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Supplemental information

Crosstalk between Drp1 phosphorylation sites during mitochondrial remodeling and their impact on metabolic adaptation

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Supplemental Information

Supplementary Figure 1

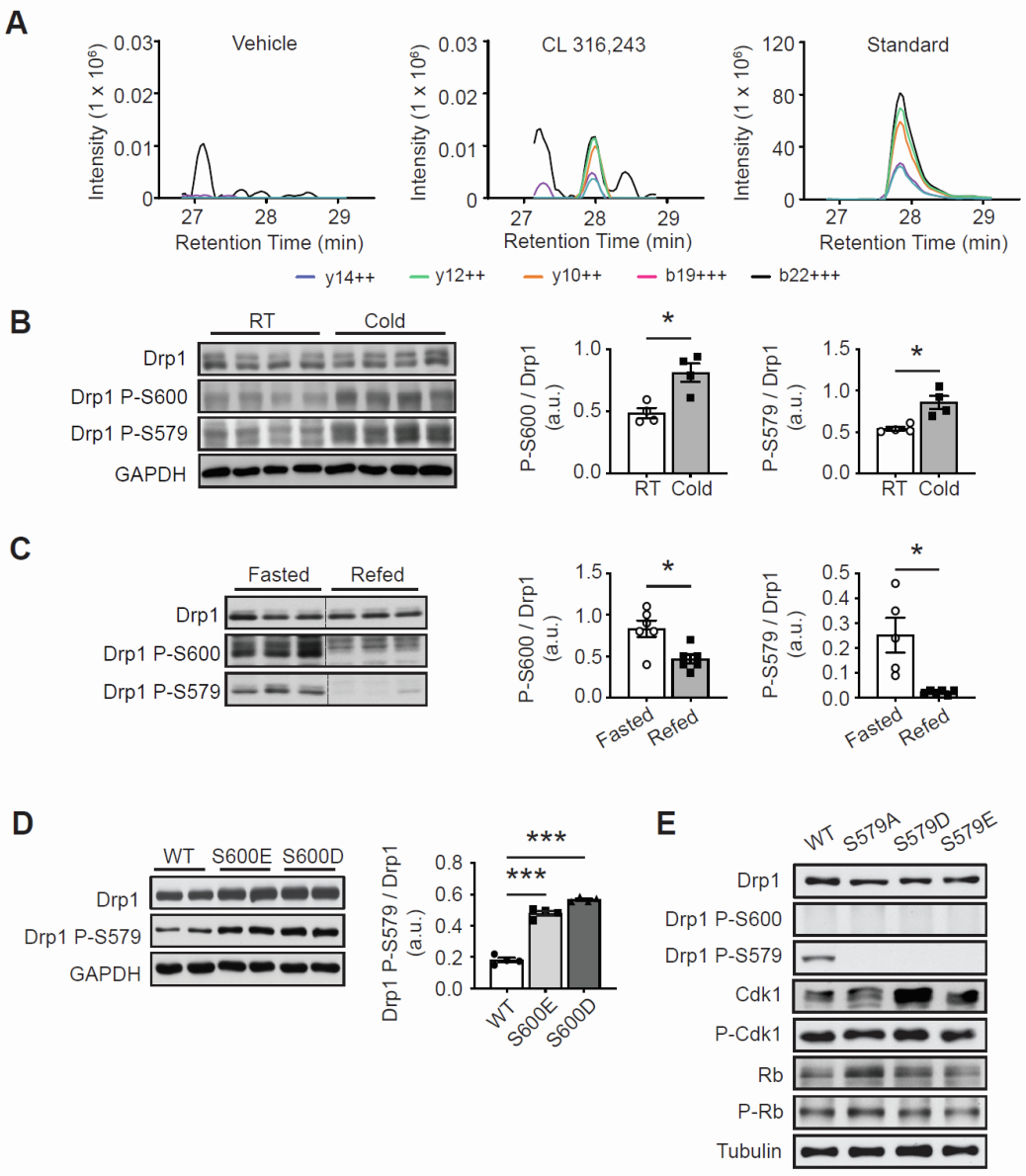

Figure S1. Crosstalk between Drp1 phosphorylation sites. Related to Figure 1. (A) 8-week old mice were intraperitoneally injected with either saline (as vehicle) or CL316,243 (1 mg/kg; 1 hour). Then, BAT was collected and flash frozen. After obtaining protein homogenates, 10 mg of proteins were used for immunoprecipitation using antibodies against Drp1 P-S579. The immunoprecipitated material was digested with Glu-C and then used for LC-MS/MS analysis. As control, the isotopically labelled form of the doubly phosphorylated peptide

