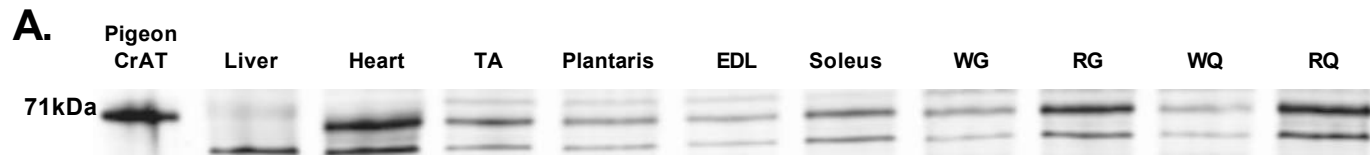
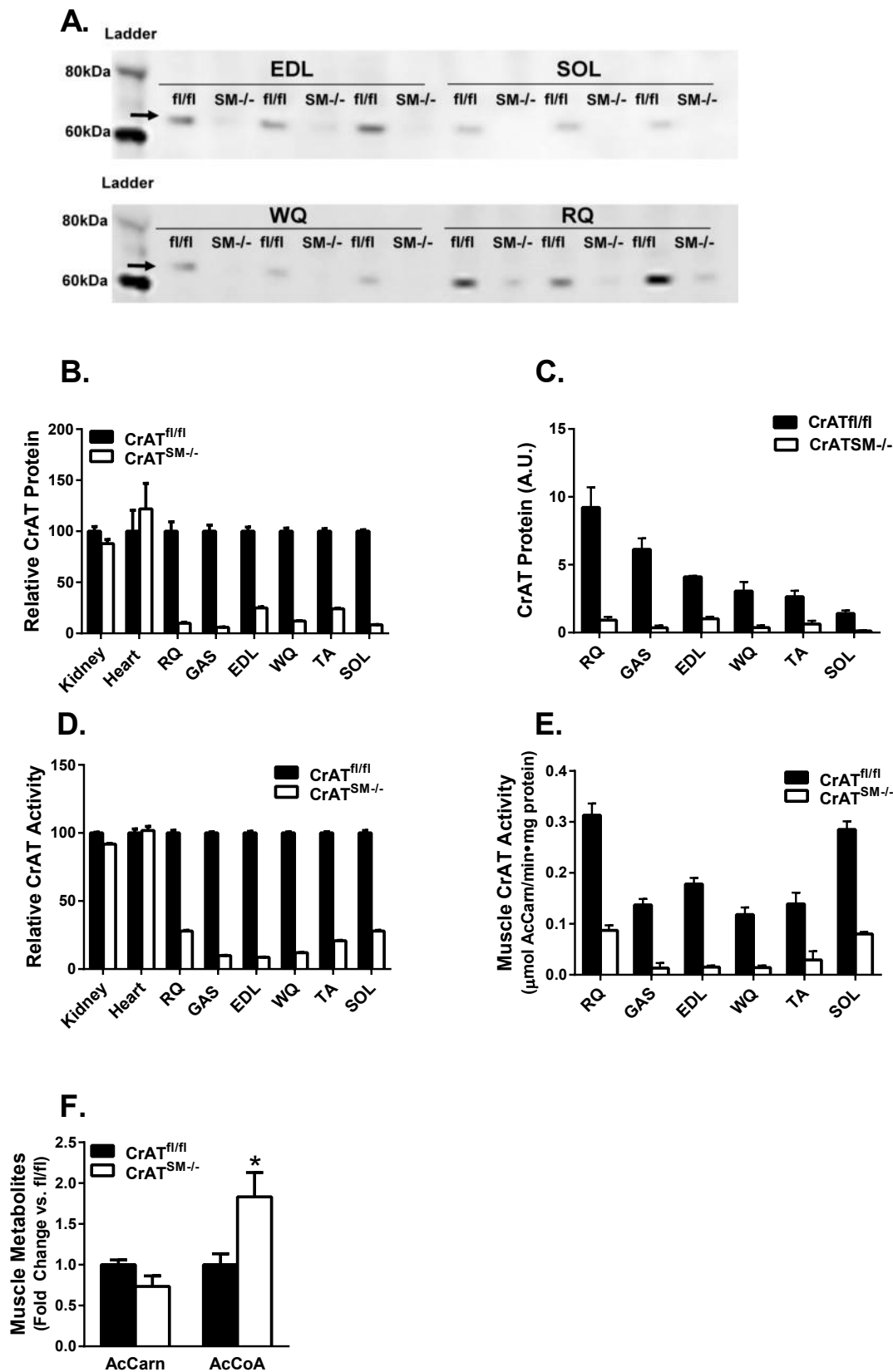


