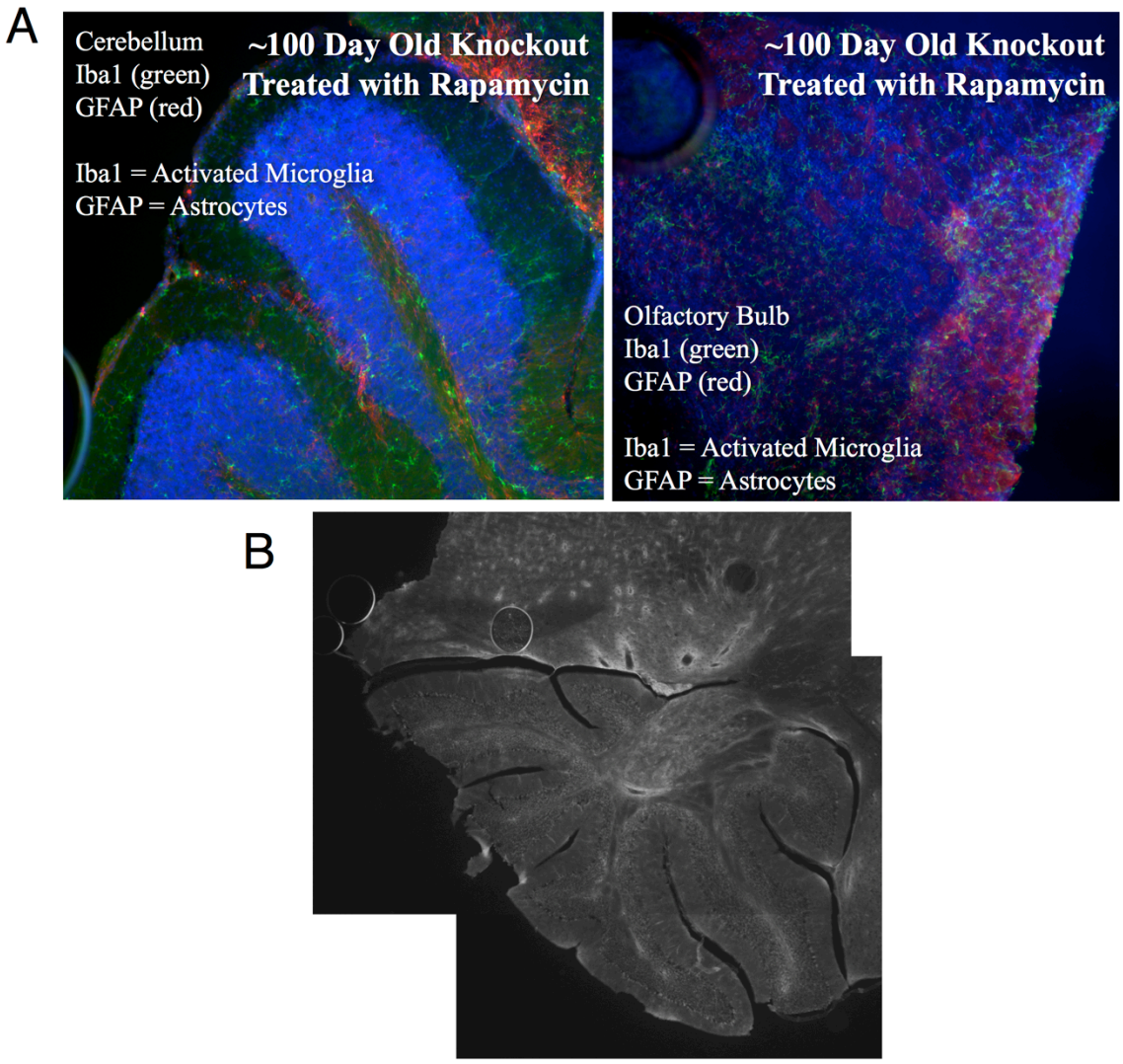


Fig. S7

Absence of Overt Lesions in Brains from Very Long-Lived Rapamycin Treated Knockout Animals. Brains from two very long-lived rapamycin treated knockout mice found dead in cage (FDIC) were removed from the corpse, fixed, stained, and imaged. The quality of stain and imaging is limited by the post-mortem condition, however in each of these cases there were no apparent lesions present at time of death. (A) A brain from an FDIC 100 day old rapamycin treated KO stained with Iba1 (green), GFAP (red), and DAPI (blue). (B) A brain from a 268 day old animal stained with Laminin. Compare to the overt lesions in Figure S6.

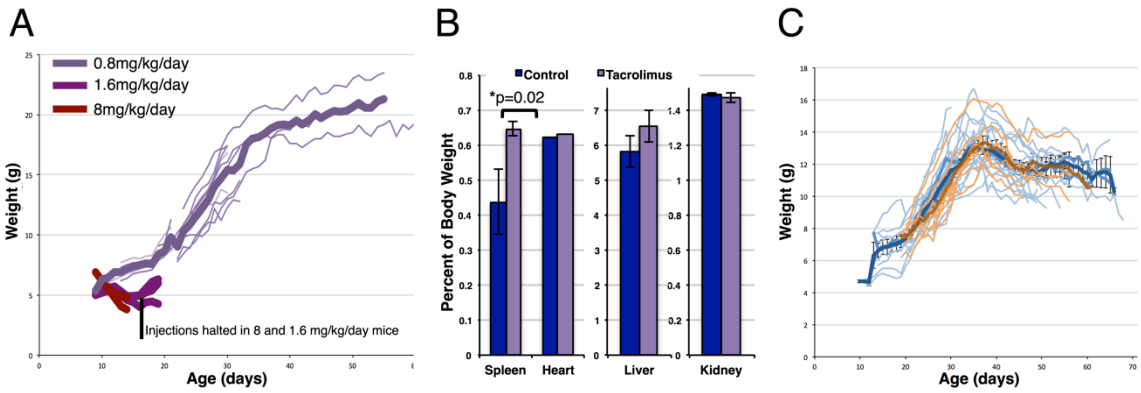


Fig. S8

Highest tolerable dose of tacrolimus (FK-506) determined in control mice. (A) Initial dose of 8 and 1.6 mg/kg/day were not tolerated by control mice as determined by weight loss during treatment. 0.8 mg/kg/day was tolerated and induced splenomegaly at 30 days (B). (C) Tacrolimus also had no effect on the weight phenotype of Ndufs4 -/- mice. Error bars represent +/- SEM, p-value determined using students t-test, n=4 per data point.

