Cell Metabolism, Volume *21*

Supplemental Information

Skeletal Muscle AMPK Activation of Autophagy Prevents Fasting-Induced Hypoglycemia and Myopathy during Aging

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SUPPLEMENTAL INFORMATION

Supplemental Figures

Figure S1, Related to Figure 1

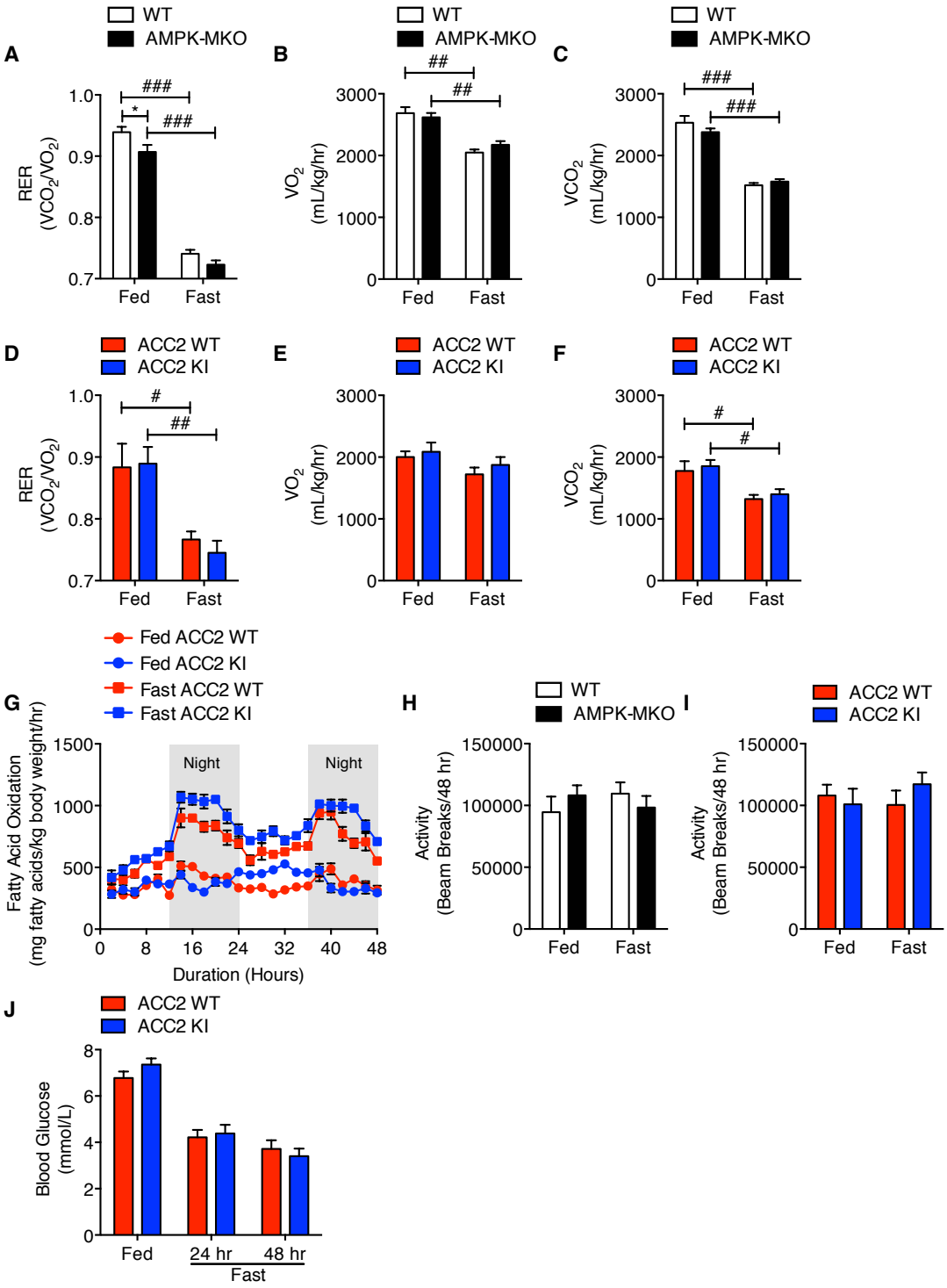

Figure S1: Serum metabolite and metabolic cage measurements. Mice were fed or fasted 48 hours. A-I) Measurements in metabolic cages over 2 days. A) RER, B) VO2 and C) VCO2 in AMPK-MKO and WT mice (*n* = 11 WT fed, *n* = 12 AMPK-MKO fed, *n* = 3 WT 48 hr fast, *n* = 6 AMPK-MKO 48 hr fast). D) RER, E) VO_2, F) VCO_2 and G) fatty acid oxidation (mg of fatty acids/ kg of body weight/ hr) in ACC2 KI and WT littermates (*n* = 3 WT fed, *n* = 4 ACC2 KI fed, *n* = 4 WT 48 hr fast, *n* = 4 ACC2 KI 48 hr fast). Activity levels in H) AMPK-MKO (*n* = 3 WT fed, *n* = 4 AMPK-MKO fed, *n* = 3 WT 48 hr fast, *n* = 6 AMPK-MKO 48 hr fast) and I) ACC2 KI mice (*n* = 3 WT fed, *n* = 4 ACC2 KI fed, *n* = 4 WT 48 hr fast, *n* = 4 ACC2 KI 48 hr fast). J) Blood glucose in ACC2 KI and WT mice at 0, 24 and 48 hours of fasting (*n* = 5 per group). Data are means ± SEM. * WT vs AMPK-MKO $P < 0.05$, $^{\#}$ fed vs fasted $P < 0.05$, $^{\#\#}$ $P < 0.01$, $^{\#\#\#}$ $P < 0.001$.