Supp. Figure 1, Related to Figure 1



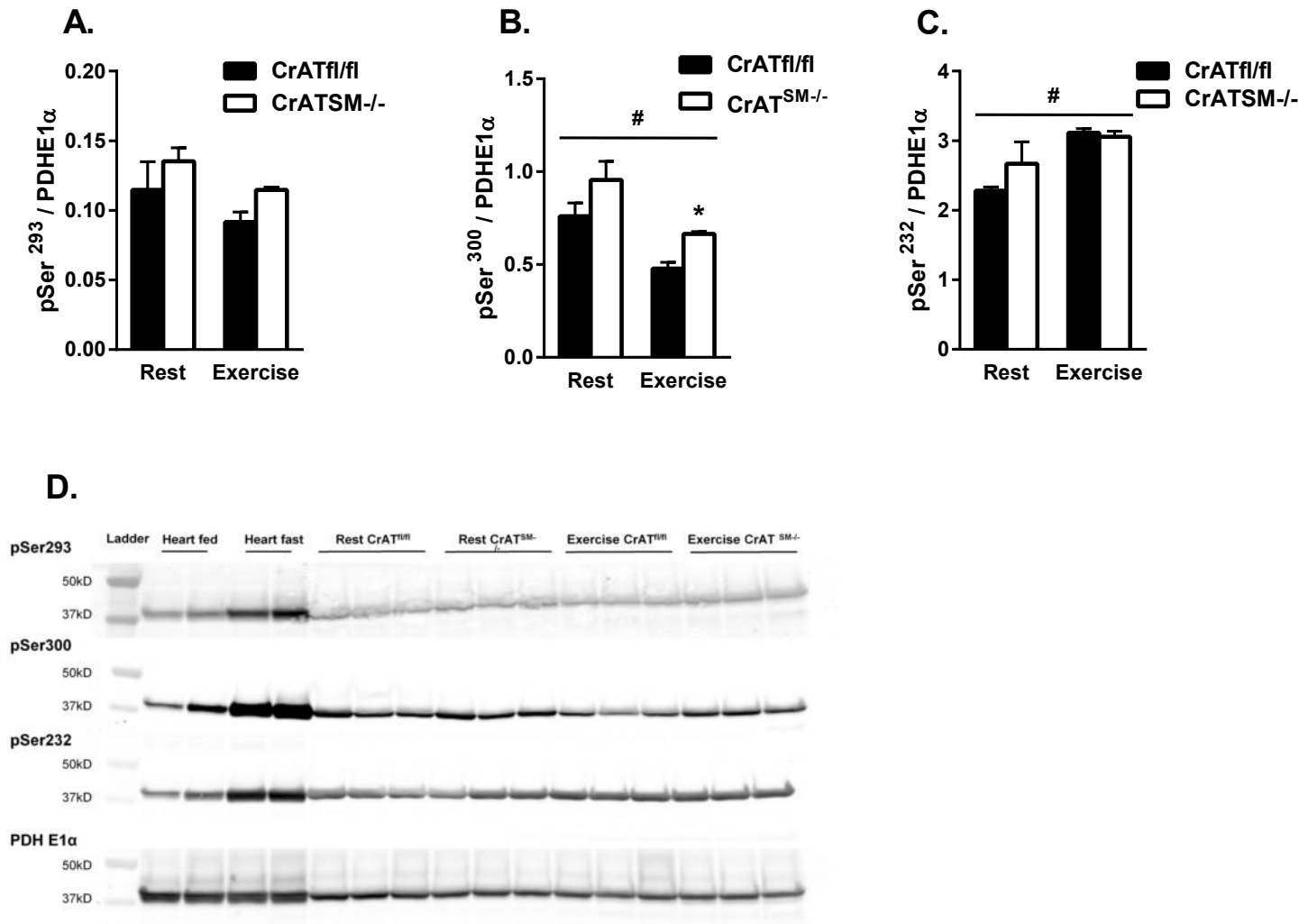
Supplemental Figure 1, related to Figure 1. Tissues were harvested from 3 month old rats. Representative Western blots show CrAT protein abundance in **(A)** tissue homogenates (50 μ g protein) prepared from rat liver, heart, tibialis anterior (TA), plantaris, extensor digitorum longus (EDL), soleus, white gastrocnemius (WG), red gastrocnemius (RG), white quadriceps (WQ), and red quadriceps (RQ) and **(B)** isolated mitochondria (50 μ g protein) harvested from respective skeletal muscles.