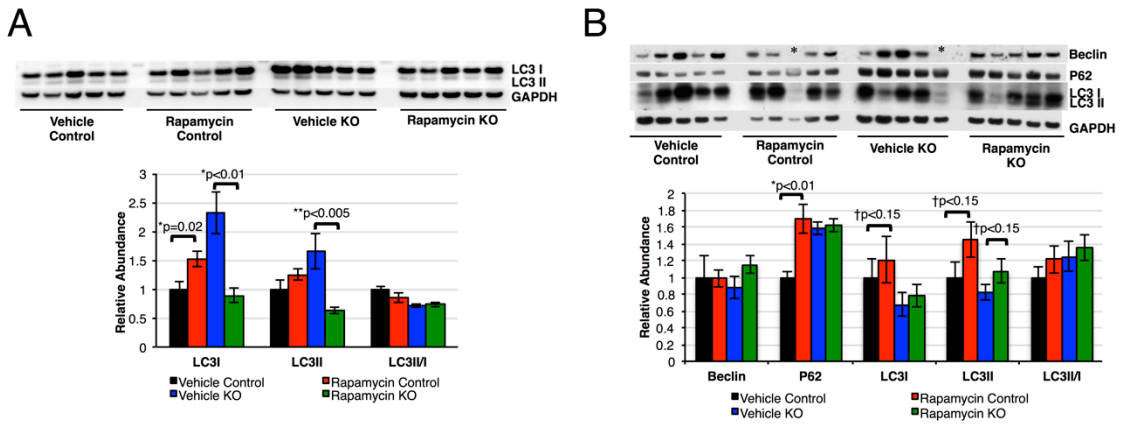


Fig. S9

Daily rapamycin increases activation of autophagy in brain and liver. (A) LC3 is increased in the brains of rapamycin treated control animals. *Ndufs4 -/-* animals show an accumulation of LC3I and II, as has been observed in models associated with overactive mTOR, while rapamycin rescues LC3I and II to WT levels. No significant changes in LC3II/I ratio were observed in brain. (B) Liver homogenates from rapamycin treated animals show increases in P62 and LC3 and a trend toward increases in LC3I and LC3II. Outliers in the Beclin blot are denoted with (*) and were excluded from the analysis, though changes were non-significant regardless of their inclusion. All values were normalized to GAPDH to account for variation in loading, with the exception of LC3II/I ratios. Error bars represent +/- SEM, p-values calculated using students t-test.

