Online Data Supplement

High CO_2 downregulates skeletal muscle protein anabolism via AMPKα2-mediated depressed ribosomal biogenesis.

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Supplementary figures and tables and other data:

Figure S1: Outline of experimental setup to interrogate protein synthesis in chronically hypercapnia mice. Animals were housed in normoxia/hypercapnic conditions for 2 months. On the day of procurement and 30 minutes before euthanasia puromycin was injected intraperitoneally. See methods section for technical details.

Figure S2: CO_2-induced anabolic attenuation occurs later than catabolic acceleration: S2-A: S2-A: Soleus or EDL **S2-B:** muscles demonstrate no significant attenuation of puromycin incorporation at three weeks of hypercapnia as indicated by (n=4).

Figure S3: Fiber-specific effects of hypercapnia: S3-A: Higher magnification of fiber-specific puromycin incorporation in normo and hypercapnia. Notice that in hypercapnia, more glycolytic fibers (black fibers, corresponding to type IIx/b) become relatively puromycin-poor (darker) compared with the more oxidative fibers, type I and IIa). **S3-B:**

Immunofluorescence in lower and higher magnification of normo and hypercapnia-exposed mice after 1 month of endurance exercise.

Figure S4: Differentiated cells demonstrate skeletal muscle (myotube) morphology: ***A,*** C2C12 cells, exposed to puromycin-containing medium and hypercapnia for 0, 4, 18 and 24h. *Scale bars*, 250µm (*n* = 3). ***B,*** primary myotubes, exposed to puromycin-containing medium and hypercapnia for 0, and 24h *Scale bars*, 100µm (*n* = 3). Neither puromycin nor hypercapnia alter the viability or phenotype of cells. **C,** C2C12 and primary myotubes express skeletal myosin.

Figure S5: High CO_2-induced anabolic attenuation occurs independently of the mTOR pathway: S5-A: C2C12 myotubes were exposed to normo (N) versus hypercapnia (H); and to vehicle (V) versus rapamycin (R) for a period of 24 h. Then, samples were processed and immunoblotted with anti phospho-mTOR antibody; total mTOR was used as a lane loading control (n=3). **S5-B:** C2C12 myotubes treated for 24 h with rapamycin (R) versus vehicle (V) demonstrate no significant reduction of 45s-Pre-RNA expression (n=7). **S5-C:** C2C12 myotubes treated with rapamycin for 24 h demonstrate a significant reduction of puromycin incorporation (n=4). **S5-D:** Mice were subcutaneously injected rapamycin for 8 weeks. Then they were euthanized 30 min after IP injection of puromycin; soleus and EDL muscles were processed to determine the regulation of phospho mTOR. Actin was used as lane loading control (n=3). **S5-E:** Mice exposed to high CO_2 for 8 weeks were processed and EDL muscles were immunoblotted and probed with anti phospho mTOR antibody. Total mTOR was used as lane loading control (n=4). **S5-F:** Rapamycin-treated mice samples were processed to determine the expression of 45s-pre-RNA (n=4). **S5-G:** Rapamycin-treated mice samples were

processed for immunoblot which showed a significant reduction of puromycin incorporation (n=4). **,p<0.01 ***,p<0.001

Figure S6: TIF-1A commercially available antibodies detect a 50kD product in cultured skeletal muscle cells. S6-A, Lysates of C1C12 cells (myoblast, MB) and myotubes at days 2, 3 and 5 post differentiation probed with antibody against TIF-1A (recognizing N terminal). Actin was used as a lane loading control. **S6-B,** Lysates of primary cells (myoblast, MB) and myotubes at days 1 and 5 post differentiation probed with antibody against TIF-1A (recognizing C terminal). Actin was used as a lane loading control. **S6-C,** C2C12 cells, immunoprecipitation using TIF-1A antibody (recognizing C terminal). Samples were Western blotted, and membrane was probed with an antibody recognizing N terminal. Actin was used as a lane loading control of the cell lysates run in parallel, and thus appears in the immunoprecipitation (IP) as a result of likely co-precipitation with the ~50kD protein. **S6-D,** C2C12 cells, immunoprecipitation using TIF-1A antibody (raised against N-terminal). Samples were Western blotted, and membrane was probed with a different antibody, raised against the C-terminal. Actin was used as a lane loading control of the cell lysates run in parallel, and thus appears in the immunoprecipitation (IP) as a result of likely co-precipitation with the ~50kD protein. **S6-E,** image of agarose gel with PCR products obtained after amplifying cDNA with TIF-1A junctional primers (5-TTTCTTCCCCTTTGACCCT-3 (forward) and 5-CCACTGTGAAATACTGCCT-3 (reverse). Upper band from Cas9+sgRNA lane was send for Sanger sequence and demonstrated adequate insertion of 3X FLAG tag at the end of TIF-1A exon 18 coding region. **S6-F,** C2C12 myotubes previously transfected with

scramble or specific TIF-1A siRNA were sampled and 45S-preRNA/GAPDH expression was determined with qPCR (n=5). **S6-G,** C2C12 myotubes previously transfected with scramble or specific KDM2A siRNA were sampled and 45S-preRNA/GAPDH expression was determined with qPCR (n=5). **S6-H,** Western blot analysis of differentiated myotubes previously transfected with KDM2A siRNA and probed with specific antibody. See methods for details. ****, $p<0.0001$

Table S1: Quantitative PCR (qPCR) primers used in this study

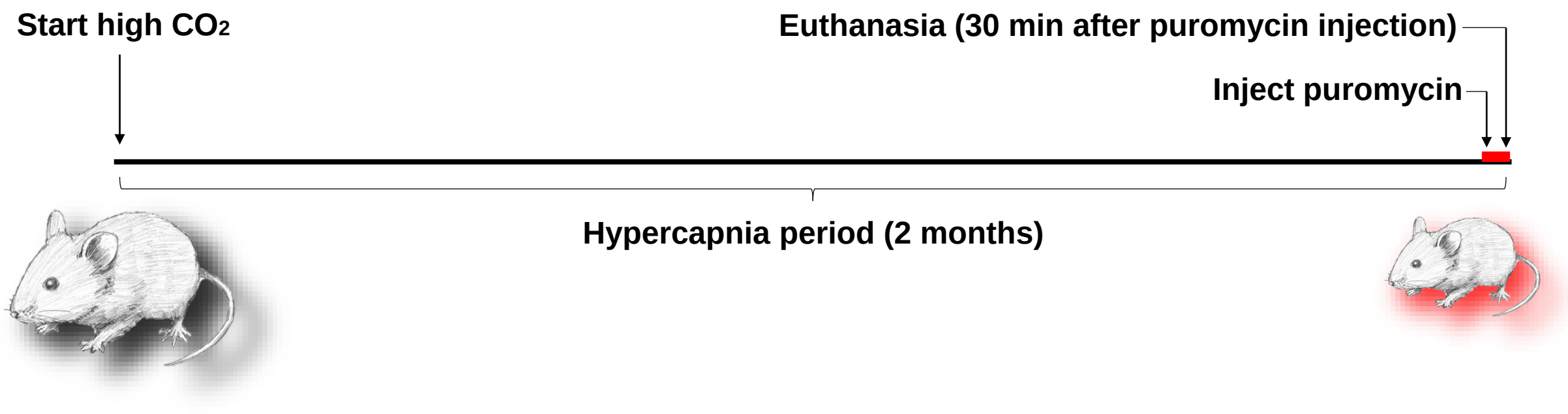
Table S2: Antibodies used in this work

Table S3: SiRNAs used in this work

File S1: Sequence of donor clone and guide RNAs (A, B and C) used for Crispr/Cas9 experiments