Supp. Figure 2, Related to Figure 1



Supplemental Figure 2, related to Figure 1: Representative western blots showing CrAT protein abundance in (A) tissue homogenates from littermate control (fl/fl) or mice with CrAT ablated in skeletal muscle (SM-/-) mice. Extensor digitorum longus (EDL), soleus (SOL), white quadriceps (WQ), and red quadriceps (RQ). Relative (B, D) and absolute (C, E) CrAT protein and activity in fl/fl and SM-/- mice for n=3 mice per group. Relative abundance of acetylcarnitine (AcCarn) and acetyl-CoA (AcCoA) (F) in fl/fl and SM-/- mice. * P<0.05 compared to fl/fl controls.

Supp. Figure 3, Related to Figure 3



Supplemental Figure 3, related to Figure 3. Quantified data (A, B, C) from quadriceps muscle (50 μ g protein) Western blot (D) showing serine phosphorylation of pyruvate dehydrogenase E1 α (PDHE1 α) regulatory sites normalized to total PDHE1 α protein abundance. Two-way ANOVA revealed main effect of genotype (*) on pSer300 (P=0.018) and main effect of exercise (#) on pSer300 (P=0.002) and pSer232 (P=0.006). Data are shown as mean \pm SE for n=3 mice per group.

	CrAT ^{fl/fl}		CrAT ^{SM-/-}	
	<i>Rest</i>	<i>Post-Exercise</i>	<i>Rest</i>	<i>Post-Exercise</i>
PCr	28.55 ± 3.45	40.87 ± 3.23 *	34.91 ± 2.29	29.22 ± 3.40
Cr	13.81 ± 0.43	10.16 ± 1.38 *	11.65 ± 0.83	11.52 ± 0.29
PCr/Cr	2.09 ± 0.32	4.19 ± 0.54 *	3.06 ± 0.33	2.53 ± 0.29

Supplemental Table 1, related to Figure 3. Muscle phosphocreatine content. Quadriceps muscles were harvested from CrAT^{fl/fl} and CrAT^{SM-/-} littermates at rest or 10 min after 12 min of high intensity exercise (Post-Exercise). Phosphocreatine (PCr) and creatinine (Cr) were measured as described in Methods. Data are expressed as $\mu\text{moles/g}$ tissue and represent means \pm SEM for n=4 muscles per group. 2-way ANOVA revealed a significant interaction between Genotype and Condition for PCr (P=0.014) and PCr/Cr (P=0.019) and main effects of Condition on Cr (P=0.033) and PCr/Cr (P=0.004). * P<0.05 by t-test between Rest and Post-Exercise within genotype.