Figure S2, Related to Figure 2

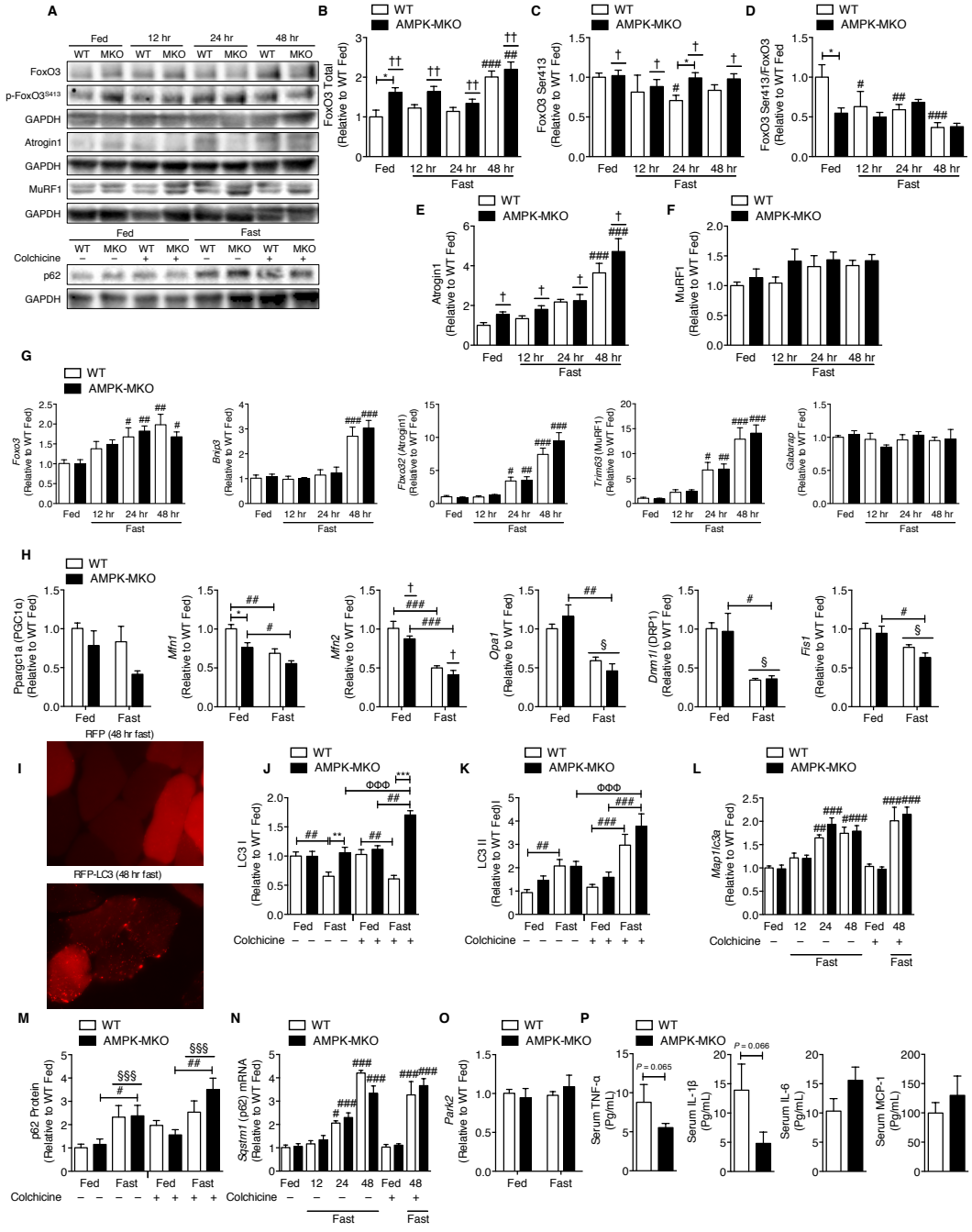

Figure S2: Expression of FoxO3 and downstream targets in AMPK-MKO and WT mice. A) Representative western blots from *tibialis anterior* muscle. Analysis of western blots for the following proteins: B) FoxO3, C) p-FoxO3 Ser413, D) p-FoxO3 Ser413/FoxO3 (n = 10 WT fed, n = 12 AMPK-MKO fed, n = 5 WT 12 hr fast, n = 5

AMPK-MKO 12 hr fast, *n* = 10 WT 24 hr fast, *n* = 12 AMPK-MKO 24 hr fast, *n* = 10 WT 48 hr fast, *n* = 12 AMPK-MKO 48 hr fast over 2 independent experiments), E) Atrogin1 and F) MuRF1 (*n* = 8 WT fed, *n* = 9 AMPK-MKO fed, *n* = 5 WT 12 hr fast, *n* = 5 AMPK-MKO 12 hr fast, *n* = 10 WT 24 hr fast, *n* = 12 AMPK-MKO 24 hr fast, *n* = 8 WT 48 hr fast, *n* = 9 AMPK-MKO 48 hr fast over 2 independent experiments). G) mRNA expression in mixed *gastrocnemius* muscle of several genes in the FoxO3 pathway (*n* = 3 WT fed, *n* = 5 AMPK-MKO fed, *n* = 5 WT 12 hr fast, *n* = 5 AMPK-MKO 12 hr fast, *n* = 5 WT 24 hr fast, *n* = 5 AMPK-MKO 24 hr fast, *n* = 4 WT 48 hr fast, *n* = 4 AMPK-MKO 48 hr fast) and H) mitochondrial fission/fusion pathway (*n* = 3 WT fed, *n* = 6 AMPK-MKO fed, *n* = 