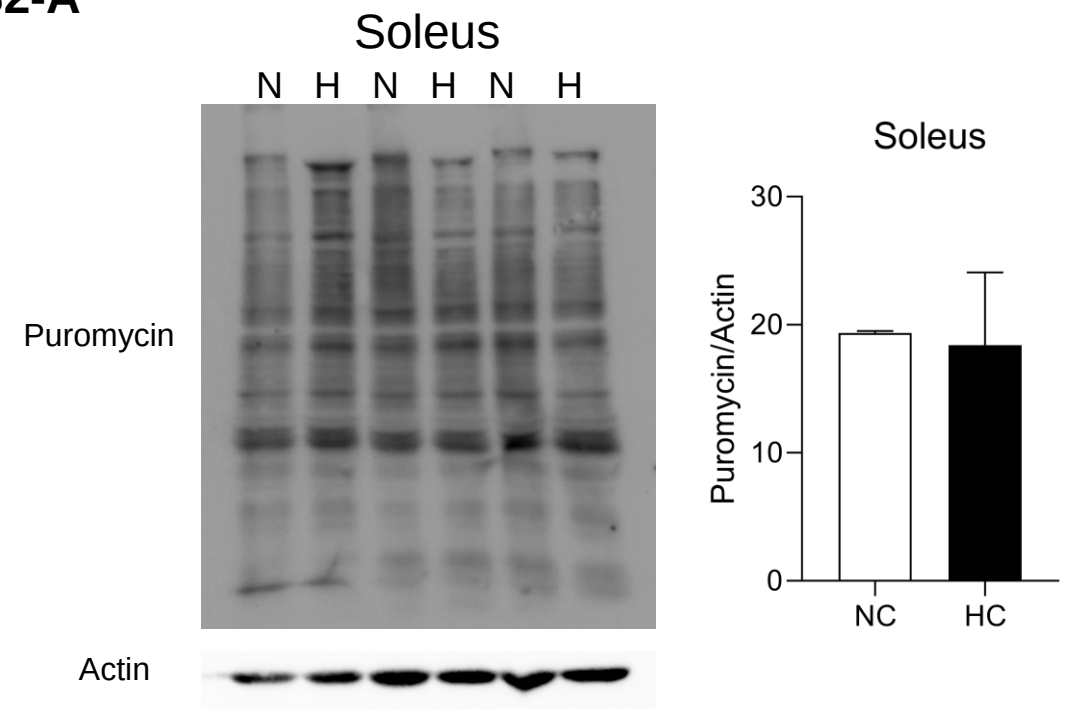
File S2: Output data from the proteomic analysis