Metabolite	<i>Rested</i>	<i>Stimulated</i>
PCr	21.3 ± 1.90	12.4 ± 1.20 *
Cr	10.6 ± 0.60	16.7 ± 0.70 *
ATP	1.8 ± 0.14	1.13 ± 0.21 *
ADP	0.2 ± 0.01	1.71 ± 0.09 *
AMP	0.07 ± 0.01	0.13 ± 0.02 *
IMP	1.0 ± 0.15	1.43 ± 0.22
PCr/Cr	2.2 ± 0.30	0.7 ± 0.09 *
ADP/ATP	0.11 ± 0.01	1.7 ± 0.24 *
AMP/ATP	0.04 ± 0.01	0.12 ± 0.02 *
IMP/ATP	0.58 ± 0.11	1.54 ± 0.44 *

Supplemental Table 2, related to Figure 4. Contraction-induced changes in muscle energy charge. Extensor digitorum longus (EDL) muscles from CrAT^{fl/fl} mice were flash frozen immediately after a four-minute incubation without (Rested) or with electrical stimulation (Stimulated) as described in the Methods. PCr and Cr content were analyzed as described in Methods. Liquid chromatography-Mass spectrometry was used to assess ATP, ADP, AMP and IMP. Data are expressed as μ moles/g tissue and represent means \pm SEM from 5-8 muscles per group. * $p < 0.05$, control vs. stimulated.

	CrAT^{fl/fl}		CrAT^{SM-/-}	
	<i>Control</i>	<i>Acetylcarnitine</i>	<i>Control</i>	<i>Acetylcarnitine</i>
PCr	11.57 ± 0.51	14.69 ± 1.16	11.29 ± 1.36	12.11 ± 0.96
Cr	16.87 ± 0.91	14.30 ± 2.41	17.62 ± 1.98	14.21 ± 1.44
PCr/Cr	0.70 ± 0.04	1.16 ± 0.11 *	0.72 ± 0.10	0.88 ± 0.05
AMP	0.13 ± 0.02	0.07 ± 0.01	0.09 ± 0.02	0.10 ± 0.03
ATP	1.13 ± 0.21	1.26 ± 0.18	0.95 ± 0.21	0.88 ± 0.23
AMP/ATP	0.12 ± 0.02	0.06 ± 0.01 *	0.09 ± 0.01	0.12 ± 0.03
IMP	1.43 ± 0.22	1.11 ± 0.16	1.53 ± 0.30	1.35 ± 0.26
IMP/ATP	1.54 ± 0.44	1.01 ± 0.25 #	2.50 ± 1.37	2.85 ± 1.67

Supplemental Table 3, related to Figure 4. Acetylcarnitine-mediated improvements in muscle bioenergetics require CrAT. Contralateral EDL muscles from CrAT^{fl/fl} and CrAT^{SM-/-} littermates were incubated ± 5mM acetylcarnitine and flash frozen immediately after 4 min of electrical stimulation as described in the Methods. Data are expressed as μmoles/g tissue and represent means ± SEM for n=4-10 muscles per group. 2-way ANOVA revealed significant interaction on AMP/ATP ratio (P=0.029) and main effects of AC on PCr (P=0.048) and PCr/Cr (P=0.001). * P<0.05, # P=0.056 between Control and 5mM AC within the same genotype by paired t-tests.

Supplemental Methods.