KSKPIPIMPAS[Phos]PQKGHAVNLLDVPVPVARKLS[Phos]ARE was spiked in the samples. The graph illustrates the profile of the 5 most intensely detected fragments from peptide precursors with charge +6 and how CL-treatment exhibits similar detection patterns to the reference standard peptide. **(B)** Control C57Bl/6NTac male mice were subjected to a cold challenge for 4 h (6°C; Cold). Then, BAT was collected and snap frozen to evaluate the markers indicated. These samples were compared to BAT of control WT mice regularly housed at ambient room temperature (22°C; RT) (n = 4 mice per condition). **(C)** Control C57Bl/6NTac male mice were single housed at 9 am and fasted for 24 h. Afterwards, half of these mice were sacrificed (fasted) and some were given back food for 6 h (refed) before sacrifice. Then, muscle (quadriceps) was collected and snap frozen to analyze Drp1 phosphorylation levels (n= 3 mice per condition). **(D)** Drp1KO MEFs were transfected with either WT Drp1 or Drp1 phospho-mimetic S600E and S600D forms. After 48 hours, total protein extracts were obtained to evaluate Drp1 P-S579 levels. Quantifications are indicated for Drp1 P-S579 normalized by total Drp1 levels. Values are mean +/- SEM from n=3 independent experiments. **(E)** Drp1KO MEFs were transfected with WT Drp1 or with S579A, S579D or S579E mutant forms. Then, after 48 hours, total protein extracts were obtained to evaluate the phosphorylation levels of S600 and S579, as well as the cell-cycle markers Rb/ P-Rb and Cdk1/P-Cdk1. All values are presented as mean ± SEM. *p < 0.05, ***p < 0.001 (two-tailed Student's t-test) for statistically significant differences between the indicated groups.