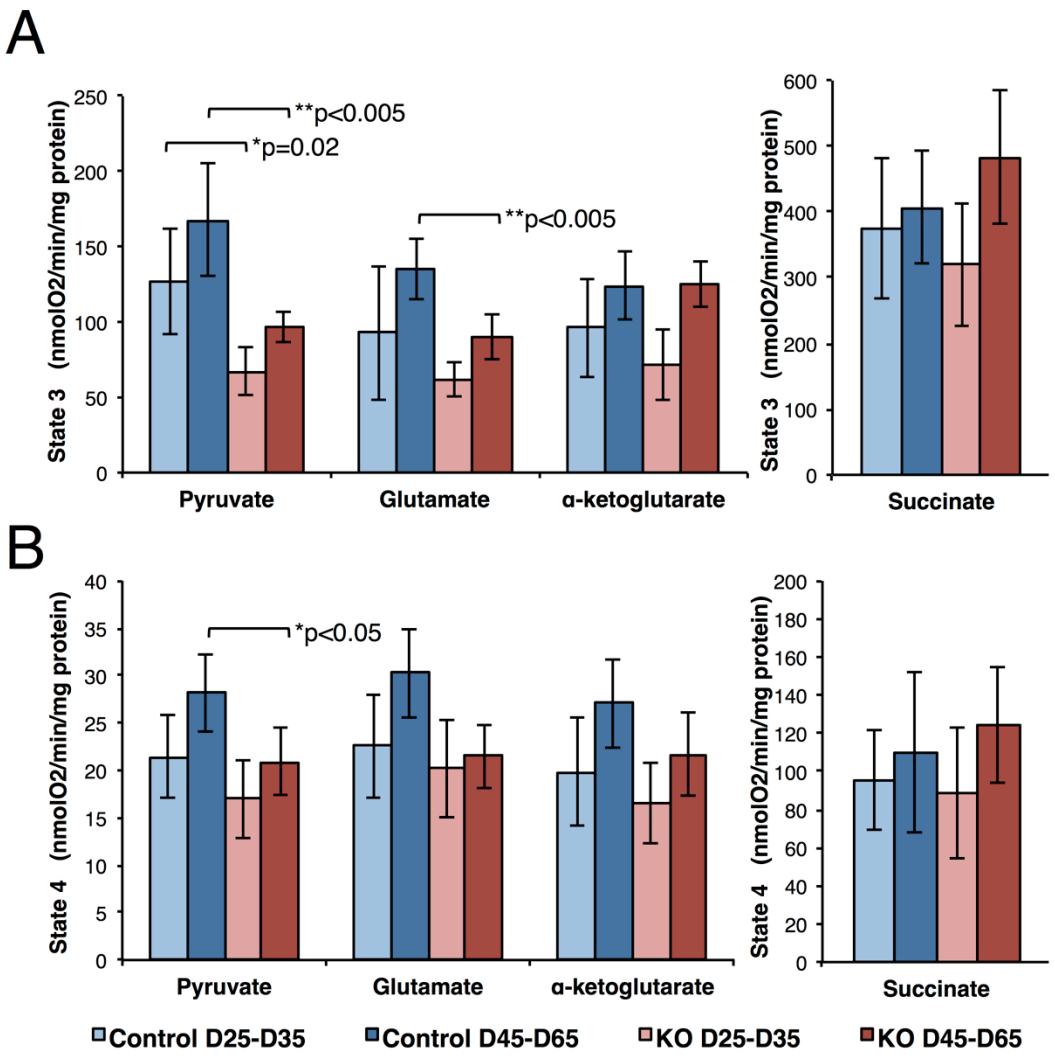


Fig. S10
State 3 and state 4 defects in complex I driven respiration in *Ndufs4 -/-* mice.
Complex I deficiency was similar when analyzed by state 3 (A) or state 4 (B), although the state 3 deficiency appeared slightly more pronounced. Mitochondrial respiration through complex I appeared to increase with age but pyruvate and glutamate driven respiration were deficient at each age. Malate provided with each complex I substrate indicated. α–ketoglutarate and succinate driven respiration were not effected by NDUFS4 deficiency. Error bars represent +/- SEM, p-values calculated using students t-test, n=4-6 animals per datapoint.

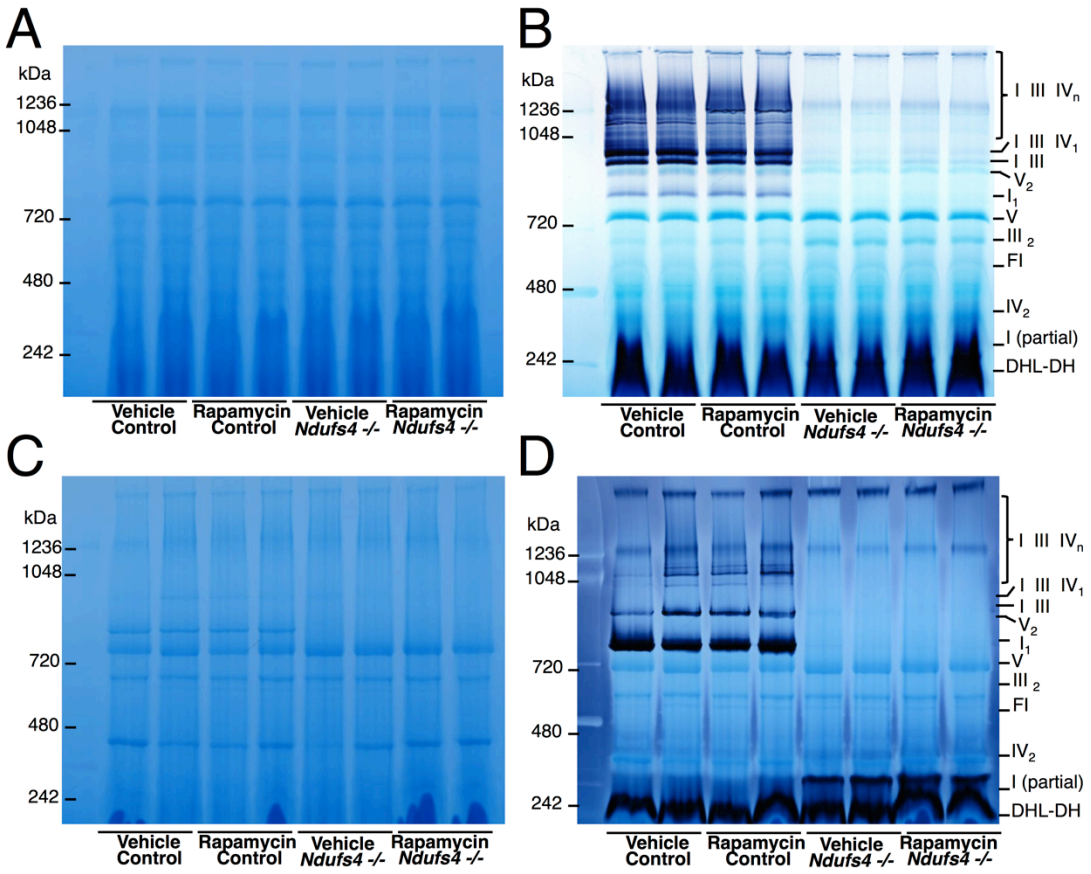


Fig. S11

Additional blue-native in gel activity assay data. Isolated brain mitochondria were analyzed by blue native gel (A,C) and stained using in gel staining for complex I activity (B,D). A mild, non-ionic detergent (digitonin) was used to analyze supercomplex formation (A-B), while a stronger detergent (Triton X-100) was used to analyze free complex I. The *Ndufs4* -/- mice had deficient complex I assembly and supercomplex incorporation and rapamycin had no effect on these features.