Mass spectrometry-based metabolite analysis. Acylcarnitine measurements were made using flow injection tandem mass spectrometry (An et al., 2004). Acyl-CoA esters were analyzed using a method in (Magnes et al., 2005) and the extraction procedure in (Deutsch et al., 1994). The CoAs were further purified by solid phase extraction (Minkler et al., 2008). To extract nucleotides, EDLs were suspended in -80°C methanol (75 mg ww/mL) and cut into small pieces before further homogenization with a tissuelyzer. Homogenates were then diluted to 2:1:3 methanol:water:hexanes, thoroughly mixed and centrifuged. The bottom aqueous layer was saved purified of particulates by centrifugation and nucleotides were measured as in (Cordell et al., 2008) with modifications. Analysis was completed on an Agilent Technologies 6410 LC-MS/MS (Santa Clara, CA). A Chromolith Fast Gradient RP-18e 50 x 2mm column (EMD Millipore, Billerica, MA, USA) was used at a flow rate of 0.3 ml/min. Mobile phase A was water:methanol (95:5), pH 7.5 with 5 mM dimethylhexylamine, and B was water:methanol (20:80), with 10 mM dimethylhexylamine. The gradient was t=0, 0% B; t=1.2, 0% B; t=22, 40% B; t=22.1, 100% B; and t=25, 100% B. SRM parameters (parent ion-product ion, collision energy) were 506-159 m/z, 33 V for ATP, 346-134 m/z, 37 V for AMP, and 347-79 m/z, 39 V for IMP. Chromatographic separations were performed using an Agilent Technologies (Santa Clara, CA) 1200 HPLC system and a Chromolith FastGradient RP-18e 50-2mm column (EMD Millipore, Billerica, MA, USA).

Acetylcarnitine measurements in endurance trained and sedentary subjects. Human studies were performed at the Maastricht University Medical Center and were approved by the institutional medical ethics committee. Written informed consent was obtained from all subjects prior to inclusion. CRAT protein abundance and activity was determined in medial vastus lateralis muscle biopsies from earlier studies, in three metabolically distinct groups: older subjects with type 2 diabetes, healthy sedentary lean subjects and endurance trained subjects (age 63.7±7.3, 22.2±2.6 and 25.2±4.5 years respectively and BMI

30.5±1.4, 21.9±1.6 and 21.1±1.6 respectively). The acute exercise protocol was conducted with four endurance trained subjects (more than 3 times weekly work outs and VO₂max > 50 ml/min/kg) and five healthy sedentary subjects (VO₂max < 40 ml/min/kg). Maximal performance and maximal oxygen uptake of all subjects was determined on a separate day in a routine incremental cycling test (Oxycon Beta, Mijnhardt, The Netherlands). Acetylcarnitine quantification was performed in the medial vastus lateralis muscle at 5:00 pm in all the subjects (after consumption of a light lunch at 12.00 PM (no food consumption or physical activity in the 5 hours preceding the measurement). After acetylcarnitine quantification, subjects performed a 30 min cycling test at a relative workload of 50% of their predetermined V_{max}. Subsequently, the same MR-protocol was used to determine acetylcarnitine concentration again. Post-exercise acquisition of spectra started approximately 15 minutes after cycling, due to transfer between the exercise room and the MR scanner and the acquisition of MR images. 1H-MRS was performed using a 3.0 T whole body MRI-scanner (Achieva, Philips Healthcare, Best, The Netherlands). A two-element flexible surface coil was placed over the vastus lateralis muscle and a Point Resolved Spectroscopy (PRESS) sequence (Volume of interest = 40 mm x 20 mm x 60 mm and TR = 6000 ms). To correct for T₂ relaxation of acetylcarnitine and t-Cr peaks in each subject individually, a series of 6 spectra with different echo times (TE = 300, 325, 350, 400, 450 and 500 ms) was acquired. NSA was adapted from 12 to 76 throughout the protocol to maintain a fairly constant SNR. To distinguish between signal changes due to T₂ relaxation and changes due to a fluctuating concentration in time, spectra with a fixed TE of 350 ms (NSA 20) were measured before and after each spectrum of the series. Total duration of the protocol was 35 minutes. Spectra were analyzed using the AMARES algorithm in the jMRUI software package. The t-Cr peak was used as internal reference, assuming a concentration of 30 mmol/kgww. The acetylcarnitine concentration is reported in mmol/kgww.

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