4 WT 48 hr fast, *n* = 4 AMPK-MKO 48 hr fast). I) Representative photomicrograph comparison between RFP construct vs the RFP-LC3 construct in 48 hr fasted mice. Analysis of western blots for J) LC3 I (*n* = 20 WT fed control, *n* = 13 AMPK-MKO fed control, *n* = 16 WT 48 hr fast control, *n* = 15 AMPK-MKO 48 hr fast control, *n* = 15 WT fed colchicine, *n* = 10 AMPK-MKO fed colchicine, *n* = 13 WT 48 hr fast colchicine, *n* = 9 AMPK-MKO 48 hr fast colchicine over 2 independent experiments) and K) LC3 II (*n* = 19 WT fed control, *n* = 14 AMPK-MKO fed control, *n* = 15 WT 48 hr fast control, *n* = 14 AMPK-MKO 48 hr fast control, *n* = 15 WT fed colchicine, *n* = 11 AMPK-MKO fed colchicine, *n* = 14 WT 48 hr fast colchicine, *n* = 9 AMPK-MKO 48 hr fast colchicine over 2 independent experiments). L) mRNA expression in mixed *gastrocnemius* muscle of *Map1lc3a* (*n* = 3 WT fed, *n* = 6 AMPK-MKO fed, *n* = 5 WT 12 hr fast, *n* = 5 AMPK-MKO 12 hr fast, *n* = 10 WT 24 hr fast, *n* = 11 AMPK-MKO 24 hr fast, *n* = 4 WT 48 hr fast, *n* = 4 AMPK-MKO 48 hr fast, *n* = 4 WT fed colchicine, *n* = 7 AMPK-MKO fed colchicine, *n* = 4 WT 48 hr fast colchicine, *n* = 5