File S3: Supplemental material and methods

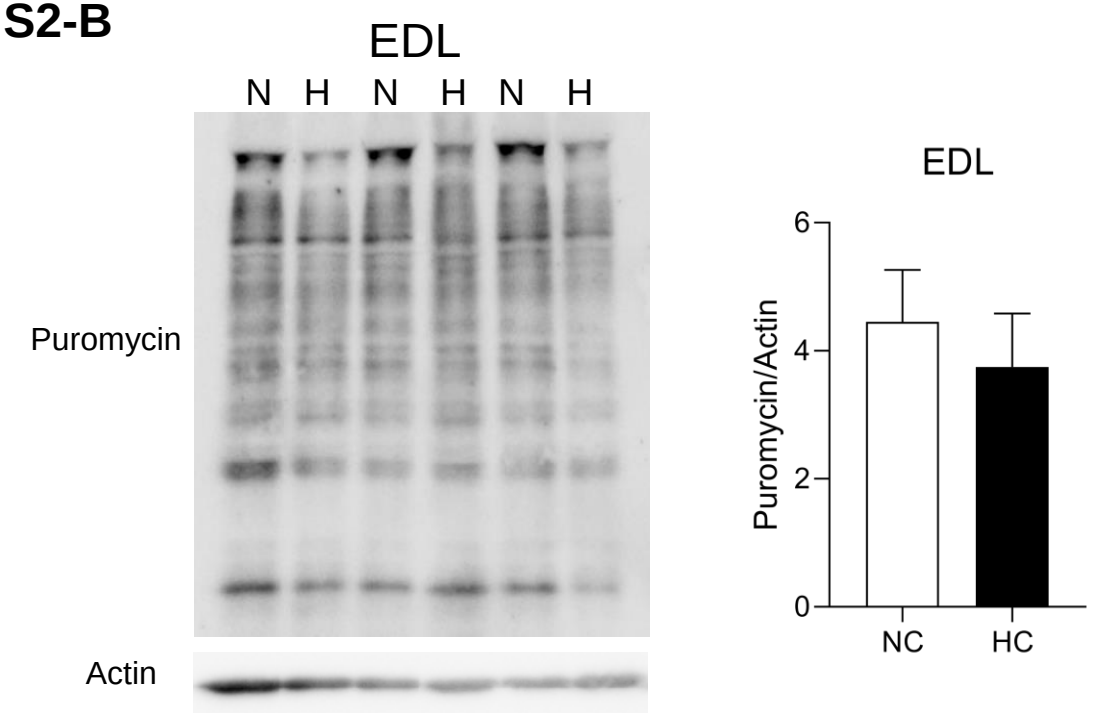


Supplementary Figure 1

S2-A

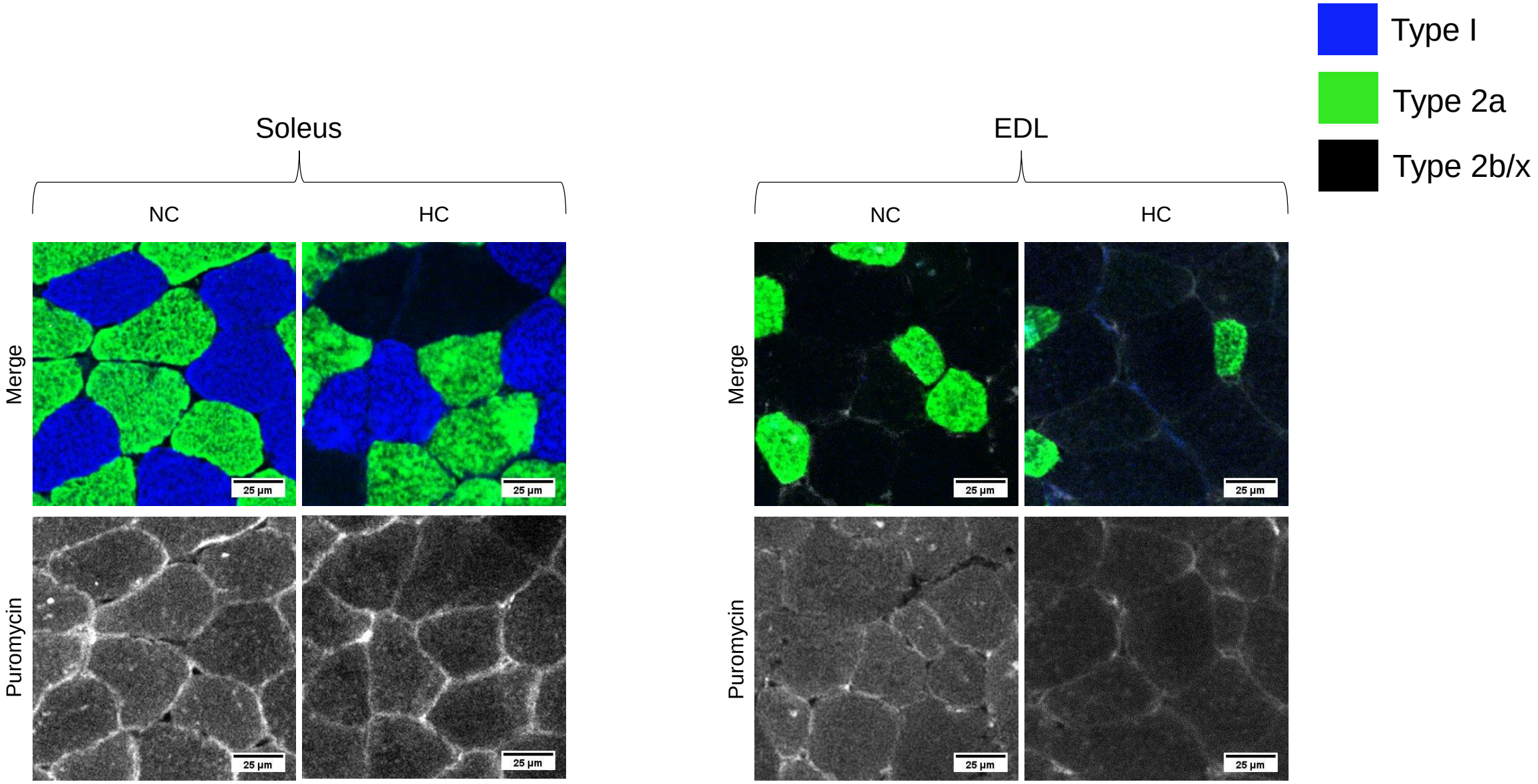


S2-B



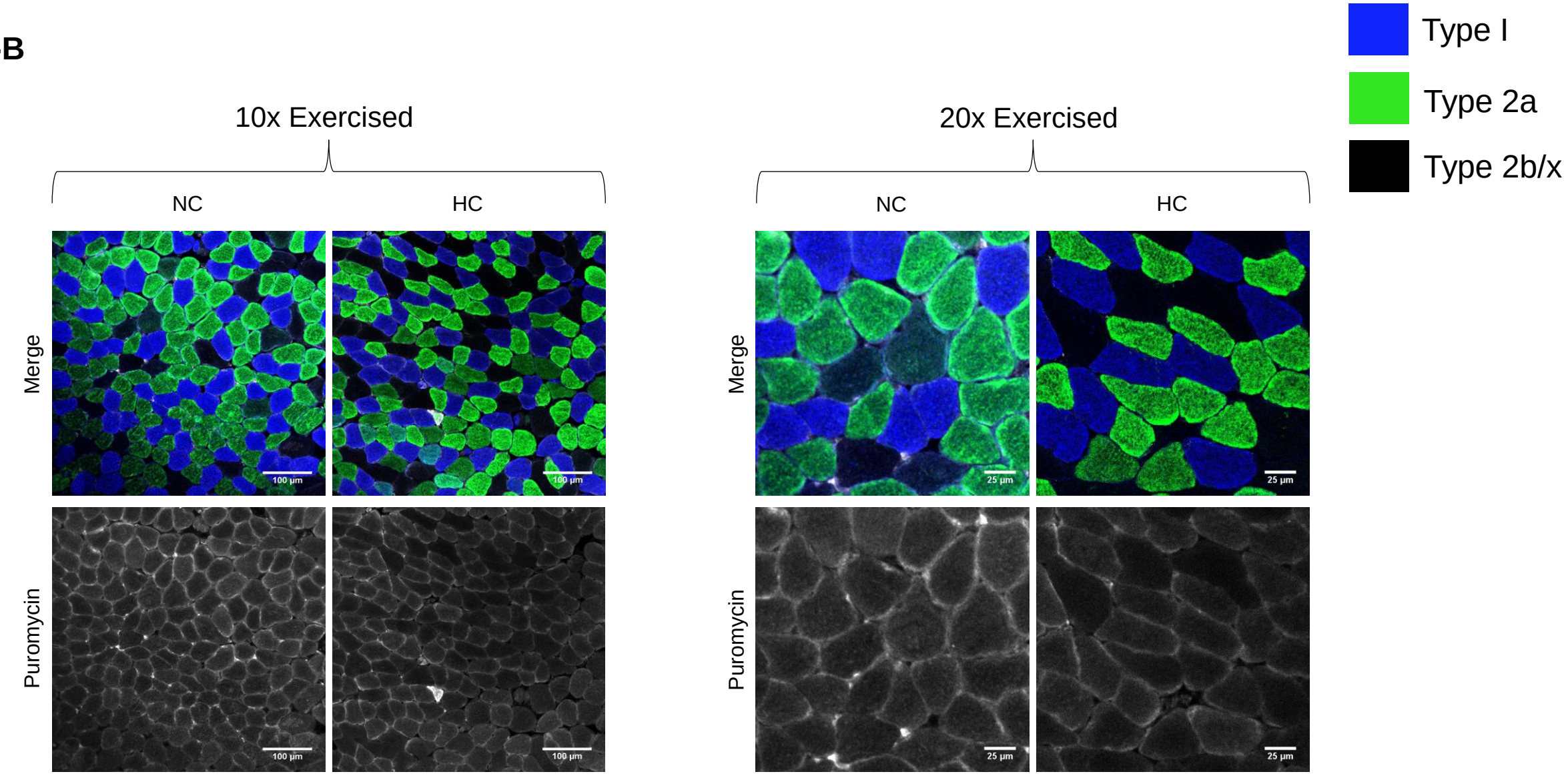
Supplementary Figure 2

S3-A



Supplementary Figure 3

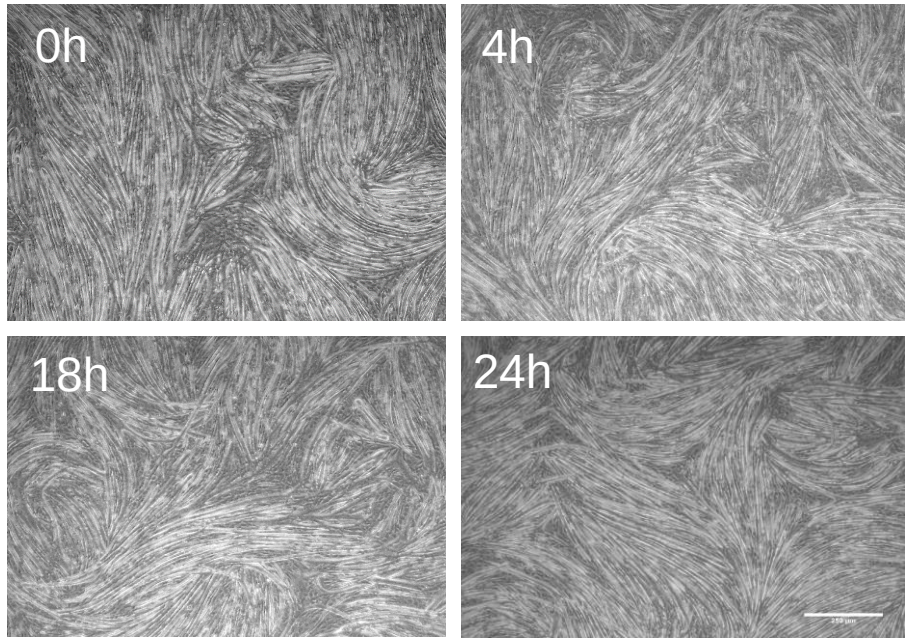
S3-B



Supplementary Figure 3 (cont)