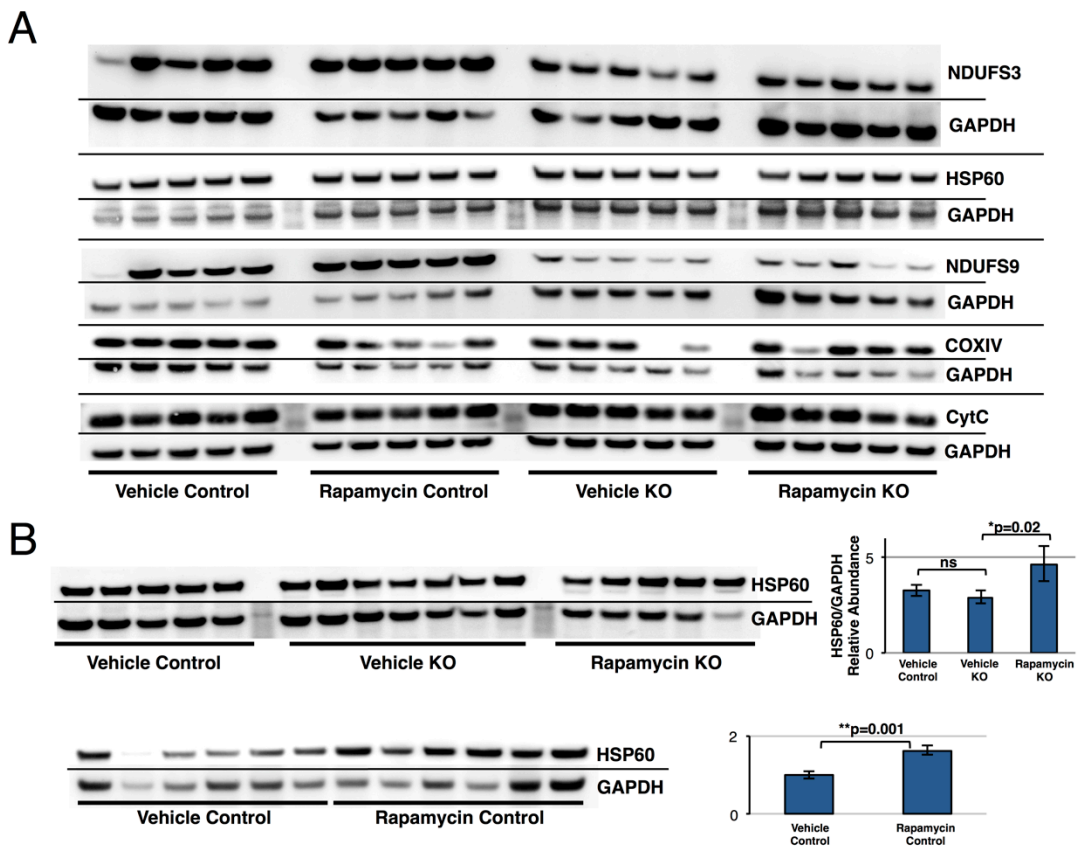


Fig. S12

Additional western blot data. (A) Western blots of whole-brain lysates corresponding to Figure 3. (B) HSP60 is induced in liver by rapamycin but is not induced in the *Ndufs4 -/-* mice without treatment. Error bars represent +/- SEM, p-values calculated using students t-test.

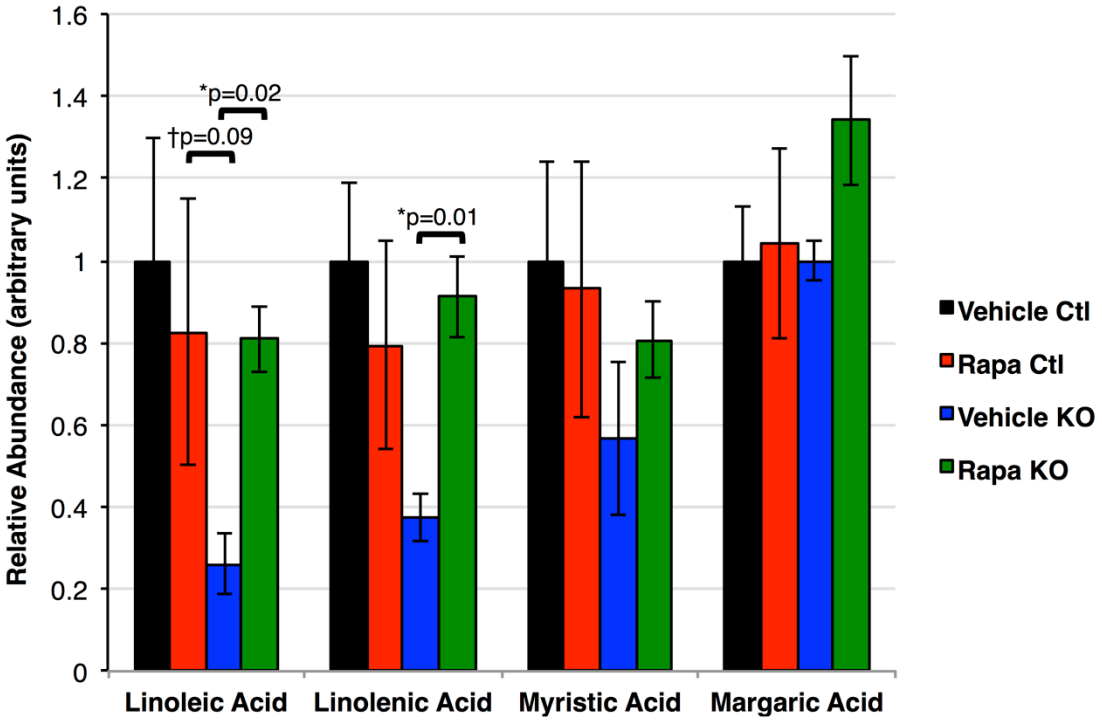


Fig. S13

Free fatty acids in liver detected by metabolomics. Free fatty acids are low in *Ndufs4* -/- animal liver and rescued by rapamycin. Error bars represent +/- SEM, p-values calculated using students t-test. n = 4 animals per treatment.