AMPK-MKO 48 hr fast colchicine). M) Analysis of p62 protein expression in *tibialis anterior* muscle (*n* = 7 WT fed control, *n* = 9 AMPK-MKO fed control, *n* = 8 WT 48 hr fast control, *n* = 9 AMPK-MKO 48 hr fast control, *n* = 4 WT fed colchicine, *n* = 7 AMPK-MKO fed colchicine, *n* = 3 WT 48 hr fast colchicine, *n* = 5 AMPK-MKO 48 hr fast colchicine over 2 independent experiments). N) mRNA expression in mixed *gastrocnemius* muscle of *Sqstm1* (*n* = 3 WT fed, *n* = 6 AMPK-MKO fed, *n* = 5 WT 12 hr fast, *n* = 5 AMPK-MKO 12 hr fast, *n* = 10 WT 24 hr fast, *n* = 12 AMPK-MKO 24 hr fast, *n* = 4 WT 48 hr fast, *n* = 4 AMPK-MKO 48 hr fast, *n* = 4 WT fed colchicine, *n* = 7 AMPK-MKO fed colchicine, *n* = 4 WT 48 hr fast colchicine, *n* = 5 AMPK-MKO 48 hr fast colchicine) and O) *Park2* (*n* = 3 WT fed, *n* = 6 AMPK-MKO fed, *n* = 4 WT 48 hr fast, *n* = 4 AMPK-MKO 48 hr fast). P) Aged AMPK-MKO and WT mice. Serum levels of TNF-α (*n* = 4 WT, *n* = 10 AMPK-MKO), IL-1β (*n* = 4 WT, *n* = 6 AMPK-MKO), IL-6 (*n* = 6 WT, *n* = 13 AMPK-MKO) and MCP-1 (*n* = 5 WT, *n* = 13 AMPK-MKO). Data are means ± SEM. * WT vs AMPK-MKO $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, $^{\#}$ fed vs fasted $P < 0.05$, $^{\#\#}$ $P < 0.01$, $^{\#\#\#}$ $P < 0.001$, † main effect of genotype $P < 0.05$, †† $P < 0.01$, § main effect of fasting $P < 0.05$, §§§ $P < 0.001$, $^{\Phi\Phi\Phi}$ fast saline vs fast colchicine $P < 0.001$.

Supplemental Tables

Table S1, Related to Figure 1. Serum metabolites in AMPK-MKO and ACC2 KI mice following 48 hour fast.

	WT				AMPK-MKO				ACC2 WT				ACC2 KI			
	Fed		Fast		Fed		Fast		Fed		Fast		Fed		Fast	
	Average	± SEM	Average	± SEM	Average	± SEM	Average	± SEM	Average	± SEM	Average	± SEM	Average	± SEM	Average	± SEM
Lactate (mM)	5.3	0.3	6.0	0.4	5.7	0.6	5.8	0.3	5.3	0.2	6.8	0.9	5.4	0.3	5.0	0.2
Glycerol (μM)	516.0	84.1	513.5	128.9	441.0	62.6	613.0	235.0	383.5	42.7	843.0	200.8	398.5	48.7	324.3 *	52.4
NEFA (μM)	647.2	35.7	866.4 §§	109.9	727.7	65.8	897.7 §§	39.2	537.8	38.4	876.9	376.3	387.8	38.4	487.3	16.7