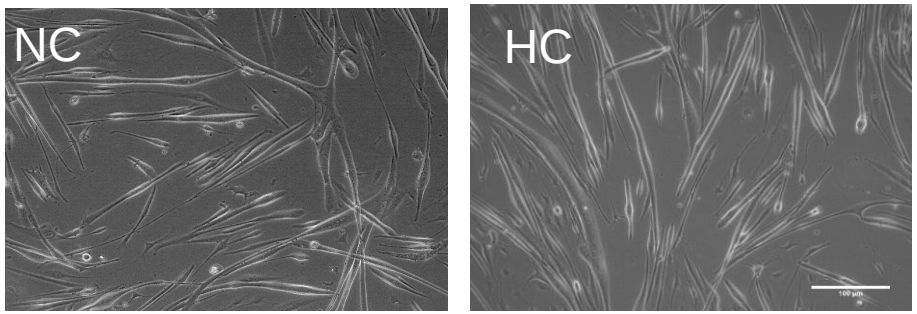
C2C12 myotubes

S4-A

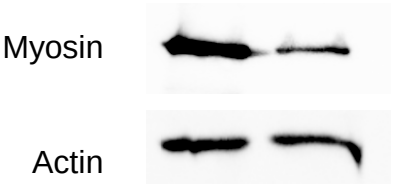


Primary myotubes

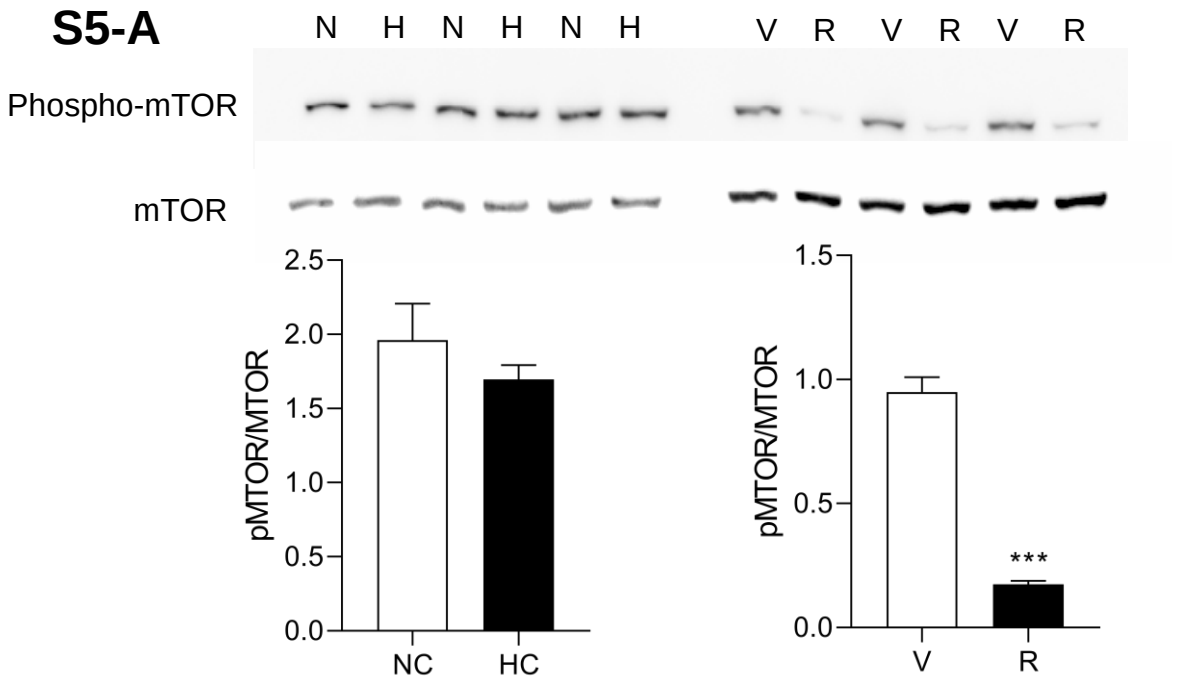
S4-B

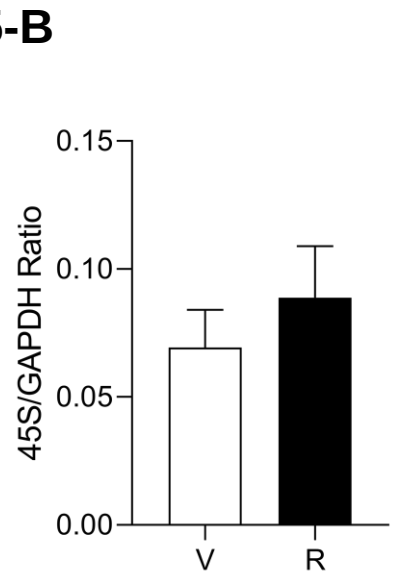


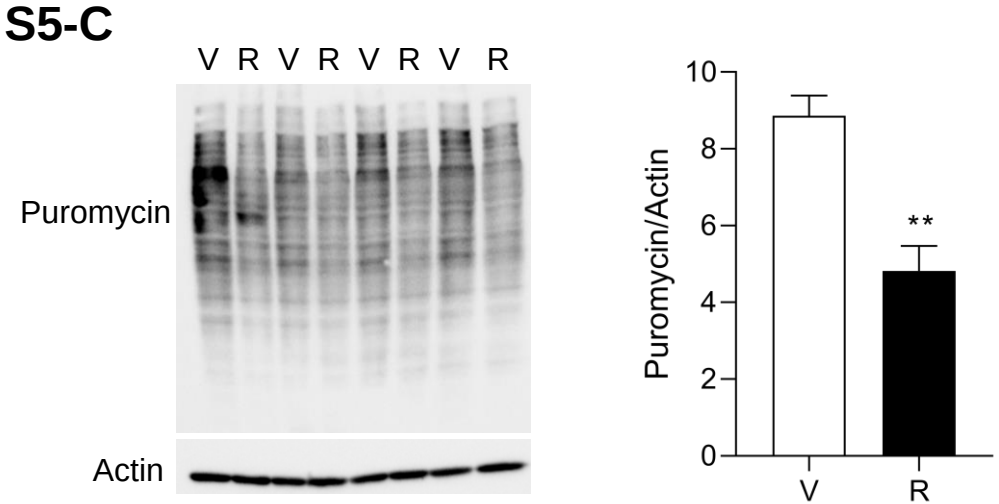
S4-C



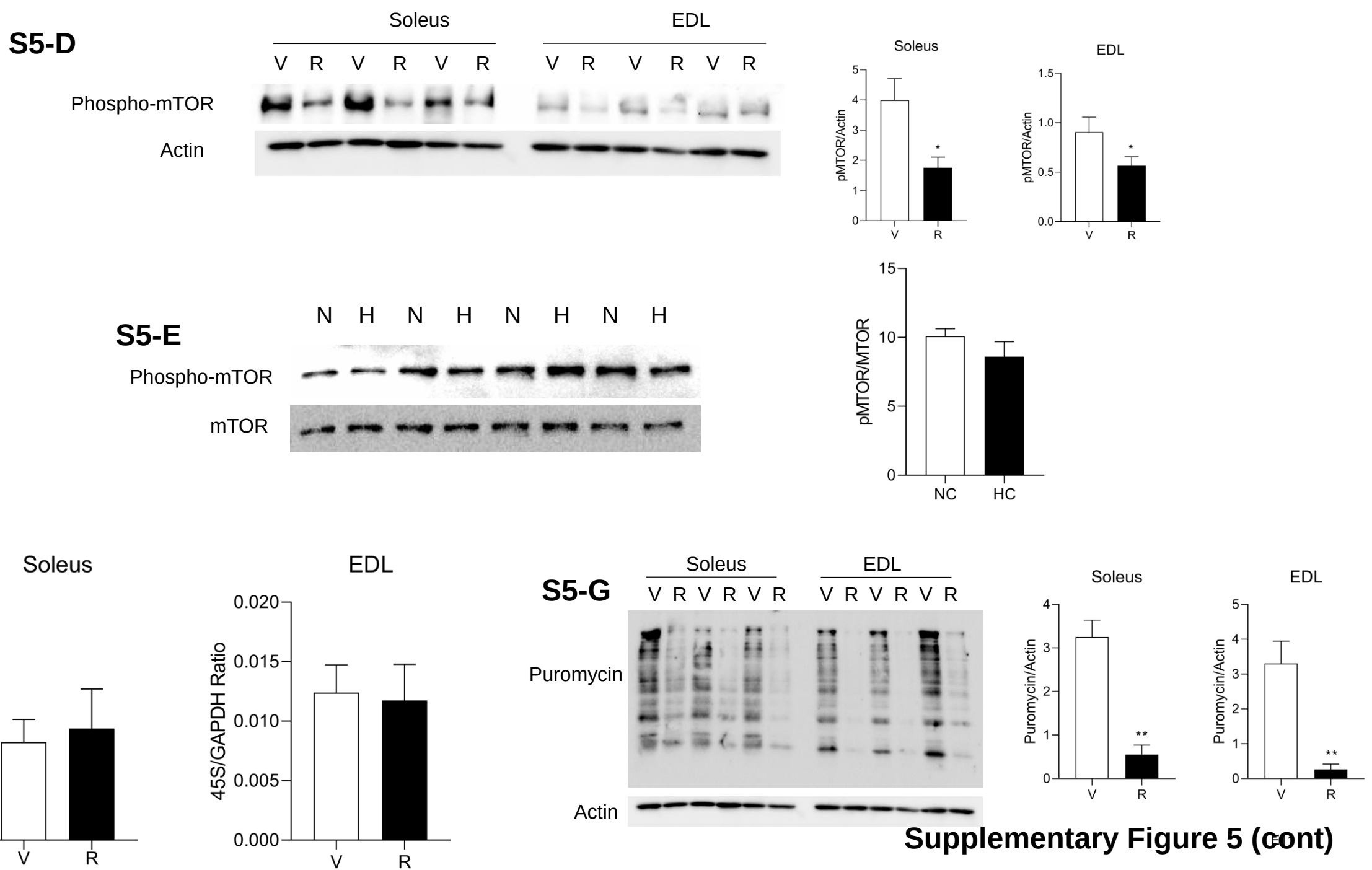
Supplementary Figure 4





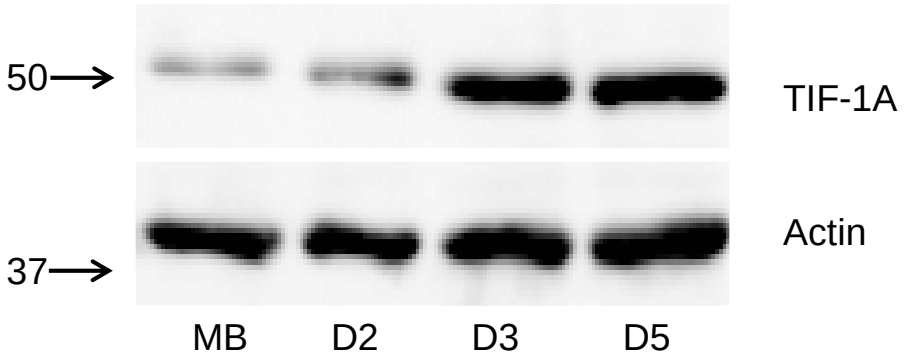


Supplementary Figure 5