Supplementary Figure 2

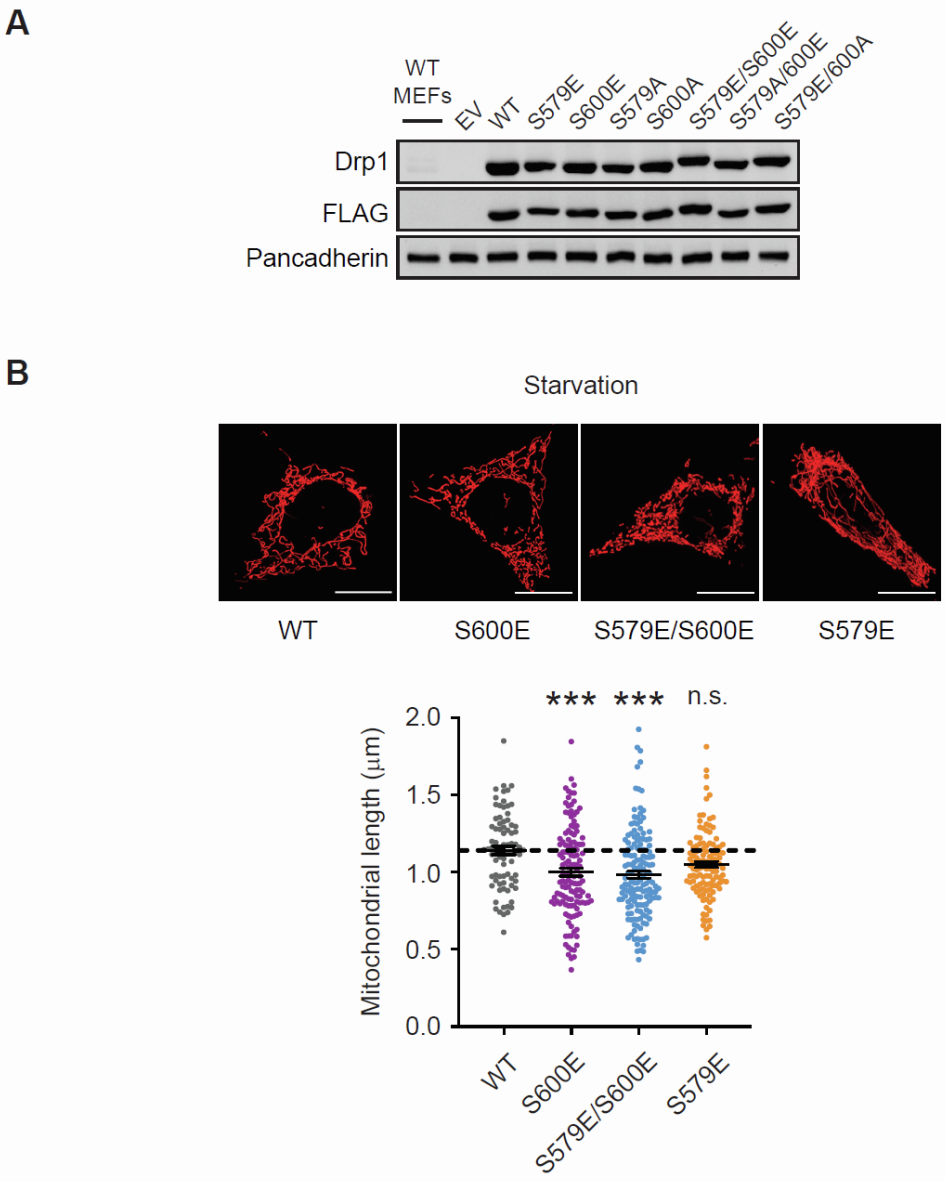

Figure S2. Crosstalk between Drp1 phosphorylation sites. Related to Figure 2. (A) Drp1KO MEFs were transfected with the different Drp1 constructs indicated. Then, after 48 hours, total protein extracts were obtained to evaluate the levels of Drp1 and compare them to Drp1 levels in WT cells. **(B)** Drp1 KO MEFs were transfected with the indicated phospho-mimetic plasmids tagged to mtDsRed. After 24h, mitochondrial morphology was evaluated by live fluorescence imaging. Cells were incubated with HBSS media for 2 h prior to imaging to induce starvation (scale bar: 20 µm). Quantifications are shown as the mean of n=3

independent experiments, counting approximately 50 different cells per experiment. All values are presented as mean ± SEM. ***p < 0.001 (two-tailed Student's t-test) for statistically significant differences vs. the WT group.