Lactate (n = 8 WT fed, n = 11 AMPK-MKO fed, n = 7 WT 48 hr fast, n = 10 AMPK-MKO 48 hr fast, n = 4 ACC2 WT fed, n = 4 ACC2 KI fed, n = 5 ACC2 WT 48 hr fast, n = 3 ACC2 KI 48 hr fast). Glycerol (n = 4 WT fed, n = 4 AMPK-MKO fed, n = 4 WT 48 hr fast, n = 5 AMPK-MKO 48 hr fast, n = 4 ACC2 WT fed, n = 4 ACC2 KI fed, n = 5 ACC2 WT 48 hr fast, n = 3 ACC2 KI 48 hr fast). NEFA (n = 10 WT fed, n = 11 AMPK-MKO fed, n = 8 WT 48 hr fast, n = 9 AMPK-MKO 48 hr fast, n = 4 ACC2 WT fed, n = 4 ACC2 KI fed, n = 5 ACC2 WT 48 hr fast, n = 3 ACC2 KI 48 hr fast). * WT vs AMPK-MKO $P < 0.05$. §§ $P < 0.01$ for main effect of fasting. Data are means ± SEM.

Table S2, Related to Figure 2. Quantification of protein expression in AMPK-MKO mice following 48 hour fast.

	WT				AMPK-MKO			
	Fed		Fast		Fed		Fast	
	Average	± SEM	Average	± SEM	Average	± SEM	Average	± SEM
AMPK	1.00	0.11	1.16	0.15	0.02 ***,†††	0.01	0.03 ***,†††	0.01
p-ACC^{S212}	1.00	0.22	1.12	0.24	0.04 ***,†††	0.02	0.24 **,†††	0.08
ACC	1.00	0.22	0.86	0.25	0.73	0.13	1.05	0.17
p-Ulk1^{S757}	1.00	0.23	1.22	0.31	2.71 **,†††	0.41	2.68 *,†††	0.25
p-Ulk1^{S317}	1.00	0.14	1.17	0.07	1.62 †	0.09	1.56 †	0.36
p-Ulk1^{S317}/Ulk1	1.00	0.19	0.67	0.17	1.42	0.40	0.88	0.24

AMPK (n = 5 WT fed, n = 4 AMPK-MKO fed, n = 3 WT 48 hr fast, n = 6 AMPK-MKO 48 hr fast). p-ACC Ser212 and ACC (n = 7 WT fed, n = 7 AMPK-MKO fed, n = 6 WT 48 hr fast, n = 8 AMPK-MKO 48 hr fast). p-Ulk1 Ser757 (n = 7 WT fed, n = 8 AMPK-MKO fed, n = 5 WT 48 hr fast, n = 8 AMPK-MKO 48 hr fast). p-Ulk1 Ser317 (n = 3 WT fed, n = 5 AMPK-MKO fed, n = 4 WT 48 hr fast, n = 4 AMPK-MKO 48 hr fast). p-Ulk1 Ser317/Ulk1 (n = 3 WT fed, n = 4 AMPK-MKO fed, n = 4 WT 48 hr fast, n = 4 AMPK-MKO 48 hr fast). * WT vs AMPK-MKO $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$, † WT vs AMPK-MKO $P < 0.05$, ††† $P < 0.001$. Data are means ± SEM.

Supplemental Experimental Procedures

In Vivo Experiments

Mice were housed in the McMaster Animal Facilities on a 12 hour light/dark cycle. During fasting experiments, food was removed from mice at 7 am (the end of the active dark cycle). Fed control mice maintained *ad libitum* access to food and were sacrificed at 11 pm (4 hours into the active dark cycle). Metabolic measures were generated using the Columbus Laboratory Animal Monitoring System as previously described (Galic et al., 2011), with an exception being that mice were either fed or fasted 48 hours during the measurements. Blood glucose levels were measured using an Accu-Chek Aviva blood glucometer. To measure autophagic flux, mice were injected with 100μl of either sterile water (control) or colchicine (Sigma) at a dose of 0.4mg/kg/day (once daily for two days prior to sacrifice).