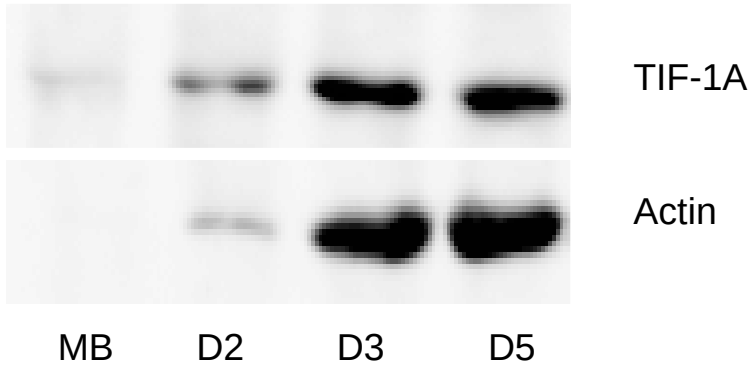
Supplementary Figure 5 (cont)

S6-A Cell Lysates C2C12 cells

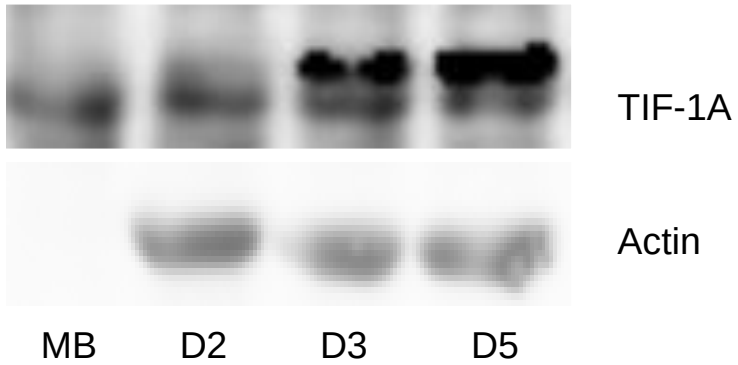


S6-B Cell Lysates Primary myoblasts

S6-C IP-1

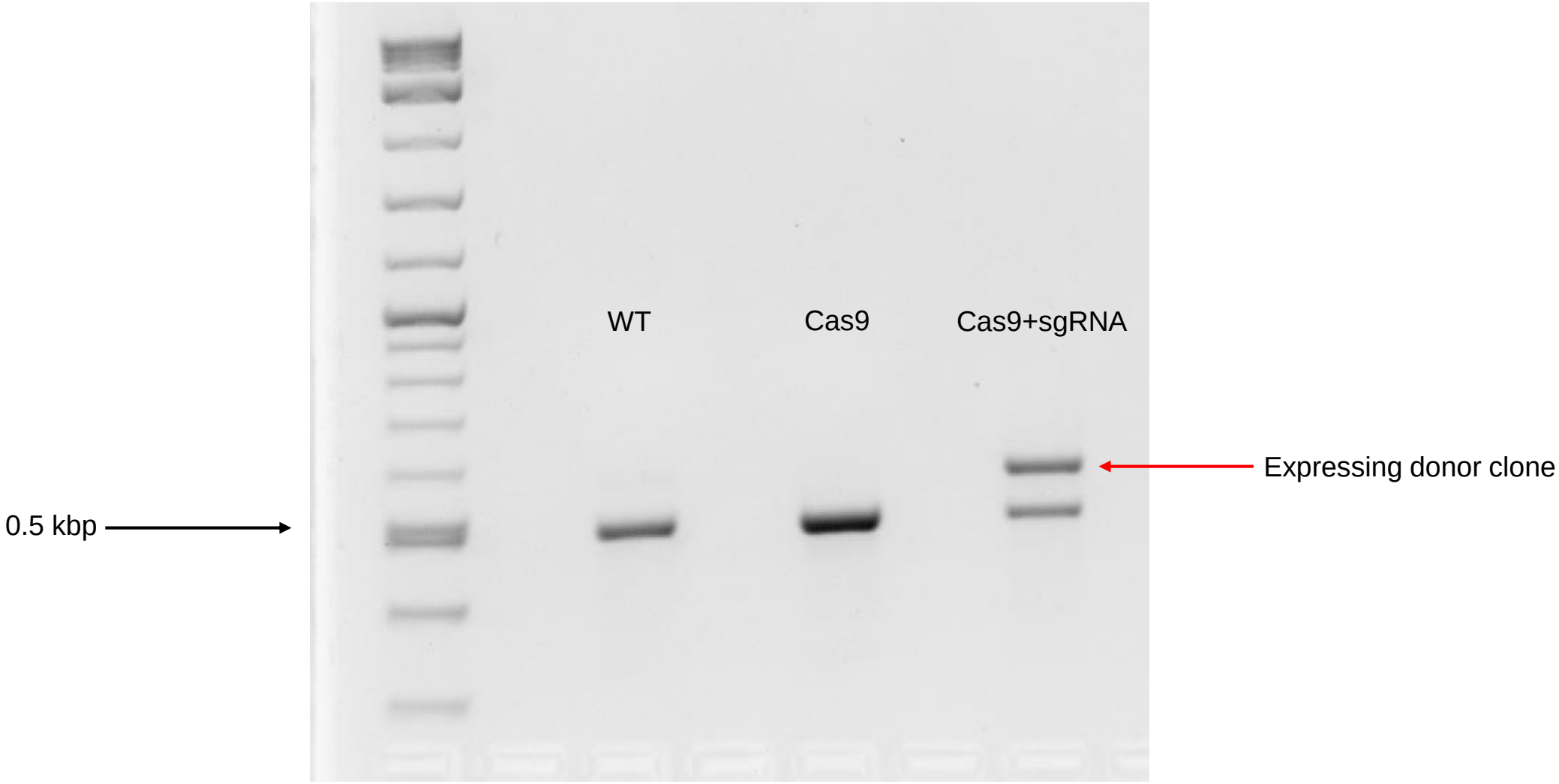


S6-D IP-2



Supplementary Figure 6

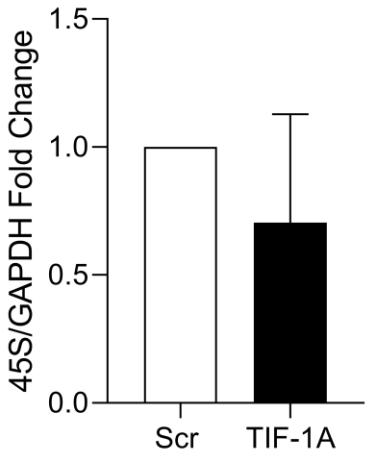
S6-E



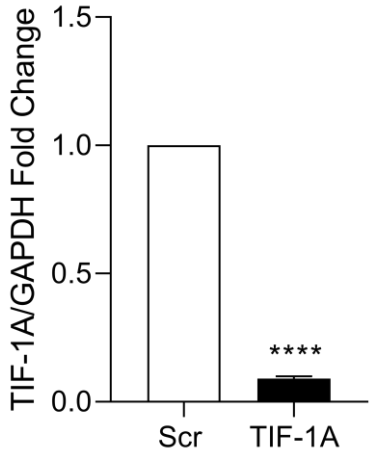
Supplementary Figure 6 (cont)