Supplemental Figure 3

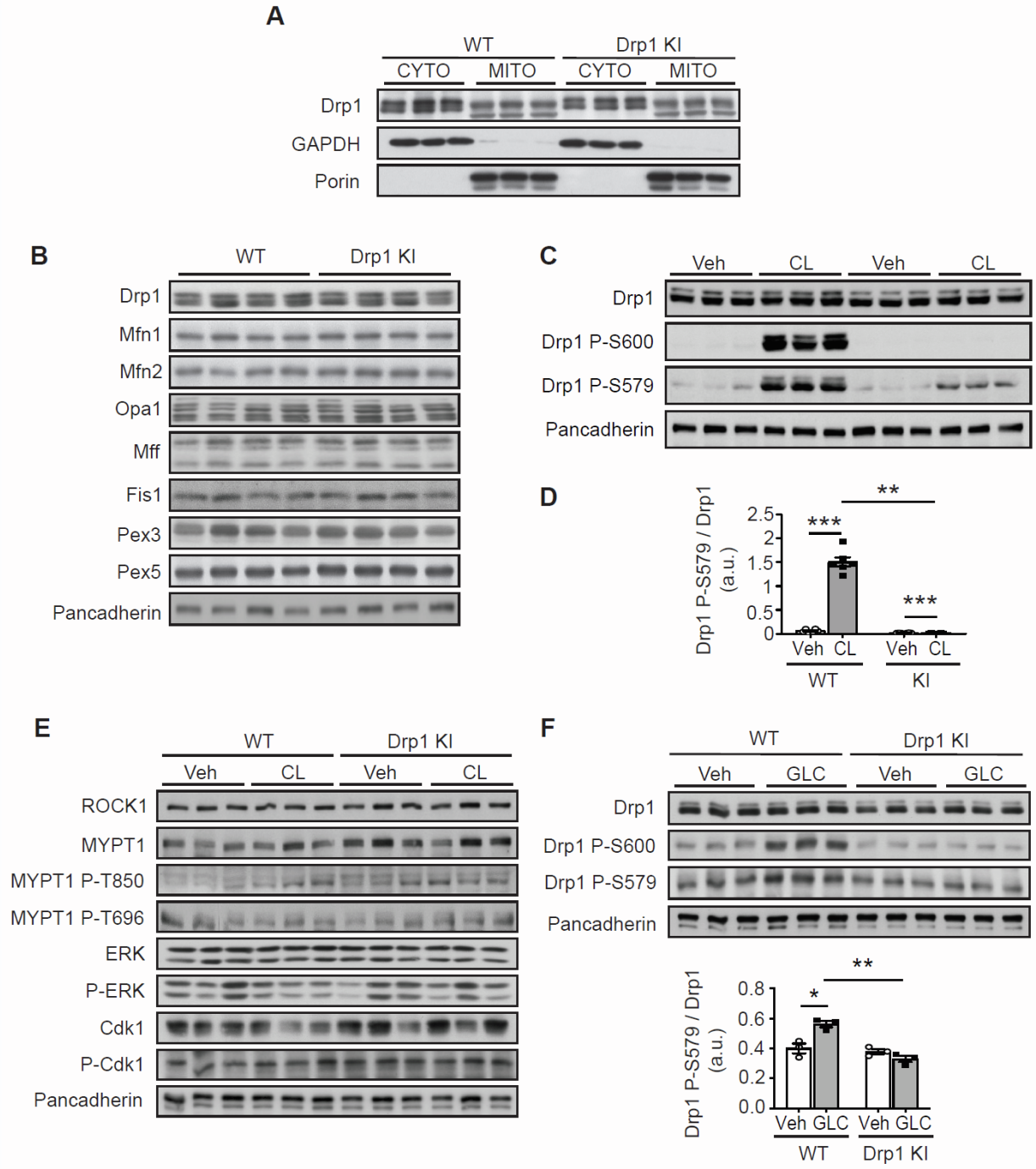

Figure S3. Characterization of the Drp1 S600A knock-in (KI) mice. Related to Figure 3. (A) Cellular subfractionation methods were applied on BAT from WT and Drp1 KI mice, and then Drp1 localization was analyzed in the cytosolic (CYTO) and mitochondrial (MITO) fractions.

GAPDH and porin were used as cytosolic and mitochondrial markers, respectively. **(B)** Mitochondrial dynamics (Drp1, Mfn1, Mfn2, Opa1), mitochondrial receptors (Mff and Fis1) and peroxisomal proteins (Pex3, Pex5) were analyzed in total homogenates from BAT of WT and Drp1 KI mice (n = 4 mice per genotype). **(C)** WT and Drp1 KI female mice were treated with CL316,243 (1mg/kg; 1 hour) before collecting BAT and evaluating the protein lysates for total Drp1 and Drp1 phosphorylation levels. **(D)** Quantification of Drp1 S579 phosphorylation levels in response to CL316,243 treatment, including male and female mice from Fig.3C and Fig.S3C (n= 3 male and 3 female mice per condition). **(E)** WT and Drp1 KI mice were treated with CL316,243 (1 mg/kg) for 1 h before collecting BAT and evaluating the protein lysates for the indicated markers. **(F)** WT and Drp1 KI mice were fasted for 2 h and then treated with glucagon (1 mg/kg) for 15 min before collecting liver and evaluating the protein lysates for Drp1 total and Drp1 phosphorylation levels (n=3 mice per condition). Quantifications of Drp1 P-S579 levels normalized to total Drp1 are also indicated. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ (two-tailed Student's t-test) statistically significant differences between the indicated groups.

Supplemental Figure 4

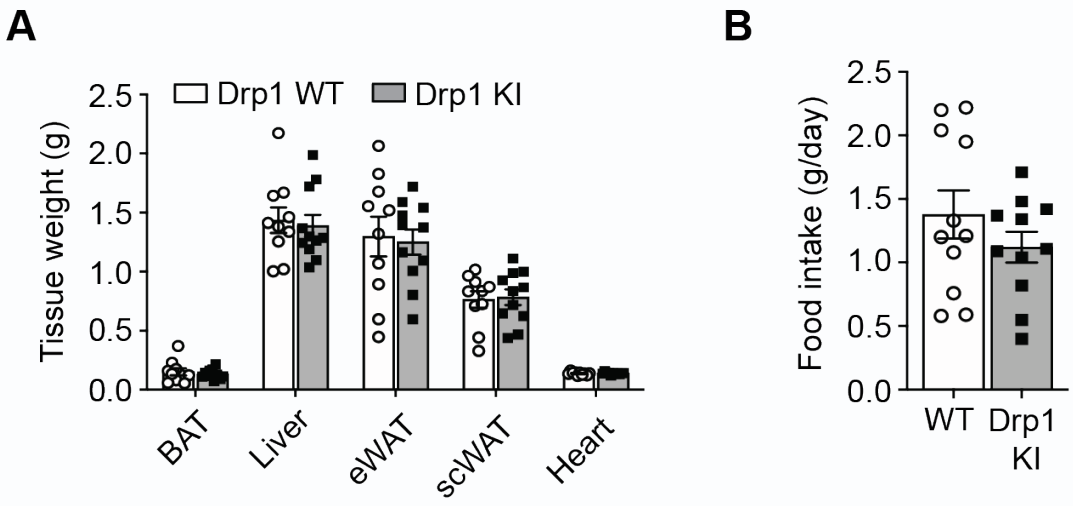

Figure S4. Tissue weights and food intake in WT and Drp1 KI mice. Related to Figure 4. **(A)** Tissues from WT and Drp1 KI mice were collected after sacrifice and weighted. **(B)** Food intake was monitored using a comprehensive laboratory animal monitoring system (CLAMS). All values are presented as mean +/- SEM of n=10 WT and 11 KI mice. WT mice are represented as white bars and white circles, while grey bars and black squares are used for Drp1 KI mice.