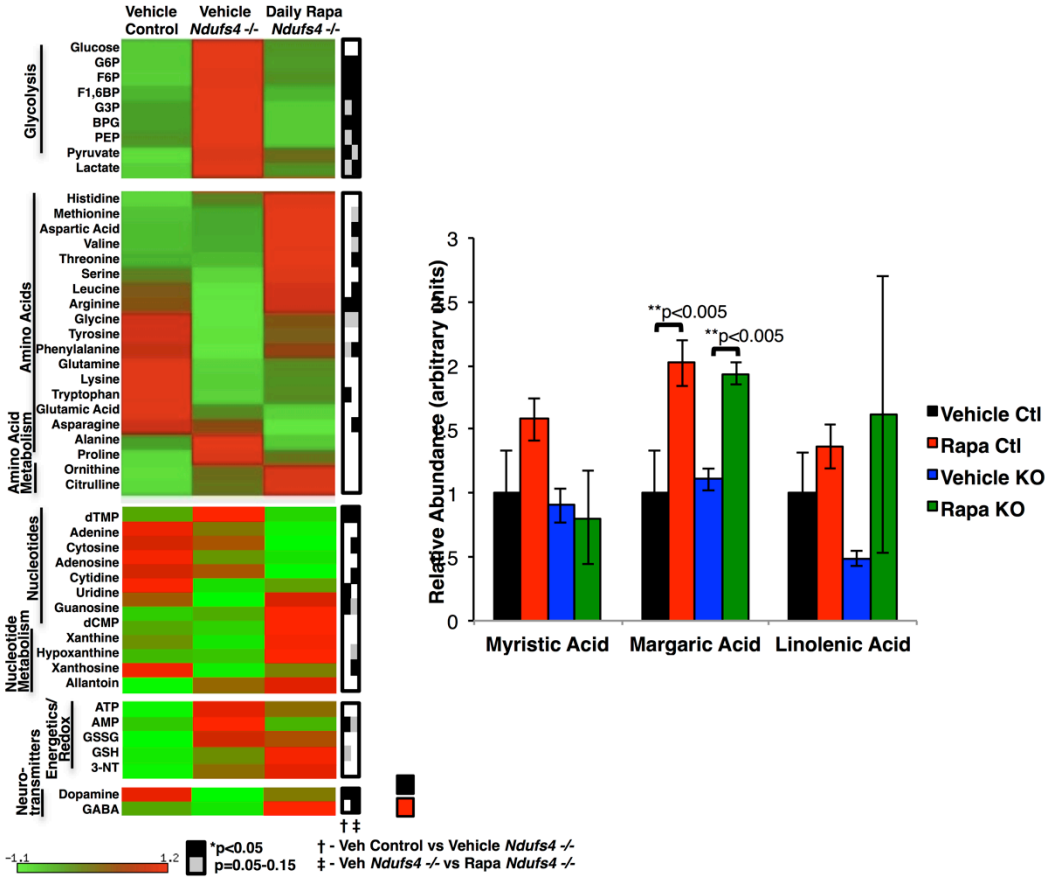


Fig. S14

Metabolic profiling of *Ndufs4 -/-* brains. Metabolomic analysis of *Ndufs4 -/-* mouse brains revealed an accumulation of glycolytic intermediates that is rescued by rapamycin. Rapamycin treatment also increases levels of free-amino acids and free-fatty acids, overall consistent with a shift from glycolysis to amino acid and fat catabolism pathways. The effects of rapamycin on KO animals is distinct from those on control animals and the effects on control mice are not intuitively informative in the setting of the KO animals and will require further study (See also **Supplemental Table 1** for data, statistics, and rapamycin treated control mouse data). Error bars represent +/- SEM, p-values calculated using students t-test. n = 4 animals per treatment.

	Control		KO	
	Vehicle	Rapamycin	Vehicle	Rapamycin
Pyruvate	5763	10200	13385	9491
lactate	1787418	2929369	2647574	2034107
Acetoacetate	4465	7495	6379	3588
Fumaric	2458	2698	2456	3478
Succinate	1910182	2663515	2350145	1206851
Nicotinate (Niacin)	20471	28959	23267	18681
Glutaric Acid	9177	13057	9921	8355
Malate	1867961	3843908	3527238	3582776
Hypoxanthine	1141017	2206619	752462	1786635
Alpha-Ketoglutaric Acid	54218	122628	52141	76741
Xanthine	115990	280033	87015	239200
Urate	5717	7673	4094	2634
PEP	21578	48057	39046	16265
D-GA3P	310469	629060	472542	502060
Glycerol-3-P	1127563	935167	406639	187431
Hyppuric Acid	4190	7588	5394	7918
Glucose	21030	29715	26093	22220
4-Pyridoxic acid	2106	2713	973	1656
2/3-Phosphoglyceric Acid	51335	77067	130036	33025
Erythrose	18885	35698	26111	137244
Cystathionine	5308	8638	5449	6200
G1P/G6P	632620	1486201	1157583	738145
Reduced glutatione	25534	52063	41382	63320
F16BP/F26BP	350646	618019	831547	351717
Sucrose	4418	6170	5786	4864
Oxidized glutathione	1600865	2575830	2115551	2026434
gama-Aminobutyrate	2149	3787	2008	2551
Malonic Acid/3HBA	58393	75980	40433	28706
Citraconic Acid	38571	66620	59710	43861
Adenine	95109	124365	83608	70607
Aconitate	11259	11177	10767	8800
Citrulline	22542	67477	28933	37208
Citric Acid	1633612	2841223	2135206	2228926
Cystine	27606	39241	34250	32160
Xanthosine	55786	86542	53100	108769
Uracil	4574	4339	6778	6161
OH-Phenylpyruvate	9576	11600	12160	10960
Anthranilate	8501	13020	11704	6466
Glucoronate	22253	36441	32409	31890
Oxaloacetate	8244	11027	7879	7923
Propionate	540	1542	1198	420
2-Aminoadipate	12283	25496	17633	14105
3-Nitro-tyrosine	441	803	584	679
Ribose-5-P	271347	493242	219540	362983
Adenylosuccinate	30028	40834	29132	28344
D-Leucic Acid	42577	82250	81256	84569
Pyridoxal-5-P	3162	4400	3827	3904
Adipic Acid	1904	3872	4051	3874
Maleic Acid	2262	3359	2244	3716
Methylmalonate	1898008	2531103	2316718	1253120
DHAP	313188	625799	472129	536446
Chenodeoxycholate	0	505	428	586
G16BP	315617	580765	774977	343008
F6P/F1P	583396	1434819	1092519	728613
Oxalic Acid	4141	6021	5992	4236
Glyceraldehyde	7061	10828	10167	7607
Glycerate	50939	62087	55408	91575

Table S1 (page 1 of 2)

Metabolomics data. Values are averages of four biological replicates per treatment group. Standard error of mean (SEM) provided in Table S2 and statistical significance (p-values and multiple-testing corrected q-values) are provided in Table S3.