S6-F

Effect on ribosomal gene expression



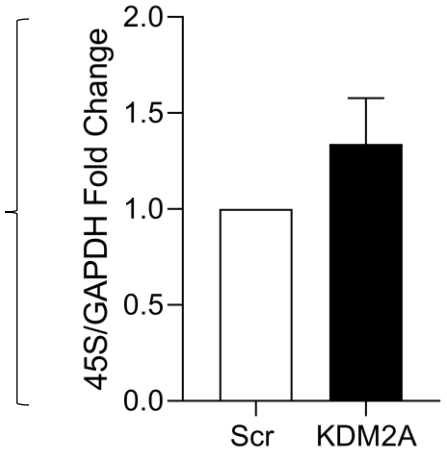
Transfection control



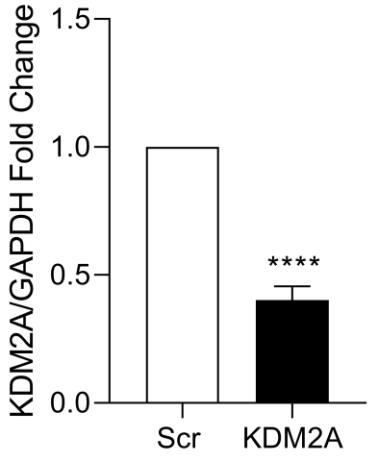
Supplementary Figure 6 (cont)

S6-G

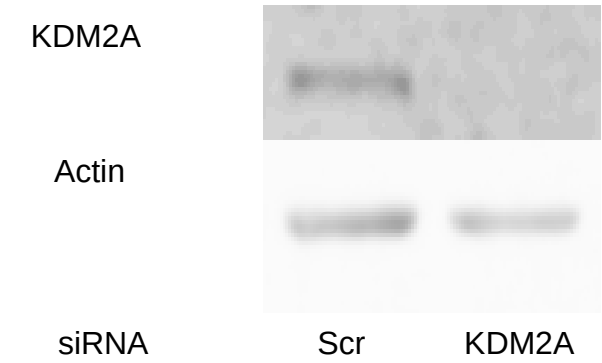
Effect on ribosomal gene expression



Transfection control



S6-H



Supplementary Figure 6 (cont)

Table S1: qPCR primers		
Target	Forward	Reverse
GAPDH	CTTTGTCAAGCTCATTTCCTGG	TCTTGCTCAGTGTCCTTGC
45S Pre-RNA	CCAAGTGTTCATGCCACGTG	CGAGCGACTGCCACAAAAA
RRN3	ATTTTGAGCGCATTGTGTTGAGC	GGGAGCATCTGGCGACTGTTC
KDM2A	GCAACGAGATAGTTCATCCTGGC	CGAGCTGTCTTCCTGGTAACAC
Cas9	CCAAAGAGGTGCTGGACG	GCTCTTTCAATGAGGGTGGA
RRN3 Exon 18	TTTCTTCCCCTTTGACCCT	CCACTGTGAAATACTGCCT

Table S2: List of antibodies		
Target	Working concentration	Company (Cat #)
Puromycin	1:3000	Millipore (MABE343)
Actin	1:3000	Sigma (A2066)
Actin (Pre-conjugated)	1:10000	Sigma (A3854)
Myosin	1:2000	Sigma (M4276)
P-mTOR (Ser2448)	1:1000	Cell Signaling (2971)
mTOR	1:1000	Cell Signaling (2983)
P-AMPK (Thr172)	1:1000	Cell Signaling (2535)
AMPKα1	1:1000	Novus (NBP2-22127)
AMPKα2	1:1000	Novus (NB100-238)
AMPK	1:1000	Cell Signaling (2532)
TIF-1A	1:1000	ThermoFisher (PA5-30872)
TIF-1A	1:1000	Sigma (SAB4502266)
3X-Flag	1:2000	Sigma (F1804)
KDM2A	1:1000	LifeSpan Biosciences (LS-C30281)
HRP Mouse	1:3000	Bio-Rad (170-6516)
HRP Mouse 2a	1:10000	Jackson IRL (115-035-206)
HRP Rabbit	1:3000	Bio-Rad (170-6515)
HRP Rabbit	1:3000	Cell Signaling (7074)
HRP Rabbit IgG Light Chain	1:3000	Abcam (ab99697)
BA-D5	1:100	DSHB (BA-D5, supernatent)
SC-71	1:100	DSHB (SC-71), supernatent)
Anti-mouse IgG2b-DyLight 405	1:250	Jackson IRL (115-475-207)
Anti-mouse IgG1-Alexa 488	1:250	Jackson IRL (115-545-205)
Anti-mouse IgG2a-Alexa 647	1:250	Jackson IRL (115-605-206)

Table S3: List of siRNAs		
Target	Sequence	Company (Cat #)
Control siRNA-A		Santa Cruz Biotechnology (sc-37007)
AMPKα1		Santa Cruz Biotechnology (sc-29674)
AMPKα2		Santa Cruz Biotechnology (sc-38924)
RRN3 SMARTpool	GCUGUCUGAUCCUGAUAUA CCGAUAUGCUCGCGUUAGA UGCCGCAAGUGGUUAAUUU GAAGUCCGAGAGAACAUUG	Dharmacon (M-051638-01-0005)
KDM2A		Santa Cruz Biotechnology (sc-145085)

DC-MTN001206-D09-B and MCP001206-SG01-3-B

sgRNA design:
aagtgatcctagcagagtttcactacccagccatctgctcgttagccaccaagtacagatccctcaaatgttcactgtagagtctgctcttgtttt
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GCGCAATTCTTCCCAGGGCCCTGTGACCCTGTGTTCCACCCCGACCCCACACTGG

MCP001206-SG01-3-B-a:agacttatatctaggggtt
MCP001206-SG01-3-B-b:GCCAGTCTCCACTCCTCACA
MCP001206-SG01-3-B-C:CTCAATCATAGATCCTTGTG

Donor design:

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Immunofluorescence: Frozen muscles collected from puromycin injected mice were sectioned using (Leica, CM1860) and 10 µm sections were obtained. Then, sections were fixed for 15 minutes in acetone at -20°C, and then left at room temperature to dry for 30 minutes. Blocking was performed using the Vector MOM blocking reagent for 1 hour at room temperature. After blocking, sections were incubated for 45 minutes at 37°C with the following primary antibodies all 1:100 in 1% BSA in PBS; BA-D5 (DSHB), SC-71 (DSHB), and anti-Puromycin (EMD Millipore, MABE341). Three washes were then performed with PBS. Secondary antibodies were added all at 1:250 and incubated for 45 minutes at 37°C; anti-mouse IgG2b-DyLight 405 (Jackson IRL, 115-475-207), anti-mouse IgG1-Alexa 488 (Jackson IRL, 115-545-205), anti-mouse IgG2a-Alexa 647(Jackson IRL, 115-605-206). Three washes were then performed with PBS. Samples were mounted with Ibidi Mounting Medium (Ibidi, 50001). Images were captured on the same day using confocal microscopy (Leica, SPE). Fiber-specific puromycin uptake was determined by quantifying the pixel saturation of the puromycin staining color channel observed in grayscale using ImageJ (National Institutes of Health) software as previously described[1].

In-vitro Puromycin exposure*:* C2C12 or primary myotubes were differentiated for 4 days and then media was changed to preconditioned normal and high CO_2, 2% horse serum DMEM/F-12/Tris base/MOPS buffered for 5 or 20% CO_2 respectively, each one with the addition of puromycin 1 µM (Sigma). Cells were left in that condition for 24 h and then processed for analysis. For western blot analysis, the secondary antibody HRP anti-mouse was used at 1:10000 as opposed to 1:3000 to avoid oversaturation of the signal.