Supplemental Figure 5

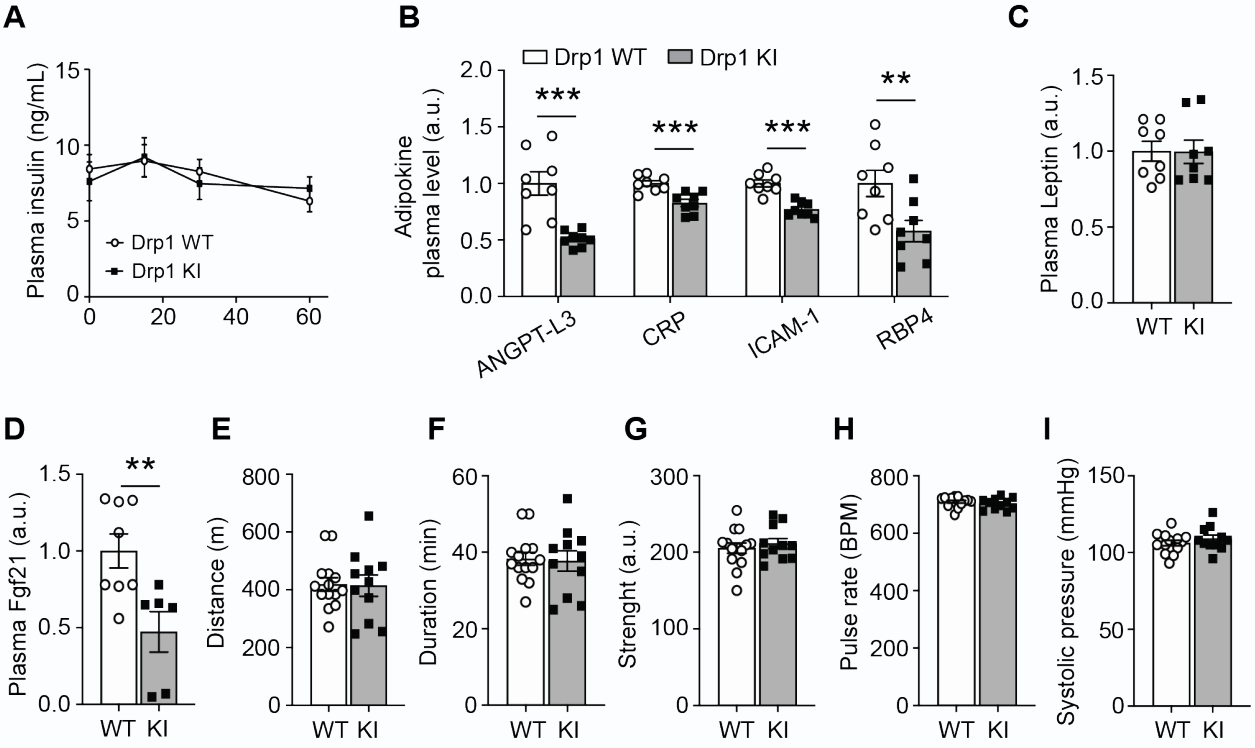

Figure S5. Plasma markers and muscle performance in WT and Drp1 KI mice fed a high-fat diet. Related to Figure 5. (A) Plasma insulin concentration was measured for the indicated times during a glucose tolerance test (See Fig 5E). **(B)** Plasma levels of the indicated cytokines in HFD-fed WT and Drp1 KI mice after a 12 hour fast (n=8 mice per genotype). **(C-D)** Circulating leptin **(C)** and Fgf21 **(D)** levels in plasma from WT and Drp1 KI mice after a 12 hour fast (n=8 mice per genotype). **(E-G)** Muscle performance in WT and Drp1 KI mice was evaluated by carrying out a treadmill test and evaluating the running distance **(E)** and duration **(F)** before exhaustion, as well as a grip test **(G)** to evaluate muscle strength. **(H-I)** Heart function was evaluated by performing a non-invasive blood pressure test and measuring pulse rate **(H)** and systolic blood pressure **(I)**. All values are presented as mean ± SEM of, unless otherwise stated, n = 14 WT and 11 KI. **, *** indicate statistically significant difference between HFD-fed control (white bars and white circles) and HFD-fed Drp1 KI mice (grey bars and black squares) at $p < 0.01$, $p < 0.001$, respectively.