Mevalonate	24496	54248	52689	69269
Allantoin	3381	5016	4463	5031
Inositol	7258	11743	10843	8717
Homovanilate	1178	1589	1451	2970
Xanthurenate	5065	7884	5283	4374
Pentothenate	345246	613383	408026	400511
Biotin	115983	144656	133023	152714
DCMP	1069	1489	1269	2349
DUMP	26853	41303	30666	22758
Geranyl Pyrophosphate	12023	22585	17169	18671
DTMP	61803	87344	101021	51884
CMP	3858	7620	6388	6348
AMP	432294	691367	829794	468492
IMP	1283594	2525042	1866841	2324187
ATP	56715	99507	84060	72888
Fructose/Galactose	42628	50748	59169	29624
Aspartic Acid	2193463	3724623	2329225	3563405
Myristic Acid	1929	3045	1741	1557
Margaric Acid	1790	3615	1989	3467
Linolenic Acid	16516	22621	8090	26683
Glycine	72050	62301	54032	65318
Trimethylamine-N-oxide	55237	22503	49005	35334
Alanine	549836	577670	604864	536832
Aminoisobutyrate	86457	77670	51559	68375
Choline	10792014	10229464	7048155	7353389
Dimethylglycine	6442326	6004327	4164721	5195609
Serine	434783	494706	417673	462394
Creatinine	164483	167101	172144	143470
Proline	1062905	1381237	1160235	1105888
Valine	45077	78230	46277	62152
Betaine	1643200	1893314	1438637	1423651
Threonine	19525	34471	18796	32952
Taurine	2648589	2687386	2629420	2455941
Creatine	6711367	8138682	6339275	6208909
Hydroxyproline	391362	415196	342951	368238
Leucine	750654	1190736	606220	846935
Ornithine	22347	39377	25310	28212
Acetylcholine	354672	347974	355276	269735
Glutamine	6166011	7677029	5569783	5751312
Glutamic acid	6762940	8014350	6368883	6169770
Methionine	53908	95055	57484	86889
Cystamine	1435	1439	1339	1471
Histidine	214232	258254	237908	281721
Carnitine	1535	1537	1789	2155
Phenylalanine	756907	907213	481623	697245
Quinolinate	1970	2013	1602	1680
Arginine	306722	685601	207167	358050
Tyrosine	143319	233594	133177	139121
Sorbitol	4531	8015	4431	4052
Epinephrine/Normetanephrin	43716	38020	30922	28071
Tryptophan	172415	177694	92256	117666
Uridine	209662	169032	156384	172332
Adenosine	5635702	3371571	3613535	2467905
Inosine	4112936	4411652	3211665	3515166
Guanosine	360272	349052	184989	417452
Lysine	6189319	7652597	5508838	5697854
Cytosine/Histamine	29415	28420	27309	19005
Homoserine	6775	8040	5846	8111
Niacinamide	3338369	3345088	2428052	2816219
1-Methylhistamine	51714	49608	53200	46846
Asparagine	52615	51016	46724	32141
Cytidine	203524	172821	187252	125115
Pyroglutamic Acid	2359902	3131725	1863864	2215769
1-Methyladenosine	132898	109103	128329	97701
1-Methylguanosine	1919	1972	1909	1227
N2,N2-Dimethylguanosine	2402	1763	2309	1740

Table S1 (page 2 of 2).

Metabolomics data. Values are averages of four biological replicates per treatment group. Standard error of mean (SEM) provided in Table S2 and statistical significance (p-values and multiple-testing corrected q-values) are provided in Table S3.

	Vehicle Ctl	Rapa Ctl	Vehicle KO	Rapa KO
Pyruvate	1717	547	983	1571
lactate	503685	248757	169015	128379
Acetoacetate	1281	796	403	885
Fumaric	723	606	363	374
Succinate	545005	245427	108037	498338
inate (Niacin)	7620	4161	2821	1722
Glutaric Acid	2956	3353	736	445
Malate	542722	982275	257468	446987
Hypoxanthine	327252	399103	134990	628841
oglutaric Acid	20184	37283	3383	2051
Xanthine	34372	105012	9148	127629
Urate	1860	2209	251	474
PEP	6796	12820	5114	4739
D-GA3P	102301	139687	55402	191577
Glycerol-3-P	345890	316081	54612	58684
Hyppuric Acid	1667	2760	647	4992
Glucose	6746	1641	2707	12379
Pyridoxic acid	684	774	232	1066
oglyceric Acid	17206	21168	23213	11359
Erythrose	6591	3530	4587	80235
Cystathionine	2094	1806	964	1384
G1P/G6P	177554	110702	76424	20239
ed glutatione	7245	11383	7818	29264
F16BP/F26BP	109323	165325	91600	137578
Sucrose	1405	274	344	534
d glutathione	463626	261441	109202	690891
minobutyrate	663	886	125	98
ic Acid/3HBA	18021	10156	4678	2763
itraconic Acid	11095	11266	4121	9396
Adenine	26719	16498	6268	18359
Aconitate	5187	2123	1151	1695
Citrulline	6571	19412	2586	8014
Citric Acid	484126	504433	94778	292631
Cystine	8033	3427	1102	1315
Xanthosine	15948	15638	3358	15076
Uracil	1299	331	481	1954
enylpyruvate	2874	579	569	650
Anthranilate	3147	1477	1819	2069
Glucoronate	6424	840	1600	1754
Oxaloacetate	2636	2104	682	103
Propionate	160	271	133	120
Aminoadipate	4834	3456	2514	719
Nitro-tyrosine	138	33	52	102
Ribose-5-P	78093	67274	14364	75349
nylosuccinate	9296	6604	2819	10421
D-Leucic Acid	12716	21838	4077	20789
Pyridoxal-5-P	914	357	175	287
Adipic Acid	624	802	1438	611
Maleic Acid	662	562	541	447
thylmalonate	543876	226401	111116	524078
DHAP	104196	121092	64933	207229
G16BP	97331	162108	101542	137072
F6P/F1P	164444	106074	59595	16815
Oxalic Acid	1182	727	361	352
yceraldehyde	2275	829	692	1540
Glycerate	20230	3630	12047	74277