In-vitro Rapamycin exposure: C2C12 cells were differentiated for up to 6 days and then exposed to differentiation media containing 100nM rapamycin[2] (LC Laboratories, Woburn, MA) that was dissolved in 10% DMSO and 90% ethanol; these cells were compared with a parallel group without rapamycin, only exposed to the drug's vehicle; and were left in that condition for 24 h and then processed for analysis.

Immunoprecipitation—C2C12 cells were differentiated for up to 6 days and cell lysates were then prepared. 100 µl SureBeads protein A magnetic beads (Bio-Rad) were washed 3 times with TBST and incubated with TIF-1A ab (1:100) or control IgG at room temperature rotating for 10 minutes. Magnetic beads bound with TIF-1A ab were then washed again with TBST three times. Aliquots containing cell lysate were rotated overnight at 4 °C with beads bound with TIF-1A antibody. Samples were then centrifuged, and the magnetic beads were washed with TBST three times and resuspended in SDS-loading buffer where the antigen-antibody complexes were eluted from the beads by incubation at 70 °C for ten minutes and separated in a 10% polyacrylamide gel. To develop the western blot, a mouse anti-rabbit IgG light chain secondary antibody (Abcam, Cambridge, MA, ab99697) targeting the light chain IgG of the primary antibody was used to avoid cross-reactivity with the rabbit IgG heavy chain.

RNA Extraction, cDNA Synthesis, and Quantitative RT-PCR—Quantification of ribosomal DNA transcription was done as previously described. RNA from muscle and cells was extracted using NucleoSpin RNA kit (Machery-Nagel, Düren, Germany). Total RNA was determined spectrophotometrically using a Nanodrop ND-1000 (Saveen & Werner, Limhamnsvägen, Sweden) at 260 nm. cDNA was synthesized using Quantitect Reverse Transcriptase Kit (Qiagen). Quantitative RT-PCR was performed using GoTaq qPCR SYBR Green (Promega) on a CFX96 Real-time PCR detection system (Bio-Rad). Primers sequences used for this paper are presented in **supplementary table 1.** Each sample was run in triplicate, and relative expression levels of transcripts of interest were calculated using the comparative Ct (ΔΔCt) method with glyceraldehyde-3-phosphate dehydrogenase (GAPDH) as housekeeping gene. Data were analyzed using the Bio-Rad CFX Maestro 1.0.

CRISPR/Cas9—C2C12 cells were infected with a concentrated lentiviral prep to express Cas9 protein and blasticidin resistance (Addgene, Cambridge, MA, 52962-LVC), and then selected with blasticidin (1:2000) (Thermo Fisher Scientific). To confirm the stable incorporation and

expression, Cas9 mRNA levels were detected by RT-qPCR from infected and non-infected C2C12. To incorporate the donor clone holding the 3XFLAG sequence, we used three different guide RNA (sgRNA) designed to target exon 18 of the TIF-1A gene (also known as Rrn3). Both the donor clone and the gRNAs were purchased from GeneCopoeia (Rockville, MD). Sequence of the guide RNAs are A: 5-AGACTTATATCTAGGGGGTT-3, B: 5-GCCAGTCTCCACTCCTCACA-3, and C: CTCAATCATAGATCCTTGTG, and the donor clone's map is shown in a **supplementary file**. Cas9-expressing C2C12 myoblasts at 75% confluency were transfected with the donor clone plasmid plus one of the three sgRNA plasmids using Lipofectamine 2000 (Thermo Scientific). 10% fetal bovine serum growth media was then added back to each dish. Transfected cells were selected by adding 2 µg/ml puromycin to the growth media for 6 days with media change every 24 hours for 6 days. Once puromycin-resistant cells were 95% confluent, the media was replaced with 2% horse serum differentiation media. 4-6 days later, myotubes were lysed to collect RNA and generate cDNA. To confirm the 3X Flag was inserted into the expected location at exon 18 of the TIF-1A gene, junctional PCR was performed with primers 5-TTTCTTCCCCTTTGACCCT-3 (forward) and 5- CCACTGTGAAATACTGCCT-3 (reverse). The product was run on a 3% agarose gel and depicted DNA 505 base pairs in length and 436 base pairs in length (**see supplementary figure 6-E**). The two bands were individually cut, and the DNA was digested using a QIAquick Gel Extraction Kit (Qiagen). The extracted DNA was sent for Sanger Sequencing (Genewiz, South Plainfield, NJ) along with the primer 5-TTTGGGAAGATGGGAGTG-3, and rendered the sequence MDYKDHHGDYKDHDIDYKDHDYK corresponding to 3XFlag, located immediately after the end of the TIF-1A exon 18 coding sequence. Once it was confirmed that exon 18 of the TIF-1A gene was tagged with 3X Flag, cells were used for further experiments.

Proteomic analyses*: Protein Lysis and Digestion.* Mouse EDL tissues (~5 – 10 mg, stored at -80 °C) were re-suspended in 500 µL of lysis buffer (8 M urea, 40 mM Tris (pH 8), 30 mM NaCl, 1

mM $CaCl_2$, 1 tab of mini EDTA-free protease inhibitor (Roche Diagnostics, Indianapolis, IN)). Samples were homogenized by bead beating (3 × 30 seconds in bead beater, ~30 seconds on ice) then centrifuged twice (22,000 × g, 10 minutes, 4 °C). Supernatants were transferred to Eppendorf microcentrifuge tubes. Protein concentration was determined using a BCA assay (Thermo Fisher Scientific, San Jose, CA).

Proteins were reduced with 5 mM dithiothreitol (incubation at 58°C for 30 minutes) and alkylated with 15 mM iodoacetamide (incubation in the dark, at ambient temperature, for 30 minutes). Alkylation was quenched by adding an additional 5 mM dithiothreitol (incubation at ambient temperature for 15 minutes). Samples were diluted to a final concentration of 1.5 M urea with a solution of 50 mM Tris (pH 8) and 5 mM $CaCl_2$ before and proteins were enzymatically digested using sequencing-grade trypsin (Promega, Madison, WI) at a ratio of 1:50 (enzyme:protein). The resulting mixtures were rocked at ambient temperature overnight (~16 hours). A second aliquot of trypsin was added the following morning at a ratio of 1:100 (enzyme:protein) and samples were incubated at ambient temperature for an hour. Digests were then quenched by bringing the pH ~2 with trifluoroacetic acid and immediately desalted using C18 solid-phase extraction columns (SepPak, Waters, Milford, MA). Peptide concentration was determined using a colorimetric peptide assay (Thermo Fisher Scientific, San Jose, CA).

LC-MS/MS analysis. All experiments were performed using a Thermo Dionex Ultimate 3000 RSLC-nano liquid chromatography instrument (Thermo Fisher Scientific, San Jose, CA) coupled to a FAIMS-enabled[1] Orbitrap Fusion Lumos Tribrid mass spectrometer (Thermo Fisher Scientific, San Jose, CA). Reverse-phase columns were made in-house by packing a fused silica capillary (75µm i.d., 360 µm o.d, with an integrated electrospray emitter (New Objective, Woburn, MA)) with 1.7µm diameter, 130 Å pore size bridged ethylene hybrid C18 particles (Waters, Milford, MA) to a final length of 30 cm.[2]

The column was heated to 50 °C for all experiments. Samples were loaded onto the column in 99:1 buffer A [water, 0.2% formic acid]:buffer B [acetonitrile, 0.2% formic acid] at a flow-rate of 0.30 µL/min. Peptides were eluted using the following gradient: an increase to 8% B over 10 minutes, followed by a 100 minute linear gradient from 8% to 55% B, followed by a 5 minute linear gradient to 100% B which was held for 4 minutes. The column was equilibrated with 1% buffer B for an additional 10 minutes. For each experiment, 2 µg of peptides were loaded onto the column. Precursor peptide cations were generated from the eluent through the utilization of a nanoESI source.

Mass spectrometry instrument methods for sample analysis consisted of MS^1 survey scans (1e6 target value; 120,000 resolution; 300 Th – 1500 Th) that were used to guide subsequent data-dependent MS/MS scans of the most intense precursors for 1 s. The MS/MS analyses were performed in the ion trap (0.7 Th isolation window, HCD fragmentation; normalized collision energy of 30; 3e4 target value, IT turbo scan). Dynamic exclusion duration was set to 20 s with an exclusion width of ±10 ppm the selected average mass. Charge states that were unknown, +1 or >+5 were excluded from the analysis. Maximum injection times were set to 50 ms for all MS^1 scans and 14 ms for MS/MS scans. FAIMs compensation voltages were alternated between -50 V and -70 V.