Supplementary Figure 6

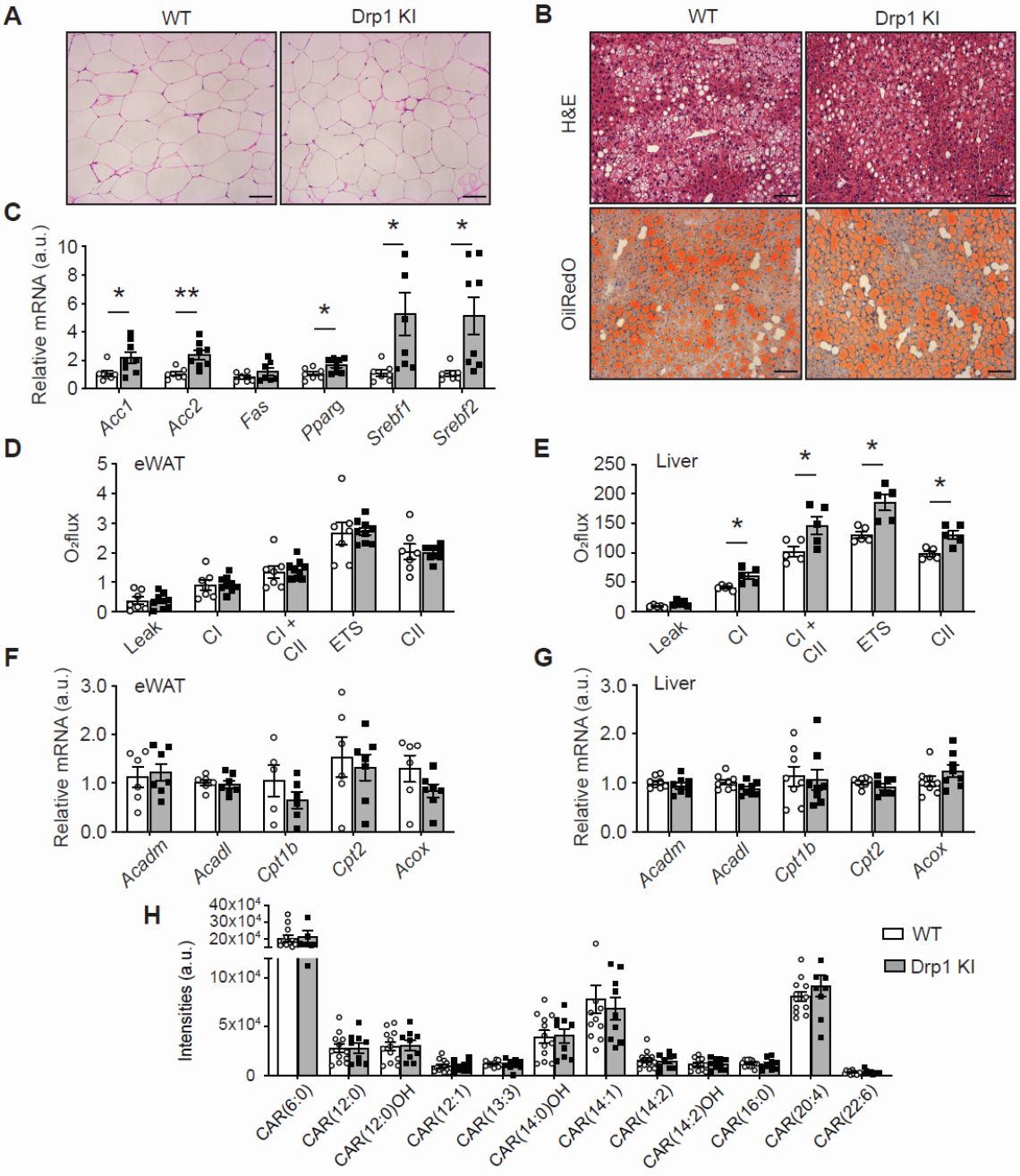

Figure S6. Characterization of eWAT and liver from WT and Drp1 KI mice under high-fat feeding. Related to Figure 6. (A) H&E staining of eWAT from wild-type (WT) and Drp1 KI HFD-fed mice (scale bar: 100 μm). **(B)** H&E staining and Oil Red'O stainings of liver from HFD-fed mice (scale bar: 100 μm). **(C)** mRNA levels of lipogenesis markers from BAT of WT and Drp1KI

mice on HFD. **(D-E)** Respirometry analyses of uncoupled respiration (Leak), Complex I respiration (CI), Complex I + Complex II respiration (CI + CII), maximal electron transfer system capacity (ETS) and maximal Complex II driven respiration (CII) in either eWAT (D) or liver homogenates (E). **(F-G)** mRNA levels from fatty acid oxidation markers in eWAT (F) and liver (G). **(H)** Acyl-carnitine levels were measured by liquid chromatography and mass-spectrometry (LC-MS) from liver of HFD-fed mice (n = 12 WT and 9 KI). All values are presented as mean +/- SEM of, unless otherwise specified, n=8-10 mice per genotype. * and ** indicate statistical difference between HFD-fed control (white bars and white circles) and HFD-fed Drp1 KI mice (grey bars and black squares) at $p<0.05$ and $p<0.01$, respectively (two-tailed Student's T-test).