Table S2 (page 1 of 2).
Standard error of the mean values for data in Table S1.

Mevalonate	8597	8754	8587	28532
Allantoin	1164	466	458	80
Inositol	2162	885	586	473
Homovanilate	343	113	85	293
Kanthurenate	1690	1199	767	841
Pentothenate	102457	41221	29400	67219
Biotin	34233	13680	3707	24456
DCMP	301	131	90	1163
DUMP	8282	3720	3355	5755
rophosphate	3463	2747	430	2813
DTMP	17798	10064	6022	14186
CMP	1157	1373	307	655
AMP	121881	203990	99176	194414
IMP	367017	454386	79923	540394
ATP	17797	9948	4625	11387
se/Galactose	15925	3386	9267	4027
Aspartic Acid	645538	541032	93887	229900
Myristic Acid	649	520	256	699
Margaric Acid	596	649	159	150
Linolenic Acid	5174	3972	995	17979
Glycine	10386	5140	3080	5762
mine-N-oxide	13809	4353	2741	12787
Alanine	72156	48809	34654	50569
oisobutyrate	12728	12757	2478	14207
Choline	1345604	1198198	535605	390119
methylglycine	447165	975847	357074	1031551
Serine	48242	43704	52422	31619
Creatinine	21586	11893	13226	10209
Proline	128243	146851	71188	61607
Valine	5537	9949	4523	6883
Betaine	141053	390049	110653	76666
Threonine	3672	4452	1149	5010
Taurine	422978	264287	169207	238261
Creatine	585999	1430521	573599	349293
droxyproline	45146	32479	10042	9709
Leucine	105423	136879	36710	78706
Ornithine	2995	2321	7442	4751
Acetylcholine	41020	34533	26048	40135
Glutamine	717539	1169078	415262	426004
Glutamic acid	802393	1064476	565065	494175
Methionine	6184	16178	5989	17327
Cystamine	385	286	463	320
Histidine	24971	25645	29648	35106
Carnitine	345	74	204	801
Phenylalanine	116729	225677	56987	22502
Quinolinate	497	296	283	34
Arginine	29360	99644	11604	49433
Tyrosine	27140	49849	5676	7705
Sorbitol	857	1599	469	221
metanephrin	4517	4160	1427	576
Tryptophan	16188	37155	10038	11409
Uridine	14086	21411	7125	13352
Adenosine	2283452	1065809	2159771	2262761
Inosine	291196	391261	153930	434854
Guanosine	57153	84216	6346	139619
Lysine	663525	1332331	388874	545520
ne/Histamine	3062	2816	1190	2483
Homoserine	1276	965	706	1120
Niacinamide	313843	370616	242911	223881
thylhistamine	9069	4169	2906	5041
Asparagine	7344	5253	1134	3769
Cytidine	34672	16578	5111	16086
glutamic Acid	259586	549921	45054	59147
hyladenosine	17625	11537	7304	14633
hylguanosine	122	450	112	118
hylguanosine	132	451	242	163

Table S2 (page 2 of 2).
Standard error of the mean values for data in Table S1.