Data Analysis. Data was processed using MaxQuant software (version 1.6.2.3). Searches were performed against a target-decoy database (Uniprot (mouse), www.uniprot.org, October 28, 2018). Searches were conducted using a 20 ppm precursor mass tolerance and a 0.04 Da product mass tolerance. A maximum of 2 missed tryptic cleavages were allowed. The fixed modifications specified were carbamidomethylation of cysteine residues. The variable modifications specified were oxidation of methionine and acetylation of the N-terminus. Within MaxQuant, peptides were filtered to a 1% unique peptide FDR. Characterized proteins were grouped based on the rules of parsimony and filtered to a 1% FDR.[3]

Label-free quantification was performed within MaxQuant using MaxLFQ.[4] Missing values were imputed using the Perseus tool available with MaxQuant.[5] Quantitative data from each experiment was log_2 transformed and mean-normalized across all tissues for each given protein. Significantly changing proteins were identified using a two-sided Student's T-test in Excel. GO enrichment was performed in R[6] using the Fisher's exact test and corrected for multiple comparisons using the Hochberg correction.[7]

Venous Blood Bicarbonate Determination—We[3] and others[4] have previously established the presently used model of chronic normoxemia/hypercapnia by directly checking arterial blood gases composition of mice housed in that setting. Chronic CO_2 retention was confirmed here by the surrogate of venous bicarbonate values, which represent the kidneys buffering capacity in that context[5] and consistently reflects chronic CO_2 retention in the mentioned model[3,4]. Blood was released by severing the vena cava and collected in 200uL EDTA-coated Micorvette capillary collection tubes (Sarstedt). Bicarbonate (HCO_3^-) levels were measured immediately with an i-STAT handheld blood analyzer (Abbott, Chicago, IL).

Protein Synthesis/Puromycin Incorporation—For measurements of protein synthesis, hypercapnic and normocapnic mice received 0.040µmol/g puromycin dissolved in PBS via intraperitoneal injection 30 minutes prior to euthanasia and tissue extraction. EDL and soleus were extracted and stored at -80°C for Western blot analysis or embedded in optimal cutting temperature (OCT) compound and frozen for immunofluorescence. For Western blot analysis, the secondary antibody Peroxidase AffiniPure Goat Anti-Mouse IgG, Fcγ subclass 2a specific was used instead of a standard HRP anti-mouse secondary antibody to avoid non-specific binding of the antibody to endogenous mouse IgG.

Muscle Fiber Cross Sectional Area Measurement—Muscles sections were stained with anti-Laminin (Sigma-Aldrich, L9393), and images were obtained with confocal microscopy (Leica,

SPE). Muscle fiber delineations at 10X magnification were analyzed with CellProfiler software (Broad Institute, Cambridge MA) to automatedly and unbiasedly measure fiber cross sectional area. Output muscle traces were reviewed to assure accuracy of measurement software's pipeline.

Muscle weight—Freshly procured muscles were weighed using an analytical balance (Sartorius Entris, Germany). To minimize the weight's variability intrinsic to small magnitudes, determinations were done using relatively larger gastrocnemius and tibialis anterior muscles.

Exercise protocol—At the 4 week point of high CO_2 exposure, both hypercapnic and normocapnic mice were started on an endurance exercise regime. Animals were exercised on a treadmill (Ugo Basile, 57630) for 1 hour per day excluding weekends at 12m/min. This protocol was continued for the final 4 weeks before collection.

Grip Strength Determination—Animal's four limb grip strength was determined using a GSM Grip Strength Meter (Ugo Basile, Gemonio, Italy, 47200) with the full grasping grid as previously established[3]. In short, the mouse was held by the tail and placed on the grasping grid; once the test was started, pressure was steadily applied to the mouse's tail until failure and release from the grasping pad occurred. The peak force was recorded, and the mouse was given a 1-minute rest; the test was repeated for a total of 5 replicates per animal.

In vivo Rapamycin experiments—11-week-old animals were started on daily injections of 8mg/kg rapamycin that was dissolved in 10% DMSO and 90% ethanol and administered subcutaneously. Injections were continually delivered for 6 weeks until the animals were euthanized, and muscles collected.

Motion Detection—Motion detection cages (Ugo Basile, Gemonio, Italy, 47420) were used for quantitative monitoring of animals housed in hypercapnic and normocapnic environments, using protocol suggested by manufacturer.

Food Intake—Food intake of normocapnic and hypercapnic animals was compared by recording the difference in the mass of food given to single animals after 3 days. When kept in the hypercapnia chamber, food would absorb excess moisture due to increased ambient humidity. To correct for this confounder, a control container of 100g of food was placed in the chamber during the same 3-day period of monitoring. The percentage of weight gain of the control food was used to correct the experimental food masses.

Reagents—All cell culture reagents were from Corning Life Sciences (Tewksbury, MA). HRP-conjugated goat anti-mouse secondary antibody was from Bio-Rad (Hercules, CA) and goat anti-rabbit secondary antibody was from Bio-Rad or Cell Signaling Technology (Danvers, MA). All other chemicals were purchased from Millipore (Billerica, MA), Sigma-Aldrich (St. Louis, MO) or Bio-Rad. Reagents for production of cDNA and quantitative real time PCR (qPCR) were from Bio-Rad and Life Technologies. The DNA and mRNA isolation kits were from Qiagen (Germantown, MD). Primers were purchased from Integrated DNA Technologies (Coralville, IA). Restriction endonucleases were obtained from Promega (Madison, WI).

Cell Culture and myoblasts isolation and differentiation—C2C12 mouse myoblasts (ATCC, CRL1772, lot # 62042837 and 63809504) were cultured and differentiated as described elsewhere[3]. In brief, cells were allowed to grow in p60 plates in 10% fetal bovine serum (Sigma, F2442, lot # 16H111) DMEM (growth media, containing 100 units/ml penicillin, and 100 µg/ml streptomycin) until they reached ~80-90% confluence, and then culture media was changed to prewarmed 2% horse serum (Hyclone, UK SH30074.02, lot # AB10135313) DMEM (differentiation media). The differentiation media was renewed every 24 h, and cells were allowed to differentiate for 4–6 days. Primary myoblasts were obtained from animals 10-12 weeks of age using MACS Miltenyi (Auburn, CA), as previously reported[6]. Quality control of myoblasts isolation was made by immunostaining for Pax7 and MyoD and by differentiation into myotubes with 2% horse serum DMEM and determination of cell morphology and also expression of skeletal myosin. Human

embryonic kidney/HEK293 cells (ATCC, CRL 1573) were cultured in p60 plates in growth media until they reached ~80% confluency and used as positive controls.

1. Goodman, C.A.*, et al.* Novel insights into the regulation of skeletal muscle protein synthesis as revealed by a new nonradioactive in vivo technique. *FASEB J* **25**, 1028-1039 (2011).
2. Erbay, E. & Chen, J. The mammalian target of rapamycin regulates C2C12 myogenesis via a kinase-independent mechanism. *J Biol Chem* **276**, 36079-36082 (2001).
3. Jaitovich, A.*, et al.* High CO2 levels cause skeletal muscle atrophy via AMP-activated kinase (AMPK), FoxO3a protein, and muscle-specific Ring finger protein 1 (MuRF1). *J Biol Chem* **290**, 9183-9194 (2015).
4. Gates, K.L.*, et al.* Hypercapnia impairs lung neutrophil function and increases mortality in murine pseudomonas pneumonia. *Am J Respir Cell Mol Biol* **49**, 821-828 (2013).
5. Weinberger, S.E., Schwartzstein, R.M. & Weiss, J.W. Hypercapnia. *N Engl J Med* **321**, 1223-1231 (1989).
6. Motohashi, N., Asakura, Y. & Asakura, A. Isolation, culture, and transplantation of muscle satellite cells. *J Vis Exp* (2014).