Supplemental Figure 7

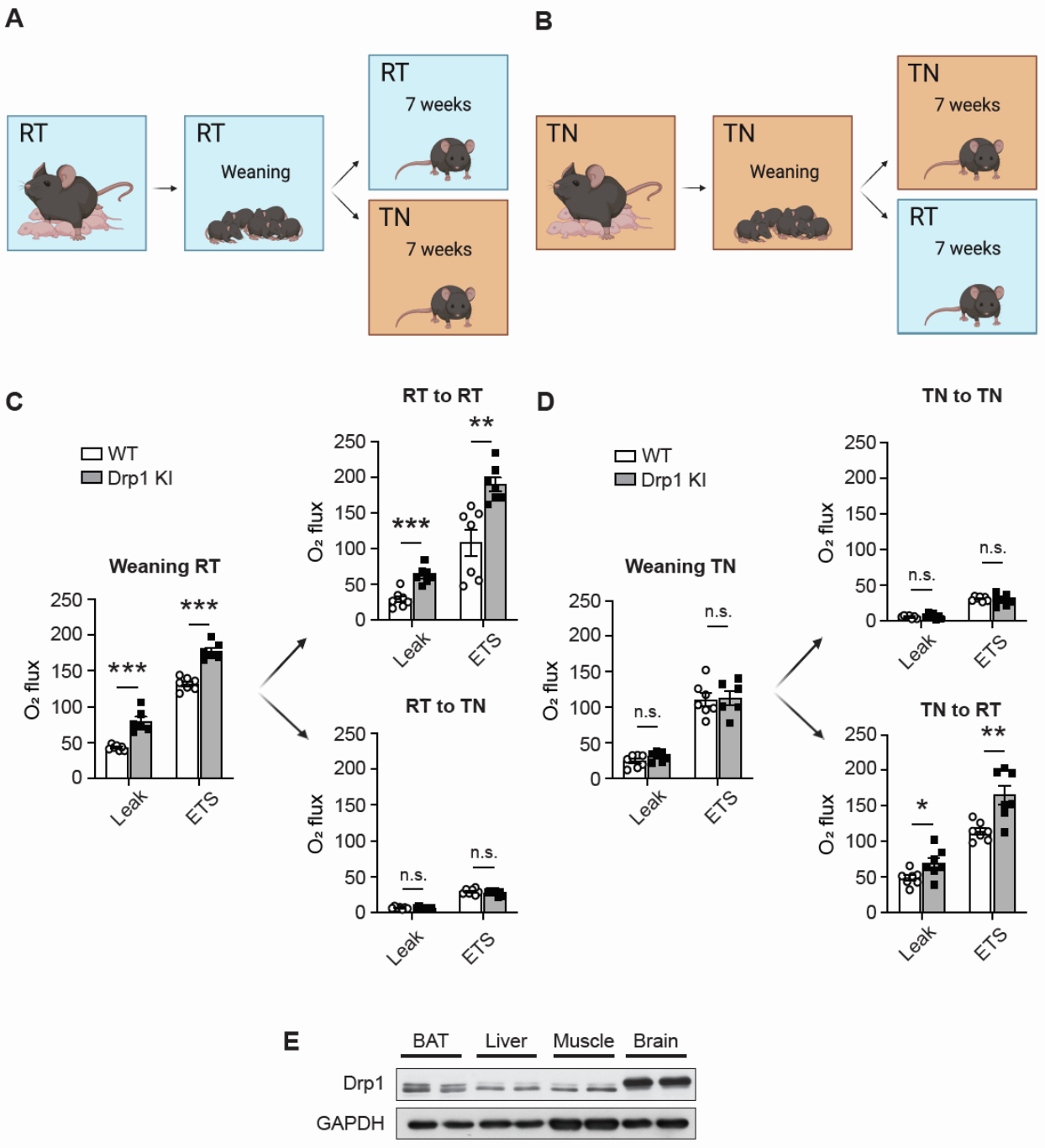

Figure S7. The increased respiration in BAT from Drp1 depends on ambient temperature. Related to Figure 7. (A) Schematic representation of the experiment in which mice were bred and maintained at room temperature (RT, 22ºC) until weaning (3 weeks of age). Then mice were