Plasmid preparation and electroporation. The tandem fluorescent-tagged LC3 (21074) and mRFP1-C1 (54765) plasmids were purchased from Addgene. The plasmid was grown and purified using endotoxin free materials and reagents with EndoFree Plasmid Giga Kit (Qiagen) by following manufacturer's instructions and as previously described (Schertzer et al., 2006a). Mice were anaesthetized with intraperitoneal injection of pentobarbitone (60 mg/kg). The anteromedial aspect of *tibialis anterior* muscle was then surgically exposed and 30 μl (0.5 U/μl) of hyaluronidase (Sigma) was injected. The incision site was kept moist with warmed isotonic saline and after 2 hours, *tibialis anterior* muscle was injected with 40 μL (2 μg/μL) of plasmid. Immediately following injection, three 20 ms transcutaneous pulses of 200 V/cm were delivered to the *tibialis*

anterior muscle at a frequency of 1 Hz using BTX caliper electrodes (Harvard Apparatus). The polarity was then reversed and 3 more pulses delivered. Anafen (5 mg/kg) and 1 mL of saline were subcutaneously injected following closure of the incision site with sutures. Mice were then fed or fasted as described above and sacrificed 5 days post-electroporation. Upon removal, *tibialis anterior* muscles were incubated in 4% paraformaldehyde (Sigma) for 4 hours, followed by 30% sucrose for 11 hours. *Tibialis anterior* muscle was then covered in OCT and frozen in liquid nitrogen-cooled isopentane.

Muscle imaging

All histology performed was in *tibialis anterior* muscles, processed and stained by the John Mayberry Histology facility at McMaster University. OCT-embedded tissues for fluorescence microscopy were cut into 5 μm sections using a Leica cryostat at -20^{o} C. Slides were then air dried for 30 min. and mounted using fluoromount (Sigma). Images were taken at random locations within the sample using a 60x magnification oil-immersion lens. RFP-LC3 puncta were counted from a minimum of 30 myofibers per mouse using Nikon Elements software (Nikon Inc.). Muscles electroporated with an empty RFP construct were used as a negative control to demonstrate the absence of puncta when processed and imaged under parallel conditions. Haemotoxylin and eosin (H&E) stained slides were used for quantifying centrally located nuclei and CSA in 200 randomly selected myofibers of the *tibialis anterior*, as described (Schertzer et al., 2006b). Measurement of collagen accumulation was performed with Masson's trichrome

staining by segmenting the green stain relative to the area of interest using Image Pro Plus (Media Cybernetics, Inc.).

Immunostaining

For imaging of Parkin and Tom20, 8 μm sections were incubated in 0.2% Triton-x-100 for 30 minutes, 5% Normal Goat Serum for 40 minutes, Parkin antibody (1:250) (ab15954, Abcam) overnight at 4˚C and Tom20 antibody (1:50) (sc-17764, Santa Cruz Biotechnology) for two hours at room temperature. To visualize Parkin and Tom20, sections were incubated in goat-anti-rabbit Alexafluor 594 (A11012,) and goat-anti-mouse Alexafluor 488 (1:250) (A121131, Life Technologies), respectively. Parkin was quantified within six random areas per animal. The negative control (no primary antibodies) was used to set exposure time. Representative images were adjusted for brightness and contrast to allow for ease of visualization by the reader.

Muscle Function

Contractile properties of the *tibialis anterior* muscle were measured *in situ*, as described (Schertzer et al., 2007). Mice were anaesthetized by inhalation anesthetic (1-2% isoflurane) and the distal tendon of the *tibialis anterior* muscle was surgically exposed, secured using a piece of 5.0 suture and severed from its attachment point. Mice were then mounted to an 809B *in situ* Mouse Apparatus platform (Aurora Scientific Inc.) maintained at 33 °C and the tendon was attached to a 300C dual mode muscle lever system (Aurora Scientific Inc.). The muscle was stimulated to contract via two small platinum hook electrodes placed around the sciatic nerve. Optimal muscle length was

determined by administering single electrical pulses (200 ms at 2-3 mA) while lengthening the muscle until optimal twitch force was attained. A force-frequency relationship was then established by administering a series of electrical pules, 300 ms in duration, at frequencies of 10, 20, 40, 80, 120, 180, 230, 300 Hz with 3 minutes of rest between contractions. Maximal isometric tetanic force was determined from the peak of the force-frequency relationship using ASI 611A Dynamic muscle analysis software (Aurora Scientific Inc). Maximal specific force was calculated by dividing the maximal isometric force by the muscle volume.