	Vehicle KO to p-value	Vehicle Control p-value	Vehicle Control to p-value	Vehicle Control to p-value
Pyruvate	0.077	0.006	0.074	0.014
lactate	0.043	0.150	0.105	0.178
Acetoacetate	0.025	0.198	0.069	0.145
Fumaric	0.113	0.998	0.591	0.727
Succinate	0.047	0.458	0.201	0.353
Nicotinate (Niacin)	0.263	0.741	0.080	0.210
Glutaric Acid	0.159	0.815	0.330	0.609
Malate	0.913	0.027	0.098	0.021
Hypoxanthine	0.119	0.307	0.087	0.424
Alpha-Ketoglutaric Acid	0.002	0.923	0.167	0.954
Xanthine	0.215	0.445	0.197	0.487
Urate	0.048	0.421	0.460	0.590
PEP	0.025	0.071	0.121	0.100
D-GA3P	0.871	0.200	0.145	0.296
Glycerol-3-P	0.043	0.081	0.938	0.157
Hyppuric Acid	0.578	0.521	0.380	0.642
Glucose	0.736	0.507	0.089	0.221
4-Pyridoxic acid	0.497	0.161	0.556	0.244
2/3-Phosphoglyceric Acid	0.021	0.022	0.407	0.042
Erythrose	0.160	0.385	0.102	0.489
Cystathionine	0.663	0.953	0.097	0.438
G1P/G6P	0.022	0.029	0.008	0.038
Reduced glutatione	0.440	0.159	0.089	0.169
F16BP/F26BP	0.029	0.010	0.273	0.024
Sucrose	0.187	0.379	0.250	0.345
Oxidized glutathione	0.886	0.319	0.119	0.303
gama-Aminobutyrate	0.024	0.842	0.164	0.959
Malonic Acid/3HBA	0.108	0.370	0.429	0.477
Citraconic Acid	0.147	0.117	0.125	0.130
Adenine	0.480	0.690	0.376	0.814
Aconitate	0.363	0.930	0.252	0.222
Citrulline	0.313	0.394	0.059	0.252
Citric Acid	0.742	0.347	0.121	0.297
Cystine	0.275	0.445	0.139	0.266
Xanthosine	0.008	0.875	0.233	0.939
Uracil	0.736	0.155	0.987	0.160
OH-Phenylpyruvate	0.224	0.411	0.397	0.318
Anthranilate	0.116	0.399	0.065	0.142
Glucoronate	0.837	0.171	0.047	0.113
Oxaloacetate	0.958	0.898	0.327	0.846
Propionate	0.009	0.013	0.014	0.013
2-Aminoadipate	0.297	0.353	0.022	0.128
3-Nitro-tyrosine	0.404	0.364	0.062	0.399
Ribose-5-P	0.079	0.539	0.086	0.648
Adenylosuccinate	0.936	0.929	0.214	0.705
D-Leucic Acid	0.862	0.023	0.115	0.013
Pyridoxal-5-P	0.819	0.502	0.223	0.421
Adipic Acid	0.925	0.173	0.108	0.218
Methylmalonate	0.104	0.983	0.163	0.762
Methylmalonate	0.068	0.479	0.256	0.372
DHAP	0.748	0.227	0.125	0.313
G16BP	0.048	0.011	0.249	0.025
F6P/F1P	0.016	0.023	0.006	0.030
Oxalic Acid	0.020	0.179	0.253	0.222
Glyceraldehyde	0.154	0.234	0.055	0.079
Glycerate	0.595	0.852	0.195	0.419
Mevalonate	0.551	0.044	0.073	0.084
Allantoin	0.346	0.414	0.091	0.176
Inositol	0.045	0.155	0.094	0.142
Homovanilate	0.002	0.468	0.338	0.499
Xanthurenate	0.464	0.909	0.276	0.950
Pentothenate	0.914	0.576	0.078	0.640
Biotin	0.390	0.640	0.422	0.564

Table S3 (page 1 of 2).

Statistical significance values for metabolomics comparisons. See methods for details.

DCMP	0.320		0.545		0.216		0.455	
DUMP	0.261		0.681		0.211		0.724	
Geranyl Pyrophosphate	0.560		0.188		0.054		0.177	
DTMP	0.016		0.076		0.296		0.112	
CMP	0.954		0.074		0.072		0.073	
AMP	0.132		0.034		0.287		0.045	
IMP	0.368		0.168		0.071		0.152	
ATP	0.356		0.183		0.122		0.271	
Fructose/Galactose	0.050		0.391		0.696		0.464	
Aspartic Acid	0.003		0.843		0.101		0.663	
Myristic Acid	0.792		0.794		0.077		0.687	
Margaric Acid	0.001		0.758		0.098		0.760	
Linolenic Acid	0.273		0.157		0.181		0.288	
Dopamine	0.027		0.010					
Glycine	0.121		0.147		0.963		0.238	
Trimethylamine-N-oxide	0.276		0.674		0.104		0.642	
Alanine	0.300		0.517		0.173		0.042	
Aminoisobutyrate	0.228		0.036		0.826		0.004	
Choline	0.686		0.041		0.607		0.013	
Dimethylglycine	0.332		0.007		0.999		0.008	
Serine	0.537		0.818		0.112		0.678	
Creatinine	0.169		0.772		0.153		0.126	
Proline	0.606		0.532		0.056		0.064	
Valine	0.099		0.872		0.025		0.337	
Betaine	0.922		0.297		0.449		0.634	
Threonine	0.024		0.856		0.019		0.169	
Taurine	0.566		0.968		0.280		0.217	
Creatine	0.867		0.666		0.291		0.779	
Hydroxyproline	0.139		0.336		0.161		0.802	
Leucine	0.028		0.243		0.024		0.495	
Ornithine	0.776		0.725		0.001		0.530	
Acetylcholine	0.119		0.990		0.516		0.331	
Glutamine	0.777		0.499		0.181		0.890	
Glutamic acid	0.810		0.702		0.172		0.604	
Methionine	0.128		0.692		0.071		0.413	
Cystamine	0.836		0.878		0.380		0.665	
Histidine	0.382		0.564		0.092		0.259	
Carnitine	0.629		0.549		0.145		0.107	
Phenylalanine	0.027		0.078		0.519		0.174	
Quinolinate	0.827		0.544		0.215		0.756	
Arginine	0.018		0.020		0.018		0.004	
Glucosamine	0.386	n/a		n/a		n/a		
Tyrosine	0.551		0.727		0.205		0.922	
Sorbitol	0.546		0.922		0.145		0.937	
ephrine/Normetanephrin	0.165		0.036		0.817		0.006	
Tryptophan	0.156		0.006		0.653		0.004	
Uridine	0.306		0.015		0.287		0.041	
Adenosine	0.733		0.544		0.870		0.978	
Inosine	0.489		0.034		0.280		0.032	
Guanosine	0.104		0.023		0.881		0.042	
Lysine	0.782		0.410		0.250		0.914	
Cytosine/Histamine	0.021		0.545		0.639		0.743	
Homoserine	0.131		0.548		0.095		0.761	
Niacinamide	0.309		0.062		0.522		0.102	
1-Methylhistamine	0.296		0.881		0.370		0.122	
Asparagine	0.008		0.458		0.427		0.589	
Cytidine	0.008		0.659		0.953		0.373	
Pyroglutamic Acid	0.005		0.109		0.198		0.165	
1-Methyladenosine	0.096		0.819		0.663		0.284	
1-Methylguanosine	0.009		0.953		0.776		0.541	
N2,N2-Dimethylguanosine	0.133		0.747		0.366		0.987	

Table S3 (page 2 of 2).

Statistical values for metabolomics comparisons. See methods for details.