either kept at RT or moved to thermoneutrality (TN; 30 °C) for the next 4 weeks, until mice were 7 weeks of age. **(B)** Schematic representation of the experiment in which mice were bred and maintained at TN until weaning. Then, mice were either kept at TN or move to RT for the next 4 weeks, until they were 7 weeks of age. **(C)** Respirometry analyses in BAT from mice at weaning or 7 weeks of age, corresponding to the different groups described in (A). The graphs illustrate uncoupled respiration driven by Complex I (leak) and maximum electron transport system (ETS) capacity, driven by both CI and CII after the addition of FCCP. **(D)** As in (C), but corresponding to the groups described in (B). **(E)** BAT, liver, muscle and brain from two control WT mice were collected and protein homogenates were used to evaluate Drp1 levels. Drp1 isoform 1 is predominantly expressed in neurons, whereas Drp1 isoform 3 is expressed ubiquitously in tissues such as BAT, liver and muscle. Isoform 1 contains two extra exons within Drp1 structure, as compared to Drp1 isoform 3. Therefore, Drp1 isoform 1 in brain displays a slightly higher molecular weight on the Western Blot. All values are presented as mean +/- SEM of 7 mice per genotype. *, ** and *** indicate statistical difference between WT (white bars, white circles) and Drp1 KI (grey bars, black squares) mice at $p<0.05$, $p<0.01$ and $p<0.001$, respectively (two-tailed Student's T-test).

Supplemental Table 1

	Primer name	Primer	Sequence 5' to 3'
Primers used for mutagenesis	Drp1 S579A	F	CCAATTATGCCAGCAGCTCCACAGAAAGGC
		R	GCCTTTCTGTGGAGCTGCTGGCATAATTGG
	Drp1 S600A	F	GTTGCAAGAAAACTGGCTGCCCGAGAACAG
		R	CTGTTCTCGGGCAGCCAGTTTTCTTGCAAC
	Drp1 S579D	F	CCAATTATGCCAGCAGATCCACAGAAAGGC
		R	GCCTTTCTGTGGATCTGCTGGCATAATTGG
	Drp1 S600D	F	GTTGCAAGAAAACTGGATGCCCGAGAACAG
		R	CTGTTCTCGGGCATCCAGTTTTCTTGCAAC
	Drp1 S579E	F	CCAATTATGCCAGCAGAACCACAGAAAGGC
		R	GCCTTTCTGTGGTTCTGCTGGCATAATTGG
	Drp1 S600E	F	GTTGCAAGAAAACTGGAAGCCCGAGAACAG
		R	CTGTTCTCGGGCTTCCAGTTTTCTTGCAAC

Table S1. List of primers used for mutagenesis. Related to STAR methods.

Supplemental Table 2

Assay	Species	Gene	Primer	Sequence 5' to 3'
Primers used for qPCR	Mouse	*b-2-microglobuline*	F	ATGGGAAGCCGAACATACTG
			R	CAGTCTCAGTGGGGGTGAAT
		cyclophilin	F	CAGGGGAGATGGCACAGGAG
			R	CGGCTGTCTGTCTTGGTGCTCTCC
		ucp1	F	CTTTGCCTCACTCAGGATTGG
			R	ACTGCCACACCTCCAGTCATT
		prdm16	F	TGGCCTTCATCACCTCTCTGAA
			R	TTTCTGATCCACGGCTCCTGTGA
		mcad	F	GGCCATTAAGACCAAAGCAGA
			R	GTGTCGGCTTCCACAATGAAT
		lcad	F	GTAGCTTATGAATGTGTGCAACTC
			R	GTCTTGCGATCAGCTCTTTCATTA
		cpt1	F	CCCATGTGCTCCTACCAGAT
			R	CCTTGAAGAAGCGACCTTTG
		cpt2	F	AGCCAGTTCAGGAAGACAGA
			R	GACAGAGTCTCGAGCAGTTA
		ACO	F	ACCGCCTATGCCTTCCACTTTC
			R	GCAAGCCATCCGACATTCTTCG
		pparg	F	ATGGGTGAAACTCTGGGAGATTCT
			R	CTTGGAGCTTCAGGTCATATTTGTA
		ACC1	F	GACAGACTGATCGCAGAGAAAG
			R	TGGAGAGCCCCACACACA
		ACC2	F	CCCAGCCGAGTTTGTCACT
			R	GGCGATGAGCACCTTCTCTA
		FAS	F	TTCCAAGACGAAAATGATGC
			R	AATTGTGGGATCAGGAGAGC
		SREBP1c	F	GGAGCCATGGATTGCACATT
			R	GCTTCCAGAGAGGAGGCCAG
		SREBP2	F	GCGTTCTGGAGACCATGGA
			R	ACAAAGTTGCTCTGAAAACAAATCA
		16S	F	CCGCAAGGGAAAGATGAAAGAC
			R	TCGTTTGGTTTCGGGGTTTC
		COX2	F	GTTGATAACCGAGTCGTTCTGC
			R	CCTGGGATGGCATCAGTTTT
		HK2	F	TCTGGCTCTGAGATCCATCTTCA
			R	CCGGCCTCTTAACCACATTCC
		UCP2	F	CTACAGATGTGGTAAAGGTCCGC
			R	GCAATGGTCTTGTAGGCTTCG

Table S2. List of primers used for qPCR. Related to STAR methods.