Western Blotting

Western blotting was used as previously described (Bujak et al., 2014), with two exceptions being that 30 μg at 1 μg/μL (15 μg at 1 μg/μL for LC3) of protein was loaded into each well and nitrocellulose membranes were used in the place of PVDF. MuRF1 (ab77577) and Atrogin1 (ab168372) were purchased from Abcam. All other antibodies were purchased from Cell Signaling and included: ACC (#3676), p-ACC Ser79/221 (#3661), AMPK α (#2532), p-AMPK Thr172 (#2532), Bnip3 (#3769), FoxO3 (#12829), p-FoxO3 Ser413 (#8174), GAPDH (#5174), LC3B (#2775), Parkin (#2132), S6K (#9202), p-S6K Thr389 (#9234), SQSTM1/p62 (#5114), Ulk1 (#8054), p-Ulk1 Ser555 (#5869), p-Ulk1 Ser317 (#12753) and p-Ulk1Ser757 (#6888). All bands were quantified relative to the loading control GAPDH.

RT-qPCR

Quantitative real-time PCR was used as previously described (Galic et al., 2011) with the

following Taqman primers obtained from Invitrogen: *Foxo3* (Mm01185722_m1), *Bnip3* (Mm01275600_g1), *Fbxo32* (Mm00499523_m1), *Trim63* (Mm01185221_m1), *Gabarap* (Mm00490680_m1), *Mfn1* (Mm00612599_m1), *Mfn2* (Mm00500120_m1), *Opa1* (Mm01349707_g1), *Dnm1l* (Mm01342903_m1), *Fis1* (Mm00481580_m1), *Park2* (Mm00450187_m1), *Ppargc1a* (Mm00447183_m1) and *Tbp* (Mm00446971_m1). All genese were normalized to the stable housekeeping gene *Tbp*.

Metabolite Analysis and Inflammatory Cytokine Analysis

Serum metabolites were measured according to manufacturer's instructions using the following kits: alanine (#MAK001, Sigma), β-hydroxybutyrate (#700190, Cayman Chemical Company), and lactate (#K627-100, BioVision), NEFA (#276-76491, Wako Chemicals), Glycerol (#F6428, Sigma). Inflammatory cytokines were measured according to the manufacturer's instructions using a Bio-Plex Pro Reagent Kit (#171-304070) to quantify the following: TNFα (#171-G5023M), IL-6 (#171-G5007M), IL-1β (#171-G5002M), MCP-1 (#171-G5019M) in comparison with the group standard (#171-I50001).

CT Imaging

Computerized tomography scanning was performed by the McMaster Center for Pre-Clinical and Translational Imaging as previously described (Galic et al., 2011).

mtDNA Copy Number and Deletions

Primer sequences utilized are as follows: *Nd1*, forward-gtggctcatctactccactga, reverse-tcgagcgatccataacaataa; *Nd4*, forward-attattattacccgatgagggaacc, reverse-attaagatgagggcaattagcagt; *β-globin*, forward-gaagcgattctagggagcag, reverse-ggagcagcgattctgagtaga

Transmission Electron Microscopy

Tibialis anterior muscles were cut longitudinally, fixed in glutaraldehyde and processed by the McMaster University Medical Center Electron Microscopy Group. Each image included SS and IMF mitochondria. Half of the images were taken near a nucleus and half were taken away from a nucleus in order to analyze a representative sample of mitochondria. An image analysis program (Nikon Elements software) was used to determine mitochondrial area and number.

Fatty Acid Oxidation

Fatty acid oxidation measurements were obtained over 2 days in the metabolic cages and are calculated using the following equation: (1.6946*VO2)-(1.7012*VCO2).

Supplemental References

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